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Original article

Peptidomimetics containing a vinyl ketone warhead as falcipain-2 inhibitors

Roberta Ettari [a,*], Maria Zappalà [a], Nicola Micale [a], Giovanni Grazioso [b], Salvatore Giofrè [a], Tanja Schirmeister [c], Silvana Grasso [a]

[a] Dipartimento Farmaco-Chimico, University of Messina, Viale Annunziata, 98168 Messina, Italy
[b] Dipartimento di Scienze Farmaceutiche "Pietro Pratesi", Università degli Studi di Milano, Via Mangiagalli 25, 20133 Milano, Italy
[c] Institute of Pharmacy and Food Chemistry, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany

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ABSTRACT

The design, chemical synthesis, and enzymatic activity evaluation of a set of falcipain-2 inhibitors are reported. These compounds contain a proven peptidomimetic recognition motif based on a benzo[1,4] diazepin-2-one (1,4-BDZ) framework built on a dipeptide sequence, and a Michael acceptor terminal moiety capable of deactivating the cysteine protease active site. Our goal is to modify the P_3 site of this motif in order to identify the structural requirements for the interaction with the target.

1. Introduction

Half of the world's population is at risk of malaria, and an estimated 247 million cases led to nearly 881 000 deaths in 2006. The World malaria report 2008 describes the global distribution of cases and deaths, how WHO-recommended control strategies have been adopted and implemented in endemic countries, sources of funding for malaria control, and recent evidence that prevention and treatment can alleviate the burden of the disease [1]. Five species of a parasite belonging to the genus *Plasmodium* can infect humans; the most serious forms of the disease are caused by *Plasmodium falciparum*. Malaria infection caused by *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae* instead, brings on a milder form of the disease in humans that is not generally fatal. A fifth species of *Plasmodium*, named *Plasmodium knowlesi*, has macaques as natural vertebrate host but recently it has been regnognized infective also for humans [2].

The increasing resistance of malaria parasites to anti-malarial drugs, the lack of widely available vaccines that provide a high level of protection for a sustained period, and the inadequate control of mosquito vectors demand new approaches to drug development [3,4]. One promising strategy to develop new drugs has been to target proteases of malaria parasites which play pivotal roles in the processes of host erythrocyte rupture, erythrocyte invasion, and hemoglobin degradation. Falcipain-2 (FP-2) of *P. falciparum* is a papain-family (clan CA, family C1) cysteine protease and is likely the major hemoglobinase in the food vacuole of erythrocytic parasites [5]. FP-2 is also able to promote host cell rupture cleaving erythrocyte membrane skeletal proteins [6]. Therefore, the inhibition of FP-2 represents a promising strategy for discovery of novel anti-malarial drugs.

Our research group has actively been involved on the synthesis of novel peptidomimetic FP-2 inhibitors containing a 1,4-benzodiazepine (1,4-BDZ) scaffold [7–10], introduced internally to a peptide sequence which mimics the dipeptide D-Ser-Gly, and different electrophilic warheads able to interact with the thiol group of the cysteine active site by forming a reversible or irreversible covalent bond. In this context, we decided to synthesize new derivatives in which the α, β unsaturated electrophilic warhead and the benzo[1,4]diazepin-2-one (1,4-BDZ) framework were left unmodified, while the P_3 site underwent profound modifications, with the aim of gaining a better insight about the characteristics of the pocket that could accommodate different functional groups positioned at this site. Vinyl ketone **1** [10] (Fig. 1) was selected as lead compound owing a good inhibitory activity against the target enzyme ($k_{2nd} = 4400\ M^{-1}\ s^{-1}$) and the strongest potency against cultured *P. falciparum* ($IC_{50} = 9.3\ \mu M$), additionally the ketone group possesses a major stability to metabolic reactions in comparison to other functions, such as the ester moiety already employed by our group [10].

* Corresponding author. Tel.: +39 090 6766466; fax: +39 090 6766402.
E-mail address: rettari@pharma.unime.it (R. Ettari).

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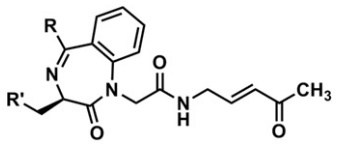


Comp	R	R'
1	C_6H_5	N-(4-Cl-2-CF_3-phenyl)carbamoyloxy
2	C_6H_5	4-morpholinyl
3	C_6H_5	1-piperidinyl
4	C_6H_5	*i*-butylamino
5	CH_3	N-(4-Cl-2-CF_3-phenyl)carbamoyloxy

Fig. 1. Structure of vinyl ketones **1**–**5**.

Specifically, the N-(4-chloro-2-trifluoromethylphenyl)-carbamoyloxy moiety of **1** linked at C-3 of the BDZ nucleus by means of a methylene group has been replaced with an alicyclic, i.e. morpholine (**2**) and piperidine (**3**), according to previously reported studies on FP-2 inhibitors [11], or aliphatic amine, i.e. *i*-butylamine (**4**), and the phenyl ring at C-5 of the 1,4-BDZ core was substituted with a less bulky group, i.e. methyl (**5**).

Herein, we report the synthesis of vinyl ketones **2**–**5** (Fig. 1), their biological evaluation against the FP-2 enzyme and molecular modeling studies. Additionally, selectivity against the target enzyme was estimated by testing the new compounds against some human cysteine proteases of the papain-family such as cathepsins B and L.

2. Results and discussion

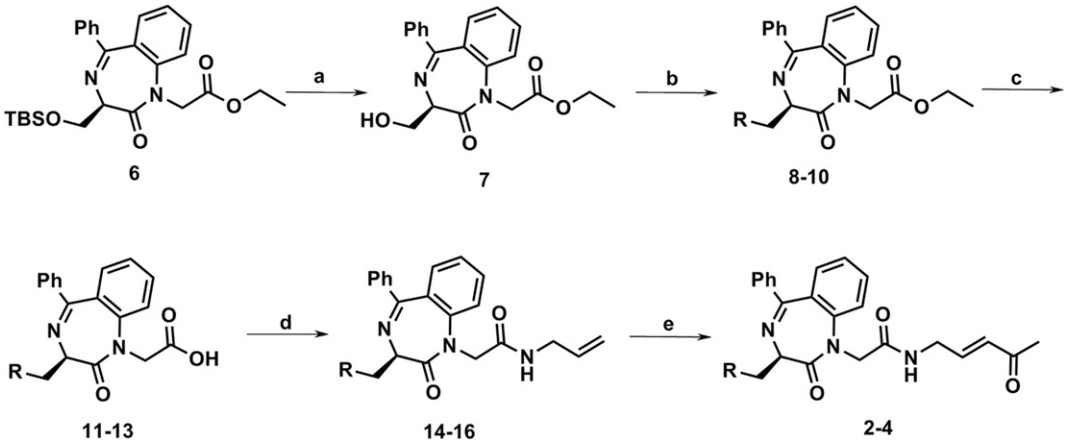
2.1. Chemistry

Synthesis of target compounds **2**–**4** was accomplished as depicted in Scheme 1. Desilylation of 1,4-BDZ derivative **6**, synthesized according to a previously reported procedure [7], provided the free alcohol **7** which was easily converted to alicyclic or aliphatic amine *via* Mitsunobu reaction [12], by using the appropriate amine as a nucleophile in the presence of diethyl azodicarboxylate (DEAD) and triphenylphosphine(TPP). The Mitsunobu reaction [12], firstly performed in standard conditions (room temperature, 12 h) was not successful and the desired products **8**–**10** were obtained under microwaves irradiation at 100 °C for 2 h. The ester functionality in **8**–**10** was then hydrolyzed and the corresponding carboxylic acids **11**–**13** were coupled to the commercially available allylamine to afford the coupling products **14**–**16**. The introduction of the required vinyl ketone warhead was realized by olefin cross-metathesis (CM) reaction, using Hoveyda–Grubbs 2nd generation catalyst, a phosphine-free N-heterocyclic carbene ruthenium complex which actually represents the catalyst of choice for these reactions [13]. Thus, CM reactions between the tripeptides **14**–**16** and methyl vinyl ketone has been carried out under microwave irradiation in an efficient and rapid manner to afford compounds **2**–**4**. The introduction of an electron-withdrawing substituent onto the olefin usually requires a relatively large amount of catalyst and a prolonged reaction time and/or results in a low conversion and yield of the expected CM product. These results are normally attributed to the fact that a relatively high temperature, used for several hours, can cause a gradual decomposition of the catalyst. This problem has been overcome by exposure of the reaction mixture to microwave heating at a high temperature (100 °C) for a short time [14].

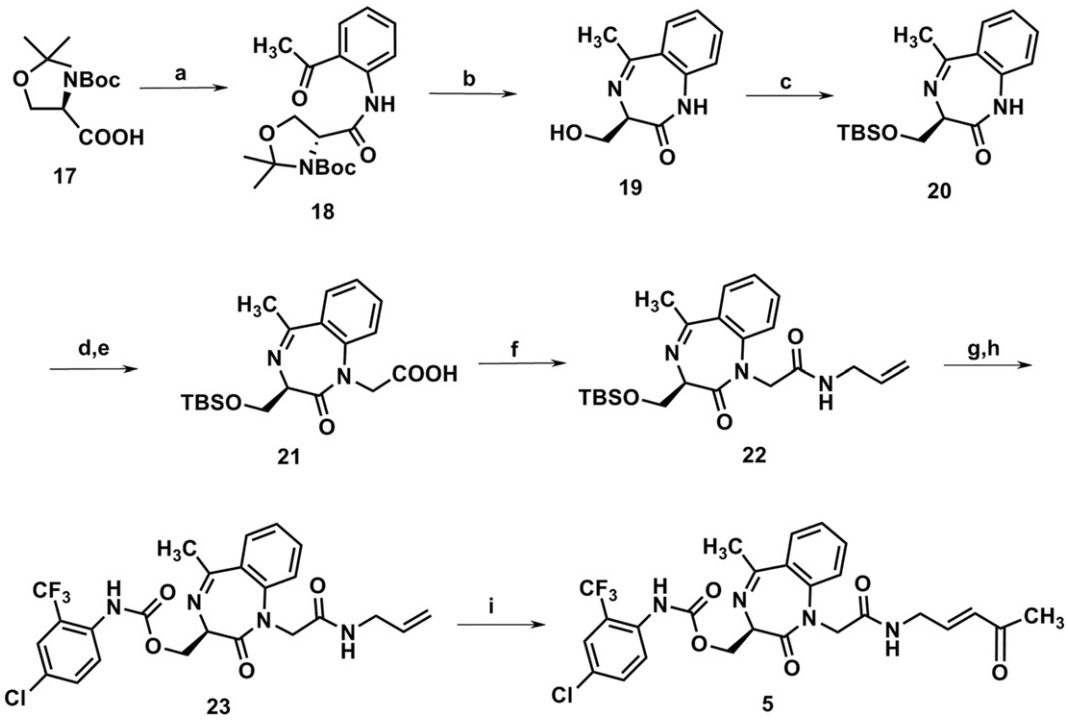
The synthesis of the methyl derivative **5** was realized starting from the carboxylic acid **17** [7], (Scheme 2), which was converted into the corresponding mixed anhydride by reaction with *i*-BuO-COCl and condensed *in situ* with 2-aminoacetophenone to afford compound **18**. According to our previously reported procedure [7], this intermediate was refluxed in acidic conditions to afford the 1,4-BDZ **19** substituted at position 3. Then, the hydroxyl group was protected and the intermediate **20** was reacted with ethyl bromoacetate to introduce the side chain at the N-1 of the 1,4-BDZ. The ester moiety was hydrolyzed to afford the corresponding acid **21** which was then coupled to the allylamine. The coupling product **22** was then deprotected to the serine hydroxyl group and treated with the required isocyanate to provide the carbamate **23**. The terminal olefin was then easily functionalized *via* CM to afford compound **5**.

2.2. Biological activity

Compounds **2**–**5** were tested against FP-2 in standard fluorescence assays as published previously [15]. The hydrolysis of Cbz-Phe-Arg-AMC (aminomethyl coumarin) in the absence or presence of the respective inhibitor was measured by following the fluorescence increase due to release of AMC. The well-known cysteine protease inhibitor E-64 [16] and DMSO were used as a positive and negative control respectively. First, initial screenings with 100 and 30 μM inhibitor concentration were performed. For inhibitors which displayed considerable activity in this screening, continuous assays (progress curve method, Fig. 2) [17] were performed to determine the kinetic parameters (Table 1). In cases where k_{obs} vs.



Scheme 1. Reagents and conditions: (a) TBAF, THF, rt, 5 h; (b) morpholine, piperidine or *i*-butylamine, TPP, DEAD, THF, MW, 100 °C, 2 h; (c) LiOH, MeOH, 0 °C-rt, 6 h; (d) allylamine, HATU, CH_2Cl_2, rt, 12 h; (e) methyl vinyl ketone, Hoveyda Catalyst, CH_2Cl_2, 100 °C, MW, 1 h.



Scheme 2. Reagents and conditions: (a) *i*-BuOCOCl, NMM, CH_2Cl_2, 0 °C-rt, 30 min, then 2-aminoacetophenone, reflux, 20 min, rt, 12 h; (b) HCl/MeOH, reflux, 5 h, then $NaHCO_3$, MeOH, rt, 12 h; (c) TBS-Cl, imidazole, CH_2Cl_2, 0 °C-rt, 12 h; (d) $BrCH_2COOEt$, NaH, 0 °C-rt, 5 h; (e) LiOH, MeOH, 0 °C-rt, 6 h; (f) allylamine, HATU, CH_2Cl_2, rt, 12 h; (g) TBAF, THF, rt, 5 h; (h) 4-Cl, 2-$CF_3C_6H_3NCO$, rt, 12 h; (i) methyl vinyl ketone, Hoveyda Catalyst, CH_2Cl_2, 100 °C, MW, 1 h.

[I] plots showed to be hyperbolic, both constants, k_{inac} and K_I, were determined (Fig. 3). In cases where k_{obs} vs. [I] plots were restricted to the linear range, only the second-order rate constants of inhibition k_{2nd} (M^{-1} s^{-1}) was determined (Fig. 4). The irreversible inhibition of this class of compounds has been confirmed in our previous studies by means of dialysis experiments [8], and can be deduced from the observed time-dependent inhibition (Fig. 2).

In order to rationalize the activity profile of compounds **1**–**5**, we performed molecular modeling calculations on a computational model of the enzyme derived from the recently published X-ray structure of the FP-2 co-crystallized with E-64 [18]. Subsequently, in agreement with the expected mechanism of inhibition, we carried out docking calculations taking for granted the formation of the Michael adducts between the β-carbon of the α, β-unsaturated-carbonyl ligands and the side chain of Cys42, present in the catalytic site of the protein.

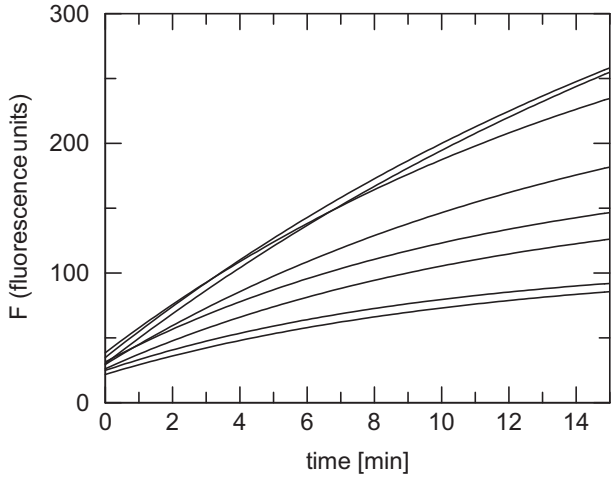


Fig. 2. Inhibition of cathepsin L by cpd. **1**. Progress curves at increasing inhibitor concentrations (0, 5, 10, 20, 25, 30, 40, 50 μM) show time-dependent inhibition, indicating an irreversible inactivation of the enzyme. The progress curves were fit to the equation: $F = A(1-\exp(-k_{obs}t)) + B$ to yield the k_{obs} values (F = fluorescence units, B = background fluorescence, t = time).

The results put in evidence that inhibitor **1** occupies entirely the catalytic crevice of the enzyme creating a remarkable H-bond network with various FP-2 residues (Fig. 5A). In particular, while the three carbonyl groups of **1** behave as H-bond acceptors with the side chain of Trp206 and the backbone of Gly83 and Ile85, the NH group of the carbamoyl moiety acts as an H-bond donor with the side chain of Asp234. Moreover, several hydrophobic contacts further stabilize the FP-2/**1** complex, i.e. the accommodation of the aryl moiety in a cleft shaped by the side chains of Phe236, Ile85, Leu84 and Asn86. On the contrary, the lack of some of these contacts is detrimental on the potency of compound **5** since the 5-phenyl ring of derivative **1** is replaced by a methyl group.

The replacement of the arylcarbamoyl moiety of **1** by an amine (e.g. compounds **2**–**4**) reduces the potency of the ligands due to the lack of the above-mentioned H-bonds and hydrophobic contacts

Table 1
Inhibition of FP-2 by **1**–**5**[a].

Compd	k_{2nd} (M^{-1} s^{-1})	k_{inac} (min^{-1})	K_I (μM)
1[b]	4400 ± 500	[c]	[c]
2	200 ± 33	[c]	[c]
3			80% inhib. at 100 μM, n.i. at 30 μM
4			>100
5	217 ± 23	[c]	[c]
E-64[d]	(26.2 ± 6.6) × 10^3	0.46 ± 0.07	0.29 ± 0.09

[a] All results are the average of at least two independent measurements, each performed in duplicate. measurement time: 15 min.
[b] Data reported in ref. 10.
[c] Since the k_{obs} vs. [I] diagrams were restricted to the linear range, only the second-order rate constant could be determined as k_{obs}/[I], for an example see Fig. 4.
[d] Data reported in Ref. [8].

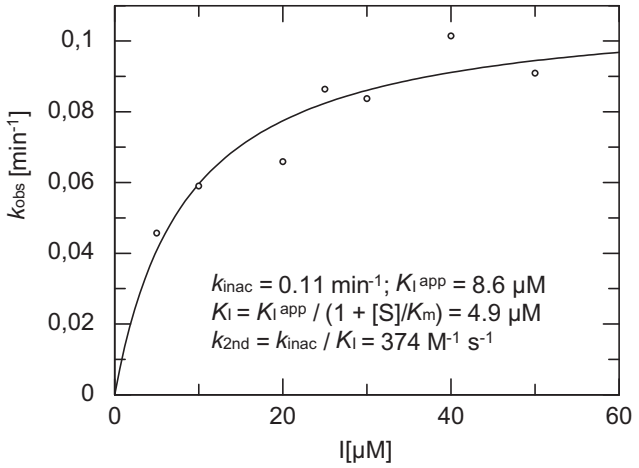


Fig. 3. k_{obs} vs. [I] plot of inhibition of cathepsin L by cpd. **1**. Non-linear regression analysis to the hyperbolic equation $k_{obs} = k_{inac}[I]/(K_I^{app} + [I])$ yields k_{inac} and the apparent K_I (K_I^{app}). The inhibition constant K_I was obtained by correction to zero-substrate concentration using the Cheng–Prusoff equation $K_I = K_I^{app}/(1 + [S]/K_m)$. The second-order rate constant was obtained by: $k_{2nd} = k_{inac}/K_I$.

created by the arylcarbamoyl moiety. Moreover, two steric clashes could be created among the cyclic amine of **2** (Fig. 5B) and **3** and the wall of the binding cleft formed by the side chain of Asp234 and Ile85. Apparently, the steric hindrance of the isobutyl chain of compound **4** is lower than the six-membered ring of derivatives **2** or **3** but, after a conformational minimization of the complex, we noted a repulsive interaction between the positively charged nitrogen of the amine and the nitrogen atom of Gly83. This outcome could account for the inactivity of derivative **4**.

For the most active of the synthesized inhibitors, i.e. compound **5**, an additional assay against the *P. falciparum* strain FCBR was performed, according to a previously published procedure [19]: compound **5** turned out to be inactive ($IC_{50} > 100$ μM), in agreement with the low enzyme inhibition.

The unsaturated ketones **2**–**5** were also tested against the papain-family human cysteine proteases cathepsins B and L (Table 2). Compounds **2** and **5**, weak inhibitors of FP-2, proved to generally possess k_{2nd} values toward cathepsins B and L 1–2 orders of magnitude lower than those reported for FP-2 inhibition.

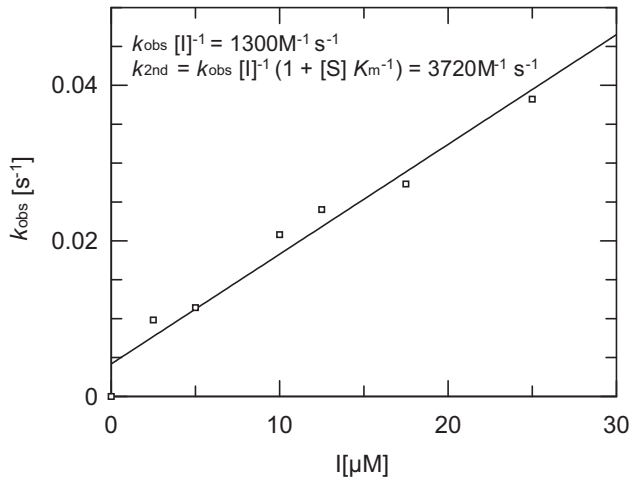


Fig. 4. k_{obs} vs. [I] plot of inhibition of falcipain-2 by cpd. **1**. Since the values are restricted to the linear range, the k_{2nd} value is calculated from the slope of the curve and correction to zero-substrate concentration: $k_{2nd} \approx k_{obs}$ [I]$^{-1}$ (1 + [S]K_m^{-1})

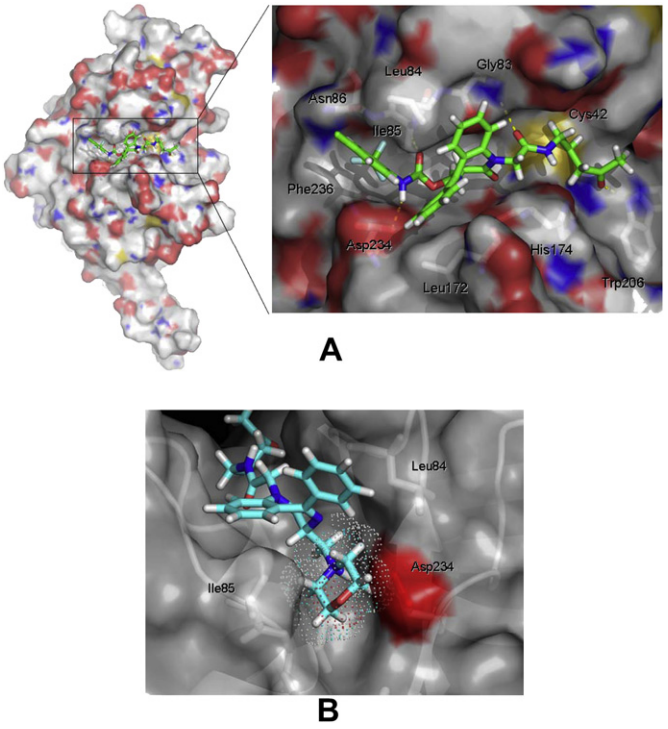


Fig. 5. Surface representation of the FP-2 together with an enlargement of the catalytic site. The predicted binding mode of **1** (A) and **2** (B) is represented. Carbon atoms are colored in green and cyan, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Compounds **3** and **4**, which do not inhibit FP-2, were also inactive toward cathepsins B and L.

In conclusion, our findings on this class of constrained peptidomimetics, supported by docking experiments, clearly indicate that the phenylcarbamoyl portion appended to the P_3 serine hydroxyl group remains a structural requirement of utmost importance in the interaction with the FP-2 enzyme and that a simplification at C-5 of the 1,4-BDZ scaffold does not fit with an enhancement of the inhibitory potency. All this information will be kept in mind for the progress of the research of new compounds with improved FP-2 inhibitory properties.

3. Experimental protocols

3.1. Chemistry

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Elemental analyses were carried out on a C. Erba Model 1106 (Elemental

Table 2
Inhibition of Cathepsins B and L by **1**–**5**[a].

	Cathepsin B			Cathepsin L		
Compd	k_{2nd} (M^{-1} s^{-1})	k_{inac} (min^{-1})	K_I (μM)	k_{2nd} (M^{-1} s^{-1})	k_{inac} (min^{-1})	K_I (μM)
1	50 ± 12	0.02 ± 0.01	6.0 ± 0.3	383 ± 2	0.12 ± 0.02	5.9 ± 3.4
2			>100	9.6 ± 0.5 [b]		[b]
3			>100			>100
4			>100			>140
5	15.5 ± 0.2 [b]		[b]	23.3 ± 9.2	0.12 ± 0.01	82 ± 16

[a] All results are the average of at least two independent measurements, each performed in duplicate.
[b] Since the k_{obs} vs. [I] diagrams were restricted to the linear range, only the second-order rate constant could be determined as k_{obs}/[I], for an example see Fig. 4.

Analyzer for C, H and N) and the results are within ±0.4% of the theoretical values. Merck Silica Gel 60 F_{254} plates were used as analytical TLC; flash column chromatography was performed on Merck Silica Gel (200–400 mesh) or was conducted using pre-packed cartridges on an MP-LC BUCHI system. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Varian Gemini 300 spectrometer. ^{1}H chemical shifts are expressed in δ (ppm) relative to TMS as internal standard and coupling constants (J) in hertz. ^{13}C chemical shifts are referenced to $CDCl_3$ (central peak, $\delta = 77.0$ ppm).

3.1.1. (3-Hydroxymethyl-2-oxo-5-phenyl-2,3-dihydro-benzo[e][1,4] diazepin-1-yl)-acetic acid ethyl ester (**7**)

To a solution of **6** (419 mg, 0.9 mmol) in dry THF (20 mL) was added TBAF (1.35 mL, 1 M in THF). The mixture was stirred until the disappearance of the starting material (TLC monitoring). After removing of THF, the mixture was diluted with EtOAc (200 mL) and washed with water (2 × 200 mL). The organic layer was dried, filtered and evaporated under reduced pressure to give the deprotected product **7** which was used for the next step without any further purification. $R_f = 0.19$ (light petroleum/EtOAc 6:4). Yield 95%. ^{1}H NMR (300 MHz, $CDCl_3$): 1.18 (t, 3H, $J = 7.1$ Hz,), 2.79 (bs, 1H), 3.88 (t, 1H, $J = 5.7$ Hz,), 4.08–4.27 (m, 3H), 4.41 (m, 1H), 4.49 (d, $J = 14.8$ Hz, 1H), 4.58 (d, 1H,$J = 14.8$ Hz), 7.20–7.65 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): 14.30, 49.96, 61.92, 63.25, 64.04, 121.75, 124.93, 128.50, 128.68, 129.94, 130.70, 130.75, 130.80, 131.97, 138.82, 142.38, 170.12, 170.76.

3.1.2. (3-Morpholin-4-ylmethyl-2-oxo-5-phenyl-2,3-dihydro-benzo [e][1,4] diazepin-1-yl)-acetic acid ethyl ester (**8**)

To a mixture of the alcohol **7** (100 mg, 0.29 mmol), morpholine (50 mg, 0.58 mmol), and triphenylphosphine (228 mg, 0.87 mmol) in THF (5 mL) was added diethyl azodicarboxylate (151 mg, 0.87 mmol). The reaction mixture was stirred at 100 °C for 2 h under microwaves irradiation. After this time the solvent was removed under reduced pressure. Diethyl ether was added to the residue to precipitate triphenylphosphine oxide and diethyl azodicarboxylate which were filtered off. The filtrate was evaporated and the crude was purified by flash column chromatography (light petroleum/EtOAc 6:4). $R_f = 0.33$ (light petroleum/EtOAc 6:4). Yield 93%. ^{1}H NMR (300 MHz, $CDCl_3$): 1.18 (t, 3H, $J = 7.1$ Hz,), 2.78–2.94 (m, 4H), 3.77–3.88 (m, 2H), 3.90 (t, 1H, $J = 5.5$ Hz,), 4.14–4.27 (m, 5H), 4.41 (m, 1H), 4.48 (d, 1H, $J = 17.3$), 4.58 (d, 1H, $J = 17.3$), 7.21–7.65 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): 14.30, 49.96, 53.01, 61.92, 63.25, 64.04, 66.73, 121.75, 124.94, 128.50, 128.68, 129.95, 130.71, 130.81, 131.97, 138.82, 168.60, 170.01, 171.23.

3.1.3. (2-Oxo-5-phenyl-3-piperidin-1-ylmethyl-2,3-dihydro-benzo [e][1,4] diazepin-1-yl)-acetic acid ethyl ester (**9**)

The reaction has been carried out according to the same procedure described for compound **8**, using alcohol **7** (100 mg, 0.29 mmol), piperidine (49 mg, 0.58 mmol), triphenylphosphine (228 mg, 0.87 mmol), and diethyl azodicarboxylate (151 mg, 0.87 mmol). The title compound was obtained after purification by flash column chromatography (light petroleum/EtOAc 6:4). $R_f = 0.36$ (light petroleum/EtOAc 6:4). Yield 87%. ^{1}H NMR (300 MHz, $CDCl_3$): 1.18 (t, 3H, $J = 7.1$ Hz), 1.23–1.29 (m, 6H), 2.31–2.36 (m, 4H), 3.88 (t, 1H, $J = 5.5$ Hz), 4.16–4.23 (m, 3H), 4.40 (m, 1H), 4.48 (d, 1H, $J = 17.3$), 4.58 (d, 1H, $J = 17.3$), 7.23–7.65 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): 14.26, 28.65, 49.95, 53.11, 61.90, 63.25, 64.06, 121.65, 124.93, 128.50, 128.78, 129.85, 130.51, 130.71, 131.87, 138.72, 168.56, 170.11, 171.13.

3.1.4. [3-(Isobutylamino-methyl)-2-oxo-5-phenyl-2,3-dihydro-benzo[e][1,4] diazepin-1-yl]-acetic acid ethyl ester (**10**)

The reaction has been carried out according to the same procedure described for compound **8**, using alcohol **7** (100 mg, 0.29 mmol), isobutylamine (42 mg, 0.58 mmol), triphenylphosphine (228 mg, 0.87 mmol), and diethyl azodicarboxylate (151 mg, 0.87 mmol). The title compound was obtained after purification by flash column chromatography (light petroleum/EtOAc 6:4). $R_f = 0.29$ (light petroleum/EtOAc 6:4). Yield 95%. ^{1}H NMR (300 MHz, $CDCl_3$): 1.18 (t, 3H, $J = 7.1$ Hz), 1.23 (s, 6H), 2.00 (m, 1H), 2.94–3.02 (m, 2H), 3.88 (t, 1H, $J = 5.7$ Hz), 4.08–4.27 (m, 3H), 4.36 (d, 1H, $J = 15.5$ Hz), 4.45 (m, 1H), 4.63 (d, 1H, $J = 15.5$ Hz), 7.24–7.71 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): 14.16, 14.90, 20.62, 51.57, 61.01, 62.03, 62.35, 64.10, 121.78, 124.52, 127.34, 128.97, 129.25, 129.43, 131.17, 131.38, 139.09, 140.75, 168.63, 169.66, 170.02.

3.1.5. (3-Morpholin-4-ylmethyl-2-oxo-5-phenyl-2,3-dihydro-benzo [e][1,4]diazepin-1-yl)-acetic acid (**11**)

To a solution of ester **8** (113 mg, 0.27 mmol) in a mixture methanol/water 1:1 (20 mL) was added LiOH (19 mg, 0.81 mmol) keeping the temperature at 0 °C and stirred at room temperature until the disappearance of the starting material (TLC monitoring). The solvent was concentrated under reduced pressure and the mixture was treated with 10% citric acid and extracted with EtOAc (200 mL), dried and concentrated to give the acid **11**. Yield 99%. ^{1}H NMR (300 MHz, $CDCl_3$): 2.77–2.93 (m, 4H), 3.76–3.85 (m, 2H), 3.91 (t, 1H, $J = 5.6$ Hz), 4.12–4.25 (m, 3H), 4.40 (m, 1H), 4.47 (d, 1H, $J = 17.3$), 4.56 (d, 1H, $J = 17.3$), 7.20–7.62 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): 49.96, 53.01, 63.25, 64.04, 66.80, 121.75, 124.94, 128.50, 128.68, 129.95, 130.71, 130.81, 131.97, 138.82, 168.60, 170.01, 173.25.

3.1.6. (2-Oxo-5-phenyl-3-piperidin-1-ylmethyl-2,3-dihydro-benzo [e][1,4] diazepin-1-yl)-acetic acid (**12**)

The reaction has been carried out according to the same procedure described for compound **11**, by using ester **9** (104 mg, 0.25 mmol) and LiOH (18 mg, 0.75 mmol). Yield 99%. ^{1}H NMR (300 MHz, $CDCl_3$): 1.24–1.30 (m, 6H), 2.30–2.35 (m, 4H), 3.88 (t, 1H, $J = 5.5$ Hz), 4.28–4.36 (m, 2H), 4.58 (d, 1H, $J = 17.3$), 4.80 (d, 1H, $J = 17.3$), 7.31–7.70 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): 28.11, 49.76, 53.11, 63.45, 64.14, 121.76, 124.84, 128.70, 128.68, 129.85, 130.72, 130.71, 131.87, 138.92, 168.26, 170.11, 173.05.

3.1.7. [3-(Isobutylamino-methyl)-2-oxo-5-phenyl-2,3-dihydro-benzo[e][1,4] diazepin-1-yl]-acetic acid (**13**)

The reaction has been carried out according to the same procedure described for compound **11**, by using ester **10** (112 mg, 0.27 mmol) and LiOH (855 mg, 35.7 mmol). Yield 99%. ^{1}H NMR (300 MHz, $CDCl_3$): 1.22 (s, 6H), 2.02 (m, 1H), 2.96–3.04 (m, 2H), 3.88 (t, 1H, $J = 5.7$ Hz), 4.12 (m, 1H), 4.37 (d, 1H, $J = 15.5$ Hz), 4.44 (m, 1H), 4.62 (d, 1H, $J = 15.5$ Hz), 7.25–7.70 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): 14.18, 20.82, 51.97, 61.10, 62.32, 64.05, 121.78, 124.42, 127.24, 128.67, 129.15, 129.33, 131.27, 131.35, 139.19, 140.65, 168.53, 169.86, 171.25.

3.1.8. N-Allyl-2-(3-morpholin-4-ylmethyl-2-oxo-5-phenyl-2,3-dihydro-benzo[e][1,4]diazepin-1-yl)-acetamide (**14**)

To a solution of **11** (105 mg, 0.27 mmol) in DMF (10 mL) at 0 °C was added HATU (154 mg, 0.40 mmol). After 5 min, the ice bath was removed and allylamine (31 mg, 0.54 mmol) was added. The mixture was stirred at room temperature for 12 h. After this time the mixture was diluted with EtOAc and washed with water. The organic layer was dried, filtered and evaporated under reduced pressure and the crude was purified by flash chromatography ($CHCl_3$/MeOH 9:1). $R_f = 0.58$ ($CHCl_3$/MeOH 9:1). Yield 98%. ^{1}H NMR (300 MHz, $CDCl_3$): 2.80–2.95 (m, 4H), 3.82–3.89 (m, 3H), 4.16–4.27 (m, 5H), 4.32 (d, 1H, $J = 15.4$ Hz), 4.41 (m, 1H), 4.59 (d, 1H, $J = 15.4$ Hz), 5.02–5.11 (m, 2H), 5.75 (m, 1H), 6.39 (bs, 1H), 7.11–7.69 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): 42.27, 52.49, 52.63, 62.48, 63.13, 64.22, 116.82, 122.76, 125.28, 128.58, 129.78, 129.96, 130.54, 131.03, 132.35, 133.71, 138.54, 157.01, 168.27, 170.29, 171.32.

*3.1.9. N-Allyl-2-(2-oxo-5-phenyl-3-piperidin-1-ylmethyl-2,3-dihydro-benzo[e][1,4]diazepin-1-yl)-acetamide (**15**)*

The reaction has been carried out according to the same procedure described for compound **14**, by using acid **12** (98 mg, 0.25 mmol), HATU (142 mg, 0.37 mmol) and allylamine (28 mg, 0.50 mmol). The title compound was obtained after purification by flash column chromatography ($CHCl_3$/MeOH 9:1). $R_f = 0.61$ ($CHCl_3$/MeOH 9:1). Yield 95%. ^{1}H NMR (300 MHz, $CDCl_3$): 1.25–1.31 (m, 6H), 2.31–2.36 (m, 4H), 3.82–3.89 (m, 3H), 4.25 (m, 1H), 4.32 (d, 1H, $J = 15.4$), 4.41 (m, 1H), 4.60 (d, 1H, $J = 15.4$), 5.03–5.07(m, 2H), 5.71 (m, 1H), 6.26 (m, 1H), 7.23–7.70 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): 28.68, 42.37, 52.19, 52.53, 62.28, 63.03, 116.82, 122.26, 125.48, 128.88, 129.79, 129.94, 130.52, 131.13, 132.35, 133.70, 138.53, 157.01, 168.37, 170.19, 171.22.

*3.1.10. N-Allyl-2-[3-(isobutylamino-methyl)-2-oxo-5-phenyl-2,3-dihydro-benzo[e][1,4]diazepin-1-yl]-acetamide (**16**)*

The reaction has been carried out according to the same procedure described for compound **14**, by using acid **13** (102 mg, 0.27 mmol), HATU (154 mg, 0.40 mmol) and allylamine (31 mg, 0.54 mmol). The title compound was obtained after purification by flash column chromatography ($CHCl_3$/MeOH 9:1) $R_f = 0.54$ ($CHCl_3$/MeOH 9:1). Yield 97%. ^{1}H NMR (300 MHz, $CDCl_3$): 1.24 (s, 6H), 2.01 (m, 1H), 2.94–3.03 (m, 2H), 3.89 (t, 1H, $J = 5.7$ Hz), 4.07–4.15 (m, 2H), 4.24 (m, 1H), 4.37 (d, 1H, $J = 15.5$ Hz), 4.46 (m, 1H), 4.65 (d, 1H, $J = 15.5$ Hz), 5.03–5.12 (m, 2H), 5.73 (m, 1H), 6.53 (bs, 1H), 6.77 (bs, 1H), 7.22–7.69 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): 14.67, 20.81, 42.27, 51.27, 62.49, 63.09, 64.25, 116.81, 122.73, 122.77, 128.56, 128.61, 129.94, 129.98, 130.57, 131.05, 132.37, 138.52, 142.44, 168.60, 171.10, 171.20.

*3.1.11. N-(4-oxo-pent-2-enyl)-2-(2-oxo-5-phenyl-3-morpholin-1-ylmethyl-2,3-dihydro-benzo[e][1,4]diazepin-1-yl)-acetamide (**2**)*

To a solution of **14** (115 mg, 0.27 mmol) in dry CH_2Cl_2 (5 mL) was added methyl vinyl ketone (189 mg, 2.7 mmol) followed by Hoveyda–Grubbs 2nd generation catalyst [(1,3-bis-(2,4,6-trimethylphenyl)-2-imidazolidinylidene)-dichloro-(*o*-isopropoxy phenyl methylene) ruthenium] (16.8 mg, 0.027 mmol). The resulting mixture was heated under microwave irradiation at 100 °C for 2 h. The title compound was obtained after purification by flash column chromatography ($CHCl_3$/MeOH 9:1). $R_f = 0.38$ ($CHCl_3$/MeOH 9:1). Yield 85%. ^{1}H NMR (300 MHz, $CDCl_3$): 2.21 (s, 3H, $COCH_3$), 2.80–2.97 (m, 4H, morph (CH_2)$_2$), 3.83–3.95 (m, 3H, $NCH_2CH{=}CH$ and CH-3), 4.16–4.23 (m, 5H, morph (CH_2)$_2$ and N–CH_a), 4.31 (d, 1H, $J = 16.5$ Hz, NCH_aCO), 4.41 (m, 1H, N–CH_b), 4.58 (d, 1H, $J = 16.5$ Hz, NCH_bCO), 6.06 (d, 1H, $J = 15.9$ Hz, $NCH_2CH{=}CH{-}$), 6.61 (bt, 1H, $NCH_2CH{=}CH{-}$), 7.26–7.71 (m, 9H, C*H* Ar). ^{13}C NMR (75 MHz, $CDCl_3$): 29.94 ($COCH_3$), 40.62 ($NCH_2CH{=}CH{-}$), 52.74 (morph (CH_2)$_2$), 52.80 (NCH_2CO), 63.03 (N–CH_2), 64.32 (CH-3), 65.90 (morph (CH_2)$_2$), 122.82 (*C*H Ar), 125.43 (*C*H Ar), 128.60 (*C*H Ar), 128.65 (*C*H Ar), 129.89 (*C*H Ar), 129.93 (*C*H Ar), 130.62 (*C*H Ar), 131.08 (*C*H Ar), 131.17 ($NCH_2CH{=}CH{-}$), 138.54 (*C* Ar), 140.01 (*C* Ar), 142.12 ($NCH_2CH{=}CH{-}$), 168.27 (*C* Ar), 170.31 (*C*O), 171.36 (*C*O), 195.85 ($COCH_3$). Anal. Calcd for $C_{27}H_{30}N_4O_4$: C, 68.34; H, 6.37; N, 11.81; found C, 68.59; H, 6.27; N, 12.11.

*3.1.12. N-(4-oxo-pent-2-enyl)-2-(2-oxo-5-phenyl-3-piperidin-1-ylmethyl-2,3-dihydro-benzo[e][1,4] diazepin-1-yl)-acetamide (**3**)*

Compound **15** (102 mg, 0.24 mmol) was reacted with methyl vinyl ketone (168 mg, 2.4 mmol) according to the same procedure described for **2**. The title compound was obtained after purification by flash column chromatography ($CHCl_3$/MeOH 9:1). $R_f = 0.42$ ($CHCl_3$/MeOH 9:1). Yield 83%. ^{1}H NMR (300 MHz, $CDCl_3$): 1.25–1.31 (m, 6H, piper (CH_2)$_3$), 2.21 (s, 3H, $COCH_3$), 2.30–2.35 (m, 4H, piper (CH_2)$_2$), 3.89 (t, 1H, $J = 5.6$ Hz, CH-3), 4.03–4.08 (m, 2H, $NCH_2CH{=}CH{-}$), 4.25 (m, 1H, N–CH_a), 4.32 (d, 1H, $J = 15.4$, NCH_aCO), 4.41 (m, 1H, N–CH_b.), 4.60 (d, 1H, $J = 15.4$, NCH_bCO), 6.02 (d, 1H, $J = 15.8$ Hz, $NCH_2CH{=}CH{-}$), 6.51 (bs, 1H, N*H*), 6.63 (bt, 1H, $NCH_2CH{=}CH{-}$), 7.26–7.71 (m, 9H, C*H* Ar). ^{13}C NMR (75 MHz, $CDCl_3$): 28.79 (piper (CH_2)$_2$), 29.84 ($COCH_3$), 40.76 ($NCH_2CH{=}CH{-}$), 52.64 (piper (CH_2)$_2$), 52.90 (NCH_2CO), 63.13 (N–CH_2), 64.22 (*C*H-3), 122.72 (*C*H Ar), 125.23 (*C*H Ar), 128.50 (*C*H Ar), 128.61 (*C*H Ar), 129.79 (*C*H Ar), 129.83 (*C*H Ar), 130.62 (*C*H Ar), 131.08 (*C*H Ar), 131.18 ($NCH_2CH{=}CH{-}$), 138.74 (*C* Ar), 140.10 (*C* Ar), 142.10 ($NCH_2CH{=}CH{-}$), 168.17 (*C* Ar), 170.11 (*C*O), 171.26 (*C*O), 195.96 ($COCH_3$). Anal. Calcd for $C_{28}H_{32}N_4O_3$: C, 71.16; H, 6.83; N, 11.86; found: C, 71.01; H, 7.09; N, 11.90.

*3.1.13. 2-[3-(Isobutylamino-methyl)-2-oxo-5-phenyl-2,3-dihydro-benzo[e][1,4]diazepin-1-yl]-N-(4-oxo-pent-2-enyl)-acetamide (**4**)*

Compound **16** (109 mg, 0.26 mmol) was reacted with methyl vinyl ketone (182 mg, 2.6 mmol) according to the same procedure described for **2**. The title compound was obtained after purification by flash column chromatography ($CHCl_3$/MeOH 9:1). $R_f = 0.35$ ($CHCl_3$/MeOH 9:1). Yield 87%. $[\alpha]_D^{20}$: −34.3 ($c = 0.16$, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): 1.30 (s, 6H, (CH_3)$_2$CH–), 2.22 (m, 1H, (CH_3)$_2$C*H*–), 2.26 (s, 3H, $COCH_3$), 2.84–2.92 (m, 2H, (CH_3)$_2$CH–CH_2–), 3.90 (t, 1H, $J = 5.7$ Hz, CH-3), 4.08–4.16 (m, 2H, $NCH_2CH{=}CH{-}$), 4.26 (m, 1H, CH_a–N–*i*-Bu), 4.38 (d, 1H, $J = 15.5$ Hz, NCH_aCO), 4.48 (m, 1H, CH_b–N–*i*-Bu), 4.64 (d, 1H, $J = 15.5$ Hz, NCH_bCO), 6.16 (d, 1H, $J = 15.0$ Hz, $NCH_2CH{=}CH{-}$), 6.60–6.75 (m, 2H, $NCH_2CH{=}CH{-}$ and N*H*), 7.31–7.73 (m, 9H, C*H* Ar). ^{13}C NMR (75 MHz, $CDCl_3$): 14.57 ((CH_3)$_2$CH), 20.51 ((CH_3)$_2$CH–), 29.97 ($COCH_3$), 40.59 ($NCH_2CH{=}CH{-}$), 52.69 ((CH_3)$_2$CH–CH_2), 63.04 (NCH_2CO), 64.31 (CH_2–N–*i*-Bu), 65.41 (*C*H-3), 122.80 (*C*H Ar), 125.43 (*C*H Ar), 128.63 (*C*H Ar), 129.91 (*C*H Ar), 130.59 (*C*H Ar), 131.12 (*C*H Ar), 132.43 ($NCH_2CH{=}CH{-}$), 138.48 (*C* Ar), 142.14 (*C* Ar), 142.36 ($NCH_2CH{=}CH{-}$), 168.60 (*C* Ar), 170.33 (*C*O), 171.41 (*C*O), 197.89 ($COCH_3$). Anal. Calcd for $C_{27}H_{32}N_4O_3$: C, 70.41; H, 7.00; N, 12.16; found C, 70.36; H, 7.32; N, 12.03.

*3.1.14. (R)-4-(2-Benzoyl-methylcarbamoyl)-2,2-dimethyl-oxazolidine-3-carboxylic acid tert-butyl ester (**18**)*

To a solution of acid **17** (4.7 g, 19 mmol) in dry CH_2Cl_2 (80 mL) at 0 °C was added *N*-methyl morpholine (2.31 mL, 21 mmol) followed by isobutyl chloroformate (2.72 mL, 21 mmol). After 30 min, a solution of 2-aminoacetophenone (2.56 g, 19 mmol) in CH_2Cl_2 (10 mL) was added to refluxing reaction mixture dropwise over 20 min. After stirring for 12 h at room temperature, the reaction mixture was washed with 2 N HCl (100 mL), aqueous $NaHCO_3$ (100 mL), and water (100 mL). The organic layer was dried and concentrated to a residue purified by flash chromatography (light petroleum/EtOAc 8:2). $R_f = 0.55$ (light petroleum/EtOAc 8:2) to give the product **18**. Yield 75%. $[\alpha]_D^{20}$: +90.67 ($c = 1.65$, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): 1.23–1.86 (m, 15H), 2.63 (s, 3H), 4.25–4.27 (m, 2H), 4.47 (m, 1H), 7.15 (m, 1H), 7.56 (m, 1H), 7.89 (m, 1H), 8.80 (d, 1H, $J = 8.5$ Hz), 12.18 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): 28.52, 62.30, 67.22, 80.50, 97.30, 121.03, 122.90, 123.05, 131.70, 135.08, 140.37, 152.07, 171.02, 201.09.

*3.1.15. (R)-3-Hydroxymethyl-5-methyl-1,3-dihydrobenzo[e][1,4] diazepin-2-one (**19**)*

To a solution of **18** (5.07 g, 14 mmol) in MeOH (60 mL) was added 6 N HCl (11.6 mL). The mixture was refluxed for 5 h, after this time the solvent was concentrated under reduced pressure and the crude was extracted with EtOAc (200 mL) and washed with $NaHCO_3$ (2 × 200 mL). The organic phase was dried and again concentrated. The mixture was solved in MeOH (60 mL) and stirred until complete conversion of the starting material into the desired product (12 h). The crude was purified by flash chromatography (light petroleum/EtOAc 8:2). $R_f = 0.25$ (light petroleum/EtOAc 8:2).

Yield 92%. $[\alpha]_D^{20}$: −1.95 (c = 1.95, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): 2.50 (s, 3H), 3.61 (t, 1H, J = 6.3 Hz), 4.16 (m, 1H), 4.28 (m, 1H), 7.11 (d, 1H, J = 7.9 Hz), 7.24 (m, 1H), 7.49 (m, 1H), 7.58 (d, 1H, J = 9.0 Hz,), 9.21 (bs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): 26.25, 62.83, 63.31, 121.57, 124.60, 128.73, 129.28, 131.84, 136.29, 171.02.

3.1.16. (R)-3-(tert-Butyl-dimethyl-silanyloxymethyl)-5-methyl-1,3-dihydro-benzo[e][1,4]diazepin-2-one (**20**)

To a solution of alcohol **19** (2.6 g, 12.8 mmol) in CH_2Cl_2 (70 mL) were added imidazole (1.9 g, 28 mmol) and TBS-Cl (4.8 g, 32 mmol) at 0 °C. After stirring 12 h at room temperature the mixture was washed with water and dried. The crude was purified by flash chromatography to afford the clean product (light petroleum/EtOAc 8:2) R_f = 0.37 (light petroleum/EtOAc 8:2). Yield 99%. $[\alpha]_D^{20}$: −1.95 (c = 1.95, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): 0.23 (s, 3H), 0.24 (s, 3H), 1.01 (s, 9H), 2.57 (s, 3H), 3.79 (t, 1H, J = 6.3 Hz), 4.37 (m, 1H), 4.69 (m, 1H), 7.12–7.68 (m, 4H), 10.27 (bs, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): −4.94, 18.07, 25.94, 26.27, 63.62, 63.70, 121.89, 124.10, 128.45, 129.30, 131.44, 136.93, 164.51, 171.01.

3.1.17. (R)-[3-(tert-Butyl-dimethyl-silanyloxymethyl)-2-oxo-5-methyl-2,3-dihydrobenzo[e][1,4] diazepin-1-yl]-acetic acid (**21**)

Step 1. To a suspension of NaH (368 mg, 15.3 mmol) in dry DMF (10 mL) was added a solution 0.1 M of **20** (4.07 g, 12.8 mmol) in DMF (20 mL) at 0 °C under nitrogen and then slowly reaction mixture brought into room temperature. After stirring 1 h at room temperature, ethyl bromoacetate (2.13 mL, 19.2 mmol, d = 1.506) was added *via* syringe and further stirred for 12 h. The reaction mixture was quenched with NH_4Cl and extracted with EtOAc (100 mL). The organic layer was washed with water (2 × 100 mL), dried, concentrated and the resulting residue was purified by flash chromatography (light petroleum/EtOAc 8:2) to afford the clean product. R_f = 0.51 (light petroleum/EtOAc 8:2). Yield 99%. $[\alpha]_D^{20}$: −7.42 (c = 0.35, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): 0.08 (s, 3H), 0.09 (s, 3H), 0.87 (s, 9H), 1.22 (t, 3H, J = 4.4 Hz), 2.49 (s, 3H), 3.63 (t, 1H, J = 7.1 Hz), 4.09–4.20 (m, 4H), 4.48–4.68 (m, 2H), 7.24–7.57 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): −5.02, 14.27, 18.01, 25.46, 26.15, 49.80, 61.57, 63.99, 64.72, 121.98, 125.23, 127.58, 127.62, 131.38, 140.80, 168.95.169.02.

Step 2. To solution of ester (5.1 g, 12.7 mmol) in a mixture methanol/water 1:1 (60 mL) was added LiOH (1.8 g, 76.2 mmol) keeping the temperature at 0 °C and stirred at room temperature until the disappearance of the starting material (TLC monitoring). The solvent was concentrated under reduced pressure and the mixture was treated with 10% citric acid and extracted with EtOAc, dried and concentrated to give the acid **21**. Yield 99%. $[\alpha]_D^{20}$: −3.75 (c = 0.08, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): 0.07 (s, 3H), 0.08 (s, 3H), 0.88 (s, 9H), 2.50 (s, 3H), 3.60 (t, 1H, J = 7.1 Hz), 4.10–4.21 (m, 2H), 4.48–4.69 (m, 2H), 7.25–7.58 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): −5.01, 18.02, 25.36, 26.05, 49.70, 61.37, 64.32, 121.78, 125.13, 127.38, 127.32, 131.68, 140, 80, 168.75, 173.12.

3.1.18. N-Allyl-2-[3-(tert-butyl-dimethyl-silanyloxymethyl)-5-methyl-2-oxo-2,3-dihydro-benzo[e][1,4] diazepin-1-yl]-acetamide (**22**)

To a solution of **21** (4.7 g, 12.6 mmol) in CH_2Cl_2 (60 mL) at 0 °C was added HATU (9.5 g, 25.2 mmol). After 5 min, the ice bath was removed and allylamine (1.8 mL, 25.2 mmol, d = 0.761) was added. The mixture was stirred at room temperature for 12 h. After this time the mixture was washed with water. The organic layer was dried, filtered and evaporated under reduced pressure and the crude was purified by flash chromatography ($CHCl_3$/MeOH 99:1). R_f = 0.37 ($CHCl_3$/MeOH 99:1). Yield 90%. ^{1}H NMR (300 MHz, $CDCl_3$): 0.12 (s, 3H), 0.15 (s, 3H), 0.90 (s, 9H), 2.50 (s, 3H), 3.67 (t, 1H, J = 5.7 Hz), 3.87–3.95 (m, 2H), 4.07 (d, 1H, J = 15.1 Hz), 4.15–4.33 (m, 2H), 4.60 (d, 1H, J = 15.1 Hz), 5.09–5.20 (m, 2H), 5.81 (m, 1H), 6.46 (bs, 1H), 7.30 (m, 1H), 7.48–7.66 (m, 3H).

3.1.19. (4-Chloro-2-trifluoromethyl-phenyl)-carbamic acid 1-allyl carbamoyl methyl-5-methyl-2-oxo-2,3-dihydro-1H-benzo[e][1,4] diazepin-3-ylmethyl ester (**23**)

Step 1. To a solution of **22** (4.7 g, 11.4 mmol) in dry THF (50 mL) was added TBAF (17.1 mL, 1 M in THF). The mixture was stirred until the disappearance of the starting material (TLC monitoring), then diluted with EtOAc and washed with water. The organic layer was dried, filtered and evaporated under reduced pressure to give the deprotected product which was used for the next step without further purification. R_f = 0.25 ($CHCl_3$/MeOH 99:1). Yield 99%. ^{1}H NMR (300 MHz, $CDCl_3$): 2.51 (s, 3H), 3.68 (t, 1H, J = 5.7 Hz), 3.88–3.96 (m, 2H), 4.08 (d, 1H, J = 15.1 Hz), 4.16–4.32 (m, 2H), 4.60 (d, 1H, J = 15.1 Hz), 5.10–5.21 (m, 2H), 5.82 (m, 1H), 6.48 (bs, 1H), 7.31 (m,1H), 7.48–7.65 (m, 3H).

Step 2. To a solution of deprotected alcohol (3.4 g, 11.3 mmol) in dry CH_2Cl_2 (50 mL) was added 4-Cl, 2-$CF_3C_6H_3NCO$ (3.4 mL, 22.6 mmol) and the mixture was stirred under N_2 atmosphere for 12 h. After this time, the solvent was concentrated under reduced pressure extracted with EtOAc and washed. The crude was purified by flash chromatography ($CHCl_3$/MeOH 99:1). R_f = 0.52 ($CHCl_3$/MeOH 99:1). Yield 80%. ^{1}H NMR (300 MHz, $CDCl_3$): 2.50 (s, 3H), 3.87 (t, 1H, J = 6.6 Hz), 4.10 (d, 1H, J = 14.5 Hz), 4.11–4.15 (m, 2H), 4.68 (d, 1H, J = 14.5 Hz), 4.77 (m, 1H), 4.97 (m, 1H), 5.11–5.20 (m, 2H), 5.85 (m, 1H), 7.03 (bs, 1H), 7.31–7.67 (m, 6H), 8.02 (d, 1H, J = 8.8 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): 25.15, 40.50, 53.71, 61.48, 65.22, 114.36, 116.70, 121.32, 123.12, 124.63, 126.42, 126.82, 127.65, 132.56, 133.16, 134.23, 140.21, 153.69, 164.21, 170.01, 171.12.

3.1.20. (4-Chloro-2-trifluoromethyl-phenyl)-carbamic acid 5-methyl-2-oxo-1-[(4-oxo-pent-2-enylcarbamoyl)-methyl]-2,3-dihydro-1H-benzo[e][1,4] diazepin-3-lmethyl ester (**5**)

Compound **23** (100 mg, 0.19 mmol) was reacted with methyl vinyl ketone (133 mg, 1.9 mmol) according to the same procedure described for **2**. The title compound was obtained after purification by flash column chromatography ($CHCl_3$/MeOH 99:1). R_f = 0.33 ($CHCl_3$/MeOH 99:1). Yield 80%. ^{1}H NMR (300 MHz, $CDCl_3$): 2.25 (s, 3H, $COCH_3$), 2.52 (s, 3H, CH_3-5), 3.87 (t, 1H, J = 6.6 Hz, CH-3), 4.10 (d, 1H, J = 14.5 Hz, NCH_aCO), 4.11–4.15 (m, 2H, NCH_2CH=CH−), 4.68 (d, 1H, J = 14.5 Hz, NCH_bCO), 4.77 (m, 1H, $CH_aOCONHAr$), 4.97 (m, 1H, $CH_bOCONHAr$), 6.09 (d, 1H, J = 16.0 Hz, NCH_2CH=CH−), 6.70 (bt, 1H, NCH_2CH=CH−), 7.03 (bs, 1H, NH), 7.31–7.67 (m, 6H, CH Ar), 8.02 (d, 1H, J = 8.8 Hz, CH Ar). ^{13}C NMR (75 MHz, $CDCl_3$): 25.18 (CH_3-5), 29.96 ($COCH_3$), 40.60 (NCH_2CH=CH−), 53.21 (NCH_2CO), 61.58 (CH-3), 65.73 ($CH_2OCONHAr$), 114.60 (*C* Ar), 121.65 (*C* Ar), 123.07 (*C*H Ar), 124.62 (*C*H Ar), 126.11 (*C*H Ar), 126.42 (*C*H Ar), 127.85 (*C* Ar), 131.06 (*C* Ar), 132.30 (*C*H Ar), 133.16 (NCH_2CH=CH−), 140.10 (*C* Ar), 142.25 (NCH_2CH=CH−), 153.90 (*C*O), 164.10, (*C* Ar), 170.10 (*C*O), 171.02 (*C*O), 195.95 ($COCH_3$). Anal. Calcd for $C_{30}H_{25}F_3N_4O_5$: C, 62.28; H, 4.36; N, 9.68; found C, 62.16; H, 4.45, N, 9.43.

3.2. Pharmacology

3.2.1. Enzyme assays

The preliminary screening for FP-2 inhibition was performed with 100 μM and 30 μM inhibitor concentrations using an equivalent amount of DMSO as negative control. Product release from substrate hydrolysis (Cbz-Phe-Arg-AMC, 40 μM) was determined continuously over a period of 10 min. Compounds showing at least 50% inhibition at 30 μM were subjected to detailed assays. These were performed in a 50 mM sodium acetate buffer, pH 5.5 containing 10 mM DTT with Cbz-Phe-Arg-AMC (40 or 10 μM) as substrate [16]. Inhibitor solutions were prepared from stocks in DMSO. Each assay was performed twice in 96-well plates in a total volume of 300 μL. A Varian Cary Eclipse spectrofluorometer Varian, Darmstadt, Germany with a microplate reader (excitation 365 nm, emission 460 nm) was

used. Standard units of FP-2 were added to the reaction mixture containing the fluorogenic substrate and increasing from 0 to 100 μM concentrations of compounds. To determine first-order inactivation rate constants (k_{obs}) for the time-dependent inhibition, progress curves (fluorescence (F) vs. time) were analyzed by non-linear regression analysis using the equation $F = A(1 - \exp(-k_{obs}t)) + B$ [17]. Product formation was monitored continuously for 15 min at room temperature. Pseudo-first order rate constants k_{obs} were fit to the inhibitor concentrations using the hyperbolic equation: $k_{obs} = k_{inac}[I]/(K_I^{app} + [I])$ yielding the first-order rate constant k_{inac} and the apparent inhibition constant K_I^{app}. Inhibition constants K_I were obtained by correction to zero-substrate concentration using the Cheng–Prusoff equation $K_I = K_I^{app}/(1 + [S]/K_m)$. Second-order rate constants were obtained by: $k_{2nd} = k_{inac}/K_I$. In cases where the plots of the k_{obs} values against the inhibitor concentrations [I] were restricted to the linear range it was possible to calculate, by means of the program GraFit [20], only k_{2nd} values by using the equation $k_{2nd} \approx k_{obs}[I]^{-1}(1 + [S]K_m^{-1})$. The K_m value was determined to 21.5 μM (FP-2) [21]. Assays with cathepsins B and L were performed as described previously [22]. Cbz-Phe-Arg-AMC was used as substrate (80 μM for cathepsin B, 5 μM for cathepsin L).

3.3. Molecular modeling

The structure of the FP-2, retrieved from the Protein Data Bank (code 3BPF), was geometrically refined following the molecular modeling standard procedures: minimization, equilibration and molecular dynamics simulation [23]. Ligands were built by Sybyl 8.0 [24] and preliminarily minimized by Gaussian 03 [25] at DFT/HF/6-31g(d) level. The amino group of ligands **2**, **3** and **4** were considered in the ionized form to better simulate the physiological conditions. Docking calculations were then performed with the GOLD 4.1 program [26] into the binding cleft depicted by the presence of E-64 molecule. The cavity was detected with an active site radius of 20.0 Å from the side chain sulfur atom of residue Cys42 and, accordingly to the hypothesized mechanism of reaction [8], the formation of the Michael adducts by Cys42 sulfur atom and carbon in beta position of the ligands, were simulated. The goldscore fitness function and the distribution of torsion angles were chosen to evaluate the quality of the docking results. Van der Waals and hydrogen bonding radii were set at 4.0 and 3.0 Å, respectively; genetic algorithm parameters were kept at the default value. The complexes obtained by the ligand best docked poses within FP-2 were further geometry optimized by means of molecular mechanics method (Tripos force field) implemented in Sybyl 8.0. Figures were acquired by the PyMOL software (The PyMOL Molecular Graphics System, Version 0.99).

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