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Novel β-lactam derivatives: Potent and selective inhibitors of the chymotrypsin-like activity of the human 20S proteasome

Patricia Imbach,* Marc Lang, Carlos García-Echeverría, Vito Guagnano, Maria Noorani, Johannes Roesel, Francis Bitsch, Grety Rihs and Pascal Furet*

Novartis Institutes for BioMedical Research, WKL-136.4.25, CH-4002 Basel, Switzerland

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Abstract—A series of β-lactam derivatives has been designed and synthesized to inhibit the chymotrypsin-like activity of the human 20S proteasome. The most potent compounds of this new structural class of β-subunit selective 20S proteasome inhibitors exhibit IC_{50} values in the low-nanomolar range and show good selectivity over the trypsin-like and post-glutamyl-peptide hydrolytic activities of the enzyme.

The proteasome is a multicatalytic protease complex involved in the ubiquitin (Ub)-dependent degradation of proteins that are components of critical intracellular regulatory cascades (e.g., mitotic cycle, cell growth and viability, antigen presentation or inflammatory response).[1] The proteolytic activity of this N-terminal threonine hydrolase occurs in a 700-kDa cylindrical-shaped core structure known as the 20S proteasome. This catalytic core consists of four stacked rings arrayed in an $\alpha_7\beta_7\beta_7\alpha_7$ manner, and exhibits at least three distinct peptidase activities: the chymotrypsin-like, trypsin-like, and post-glutamyl-peptide hydrolytic activities.[2] Inhibitors of the 20S proteosome have been explored for use as anti-inflammatory agents and for the treatment of cancer and autoimmune diseases. Our specific target in the search for novel cytotoxic and antiproliferative agents is the chymotrypsin-like activity of the 20S proteasome. Modulation of this enzymatic activity by β-subunit-specific inhibitors may convey an antitumor effect through induction of cell cycle arrest and apoptosis in tumor cells.[3,4] Parallel to our efforts to develop noncovalent inhibitors of the 20S proteasome,[5–8] we have also explored the possibility of designing inhibitors able to form an adduct with the hydroxyl group of the catalytic threonine residue. We report herein, the design, synthesis, and biological evaluation of a new class of 20S proteasome inhibitors that bear a C-terminal substituted β-lactam as a reactive group. These compounds exploit key interactions identified during the optimization of our noncovalent inhibitors, and exhibit high selectivity for the chymotrypsin-like activity of the 20S proteasome.

The first compound in this new series (compound **8**) was prepared as outlined in Scheme 1. A key step in our initial synthetic strategy was the formation of the β-lactam. This was accomplished by reaction of the dianion-enolate of the Cbz-protected α-amino acid ester **1** with the secondary cyanomethylamine (compound **2**).[9,10] The latter serves as the precursor of an in situ generated imine. The resolution of the racemic, protected β-lactam (compound **3**) was performed by HPLC using a semi-preparative chiral column.[11] A linear sequence of standard coupling and de-protecting reactions led to precursor **7**. The target compound **8** was obtained by oxidative de-protection of compound **7** using potassium peroxodisulfate in buffered media.[12] The absolute stereochemistry of the β-lactam ring in compound **8** and derivatives thereof (Table 1) was assigned from the X-ray structure of the synthetic precursor **7** (Fig. 1).[13] The introduction of methoxy groups in the central phenyl ring of compound **8**, motivated by their beneficial effect at this position in our previous series of noncovalent inhibitors, required to modify our initial synthetic strategy. The oxidative de-protection of the β-lactam

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* Corresponding authors. Tel.: +41 61 696 62 56; fax: +41 61 696 62 46 (P.I). Tel.: +41 61 696 79 90; fax: +41 61 696 15 67 (P.F); e-mail addresses: patricia.imbach@novartis.com; pascal.furet@novartis.com

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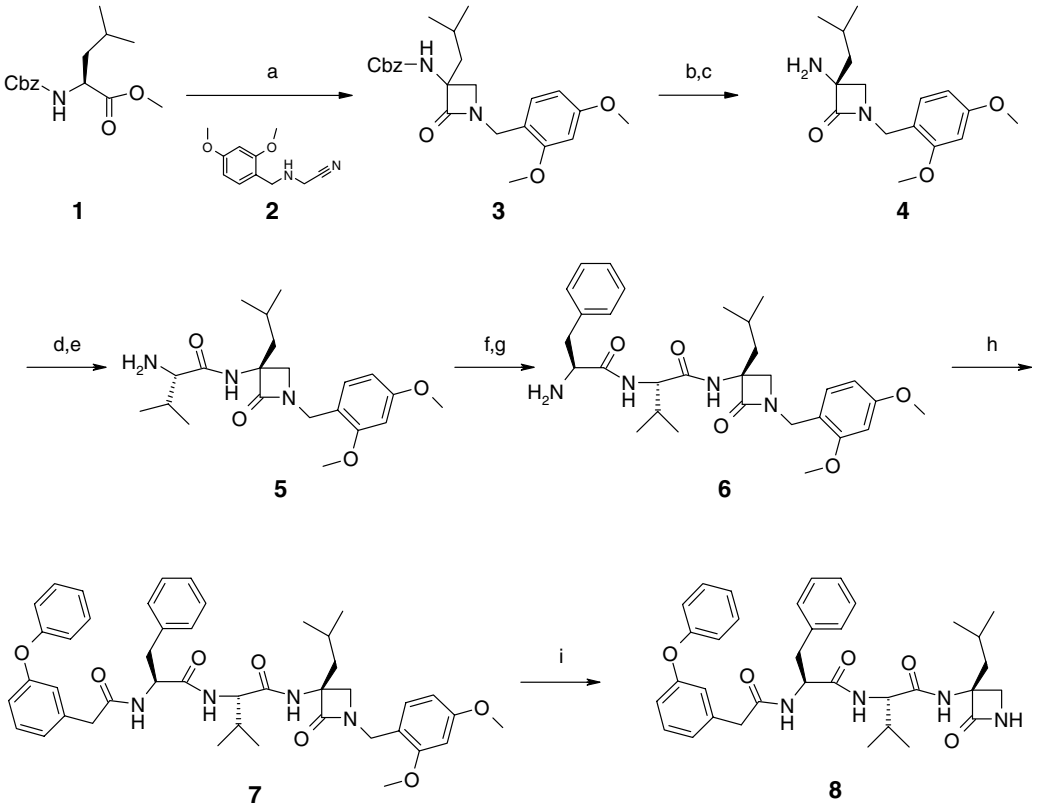


Scheme 1. Synthesis of compound **8**. Reagents and conditions: (a) LHMDS, −70 °C → rt, CH_3CN; (b) HPLC separation, CHIRALCEL OD1118; (c) H_2, Pd/C 10% in CH_3OH, rt; (d) N^{α}-Cbz-L-Val-OH, TPTU, DIEA, DMF, rt; (e) Pd/C 10% in CH_3OH, rt; (f) N^{α}-Cbz-L-Phe-OH, TPTU, DIEA, DMF, rt; (g) Pd/C 10% in CH_3OH, rt; (h) (3-phenoxyphenyl)-acetic acid, TPTU, DIEA, DMF, rt; (i) $K_2S_2O_8/Na_2HPO_4$ in CH_3CN/H_2O.

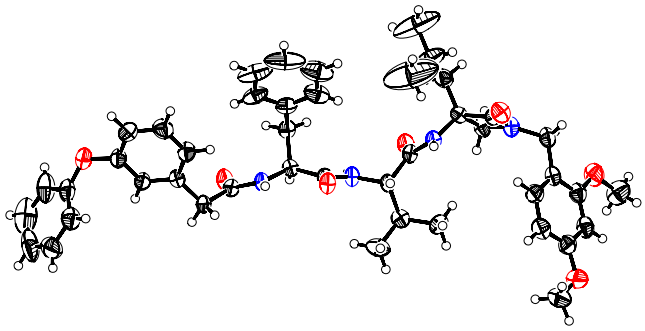


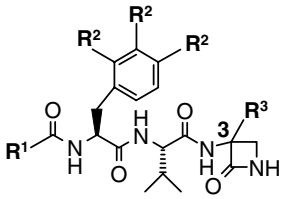
Figure 1. X-ray crystal structure of compound **7** (acetone solvate). ORTEP plot with displacement ellipsoids represented at the 30% probability level. The configuration of the chiral center in the β-lactam ring is *R*.

nitrogen at the last step in Scheme 1 was not tolerated when other methoxy groups were present in the molecule, and it had to be performed earlier in the synthetic sequence. In addition, and to facilitate the scale-up of enantiomerically pure compounds, we decided to avoid the tedious chromatographic resolution of the racemic β-lactam. Both requirements were fulfilled by following the synthetic protocol outlined in Scheme 2. In particular, the resolution of compound **3a** was straightforwardly accomplished by crystallization with (+)-mandelic acid.[14] Compounds **9**–**17** (Table 1) were prepared from compound **4a** by using the conditions described in Scheme 1.

The ability of compounds **8**–**17** (Table 1) to inhibit the proteolytic activities of the human 20S proteasome was determined in vitro using fluorogenic peptides as substrates.[15] The rates of hydrolysis were monitored by the fluorescence increase and the initial linear portions of the curves were used to calculate the IC_{50} values (Tables 1 and 2). Covalent interaction of compound **8** with the 20S proteasome protein was assessed by mass spectrometry.[16] The antiproliferative activity of compounds **8**, **10**, and **14** (Table 2) was determined using the human breast carcinoma cell line MDA-MB-435.[17]

A substantial number of 20S proteasome inhibitors described today exert their biological activity by forming a covalent bond with the side chain of the catalytic N-terminal threonine residue. These covalent, active site-directed, inhibitors belong to the following classes of compounds: epoxyketones, boronic acids, α-ketoamides, α-ketoaldehydes, β-lactones, and vinyl sulfones.[3a,b] As an alternative to the preceding reactive groups, we decided to explore the possibility of using a chiral β-lactam group to target the catalytic residue of the 20S proteasome. β-Lactams have been successfully used as war head groups in the design of covalent inhibitors of serine proteases.[18] They exert their inhibitory action on this class of enzymes by acylating the nucleophilic catalytic serine residue. We reasoned that a similar mechanism of action could be exploited to inhibit the 20S proteasome.[19] To explore this new strategy, we decided to use the molecular scaffold of a class

Table 1. Inhibition of the chymotrypsin-like activity of the 20S proteasome by β-lactam derivatives

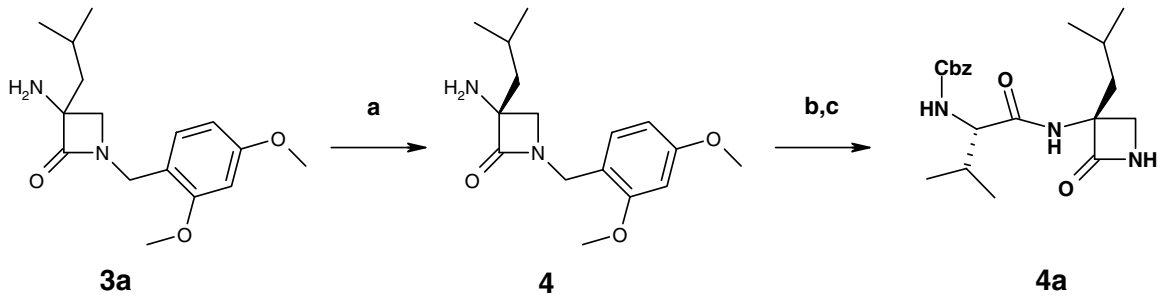


Compound	R^1	R^2	R^3	*R*/*S*[a]	IC_{50} (μM)
8	CH_2Ph-(3)–OPh	H	Isobutyl	*R*	0.020
9	CH_2Ph-(3)–OPh	H	Isobutyl	*S*	38
10	CH_2Ph-(3)–OPh	OCH_3	Isobutyl	*R*	0.0025
11	CH_2Ph-(3)-OPh	OCH_3	Isobutyl	*S*	1.3
12	O–CH_2Ph	OCH_3	Benzyl	*R*	0.0050
13	O–CH_2Ph	OCH_3	Benzyl	*S*	1.4
14	CH_2Ph-(3)–OPh	OCH_3	Benzyl	*R*	0.0014
15	CH_2Ph-(3)–OPh	OCH_3	Benzyl	*S*	0.17
16[b]	CH_2Ph-(3)–OPh	OCH_3	Benzyl	*R*	0.0040
17[b]	CH_2Ph-(3)–OPh	OCH_3	Benzyl	*S*	0.28

The IC_{50} value is the concentration of inhibitor at which the rate of the 20S proteasome catalyzed hydrolysis of the substrate Suc-Leu-Leu-Val-Tyr-AMC (chymotrypsin-like activity) is reduced by 50%.

[a] Absolute configuration at C(3).

[b] Compounds **16** and **17** contain a *tert*-leucine instead of a valine residue N-terminal to the β-lactam group.



Scheme 2. Reagents and conditions: (a) crystallization with (+)-mandelic acid; (b) N^{α}-Cbz-L-Val-OH, TPTU, DIEA, DMF, rt; (c) $K_2S_2O_8/Na_2HPO_4$ in CH_3CN/H_2O.

Table 2. 20S proteasome chymotrypsin-like (ChyL), post-glutamyl-peptide hydrolytic (PGPH), and trypsin-like (TryL) inhibitory activities as well as antiproliferative activity of some β-lactam derivatives

Compound	ChyL IC_{50} (μM)	PGPH IC_{50} (μM)	TryL IC_{50} (μM)	MDA-MB435 IC_{50} (μM)
8	0.020	>20	n.d.	1.2
10	0.0025	>100	1.1	0.034
14	0.0014	>20	19	0.032

of potent noncovalent inhibitors of the chymotrypsin-like proteolytic activity of the proteasome previously described by us.[8] Thus, we undertook molecular modeling studies[20] aimed at designing the most promising β-lactam analogues of these new 20S proteasome inhibitors. On the basis of our binding model hypotheses,[8] we determined that replacing the C-terminal benzylic group of our noncovalent inhibitors by a chiral β-lactam moiety (Fig. 2) would provide potent and covalent 20S proteasome inhibitors. The biological activity obtained with representative examples of compounds containing a C-terminal 3-substituted (isobutyl or benzyl) (*R*)-3-amino-azetidine-2-one moiety is shown in Table 1. According to our modeling studies (Fig. 3), it was predicted that the *R* configuration at position 3 of the β-lactam ring was necessary to properly orient the bulky substituent in the S1 pocket. This key structural requirement is exemplified by the 70- to 2000-fold higher inhibitory activity observed for the *R*-epimer (Table 1). As observed with our noncovalent inhibitors introduction of methoxy groups in the central phenyl ring to form hydrogen bonds with residues of the S3 pocket improves potency (compound **8** vs **10**).[21] However, less sensitivity of the inhibitory activity to the optimal filling of the S1, AS1, and AS2 pockets is observed with the covalent inhibitors. The isobutyl and benzyl groups in the S1 pocket give compounds of equal potency (compound **10** vs **14**) while compound **12** whose N-terminal group can occupy only one of the two accessory pockets AS1 and AS2 is not dramatically less active than **14** that occupies both. This comes in support of the covalent nature of the inhibitory activity of these compounds. Covalent inhibitors are less dependent on the creation of optimal intermolecular interactions to achieve potency.

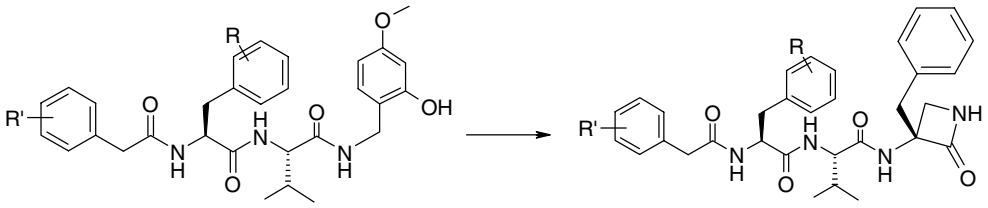


Figure 2. Schematic representation of the transformation of a noncovalent inhibitor into a covalent 20S proteasome inhibitor.

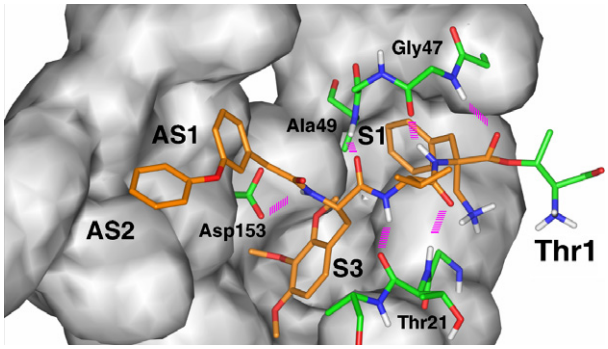


Figure 3. Model of compound **14** bound to proteasome X/HC5 subunits after acylation of the catalytic threonine (Thr1). In particular, the designed β-lactam analogues can form the same key hydrogen bond interactions (with residues Thr21, Gly 47, Ala 19, and Asp 153) as their parent noncovalent inhibitors.

Confirmation of a covalent interaction with the enzyme was obtained by mass spectrometry. The β5 subunit, which is responsible for the chymotrypsin-like activity of the 20S proteasome, was shown by LC–MS analyses to be covalently bound by one molecule of compound **8**.[22]

Compounds containing the C-terminal chiral β-lactam group are not only potent inhibitors of the chymotrypsin-like activity of the 20S proteasome but are also very selective for this catalytic site. As shown in Table 2, compounds **8**, **10**, and **14** showed at least 350-fold selectivity over the trypsin-like and post-glutamyl-peptide hydrolytic activities of the proteasome.[23] In addition, these compounds displayed a pronounced antiproliferative effect as illustrated with the inhibitory activity values obtained against the human breast carcinoma cell line MDA-MB-435 (e.g., $IC_{50} = 32$ nM for compound **14**).

In summary, the data reported in this letter demonstrate that a C-terminal chiral β-lactam group can effectively serve as the basis for designing potent and selective covalent inhibitors of the chymotrypsin-like activity of the 20S proteasome. These novel inhibitors open a new avenue for further investigation of the proteasome as a therapeutic target in oncology drug discovery.

Acknowledgment

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11. To obtain optically pure material, the racemic product **3** was separated by HPLC using a chiral column. Single peak at $t_R = 7.41$ min (CHIRALCEL OD (1118); 250 × 4.6 mm; eluting with hexane/ethanol, 90/10; flow 1 mL/min; $\lambda = 210$ nm); $[\alpha]_D = +20.4°$ ($c = 0.525$; DMSO).
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13. Suitable crystals were obtained from an acetone solution by slow evaporation of the solvent. An Enraf-Nonius CAD4 automatic diffractometer was used for data collection with CuKα radiation and a graphite monochromator. The structure was solved by direct methods (SHELXS). The parameters were refined by full-matrix least-squares calculations (SHELXL) with anisotropic displacement parameters for all non-H atoms. A subsequent difference Fourier map showed 38 of 58 hydrogen atoms. The positions of the remaining ones were calculated assuming normal geometry. Hydrogen atom parameters were idealized and not refined. Crystallographic data (excluding

structure factors) for the structure have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 620075. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [Fax: +44 (0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk].

14. Compound **3a** was crystallized with L-(+) mandelic acid (388 mg; 2.55 mM) from methanol (4 mL). After addition of diethyl ether, the crystalline product is filtered off. The crude product is taken up into ethyl acetate/saturated. $NaHCO_3$ soln and the organic layer is washed with water, brine, and dried over Na_2SO_4. After filtration, the solvent is removed by evaporation under reduced pressure to obtain 515 mg of enantiomerically pure (*S*)-2-[(*S*)-2-amino-3-(2,3,4-trimethoxy-phenyl)-propio-nylamino]-*N*-((*R*)-3-isobutyl-2-oxo-azetidin-3-yl)-3-methyl-butyramide **4**. ES-MS: 293.1 $[M+H]^+$; single peak at $t_R = 2.99$ min (System 1); $R_f = 0.56$ (CH_2Cl_2/CH_3OH 9/1); $[\alpha]_D = -22.8\,^\circ$ ($c = 1.015$; methanol).
15. The 20S proteasome was obtained in-house from human blood. Fluorogenic peptides used: Suc-Leu-Leu-Val-Tyr-AMC (substrate for chymotrypsin-like assay), Boc-Leu-Arg-Arg-AMC (substrate for trypsin-like assay), and *Z*-Leu-Leu-Glu-AMC (substrate for post-glutamyl-peptide hydrolytic-like assay). Fluorescence excitation/emission wavelengths were 355 nm/460 nm for 7-amido-4-methyl-coumarin (AMC).
16. A Q-Tof (Micromass, Manchester, UK) quadrupole time-of-flight hybrid tandem mass spectrometer equipped with a Micromass Z-type electrospray ionization source was used.
17. Inhibition of cell growth by test compounds was assessed by using a MTT-based proliferation assay. The human breast carcinoma cell line MDA-MB-435 was cultivated in MEM, supplemented with 10% FCS, 100 U/mL penicillin/streptavidin, 4 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids (1×), and 20 mM Hepes. After 24, 48 or 72 h of compound incubation, the rate of cell proliferation was assessed with the CellTiter96™ essay. The plates were read in a microplate reader (Dynatech MR5000) at 550 versus 630 nm.
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21. The most potent noncovalent inhibitors disclosed in Ref. 8 contain a 3,4,5-trimethoxy-phenyl moiety to fill the S3 pocket, whereas the corresponding group in β-lactam analogues described here is a 2,3,4-trimethoxy-phenyl moiety. The latter gives inhibitors of similar potency as the former in the noncovalent series (manuscript in preparation).
22. The 20S proteasome preparation was obtained from Dr. J. Zimmermann (Novartis Pharma Research, Oncology). The material was dissolved in a Tris buffer, pH 8.0, at a concentration of approximately 1 mg/mL for each subunit, 10 μL of 10 mM Tris–HCl, pH 8.0, buffer containing 10 mM $CaCl_2$ (Buffer A) was added to 10 μL of the proteasome solution in Tris–HCl buffer solution. Two microliters of 100 μM solution of compound **8** in Tris–HCl buffer was then added and the reaction mixture was shaken at 37 °C for 1 h using an Eppendorf thermomixer (700 min^{-1}). Final concentration of compound **8** was approximately 10 μM. A blank reaction (10 μL proteasome + 10 μL Buffer A + 2 μL DMSO) was run under the same conditions. Mass spectrometry was carried out using a Q-Tof (Micromass, Manchester, UK) quadrupole time-of-flight hybrid tandem mass spectrometer equipped with a Micromass Z-type electrospray ionization source (ESI).
23. These compounds also show high selectivity against important serine and cysteine proteases such as chymotrypsin, thrombin, and calpain. No inhibition of these enzymes was detected at a concentration as high as 25 μM.