Linear TMC-95-Based Proteasome Inhibitors

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We have designed and evaluated 45 linear analogues of the natural constrained cyclopeptide TMC-95A. These synthetically less demanding molecules are based on the tripeptide sequence Y-N-W of TMC-95A. Structural variations in the amino acid side chains and termini greatly influenced both the efficiency and selectivity of action on a given type of active site. Inhibition constants were submicromolar ($K_i \approx 300$ nM) despite the absence of the entropically favorable constrained conformation that is characteristic of TMC-95A and its cyclic analogues. These linear compounds were readily prepared and reasonably stable in culture medium and could be optimized to inhibit one, two, or all three proteasome catalytic sites. Cytotoxicity assays performed on a series of human tumor cell lines identified the most potent inhibitors in cells.

Introduction

The ubiquitin−proteasome pathway is central to intracellular protein homeostasis.[1] It is involved in processes like cell cycle regulation and cytokine-stimulated signal transduction.[2] The proteasome is a promising target for the treatment of diseases such as inflammation, immune diseases, and cancer.[3] The FDA has approved the proteasome inhibitor bortezomib (Figure 1) for anticancer therapy.[4] The eukaryotic 26S proteasome is composed of the catalytically competent 20S particle, which interacts with the regulatory 19S regulatory caps. The eukaryotic 20S proteasome is formed of four stacked $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ multiprotein rings and has two copies of each of three distinct catalytic subunits. One is responsible for chymotrypsin-like activity (CT-L, attributed to β5), the second for trypsin-like activity (T-L, β2), and the third for post-glutamyl peptide hydrolysis or post-acid activity (PGPH or PA, β1). Most proteasome inhibitors are short peptides bearing a reactive group such as aldehyde (MG132, calpain I inhibitor Ac-Leu-Leu-Nle-H, tyropeptin A, Figure 1), boronic acid (bortezomib, Figure 1), or vinyl sulfone that forms a covalent bond with the catalytic O^{γ}-Thr1 in the three catalytic sites.[5,6] Some natural molecules (epoxomycin, lactacystin, homobelactosin, salinosporamide) and their synthetic derivatives (omuralide) also form covalent adducts.[7]

Noncovalent inhibitors have been investigated less extensively, although they should have weaker side effects in therapeutic applications. A few synthetic products (ritonavir,[8] several benzylstatine derivatives[9]) and one natural product (TMC-95A,[10] Figure 1) inhibit the proteasome noncovalently. TMC-95A is a constrained cyclic tripeptide containing original N- and C-terminal functions, a tyrosine−asparagine−highly oxidized tryptophan sequence, and a side chain bridge through a phenylhydroxyoxindole (biaryl) linkage. It inhibits all three proteasome activities (K_i of 0.0054 μM for CT-L, 0.060 for PA, and 0.2 for T-L) and has antitumor activity.[11] Its binding to proteasome active sites has been characterized by X-ray crystallography.[12] The noncovalent interactions are mainly mediated by hydrogen bonds and lead to the formation of an antiparallel β sheet between the inhibitor and amino acid residues of the binding pockets with an additional hydrogen bond involving the oxygen of the oxindole group. The C-terminal (Z)-propenyl moiety acts as the P1 and the asparagine as the P3 residues. While the tyrosine residue interacts weakly with the hydrophobic S4 subsite, the hydroxyl groups of the oxidized tryptophan and the N-terminal 3-methyl-2-oxopen-

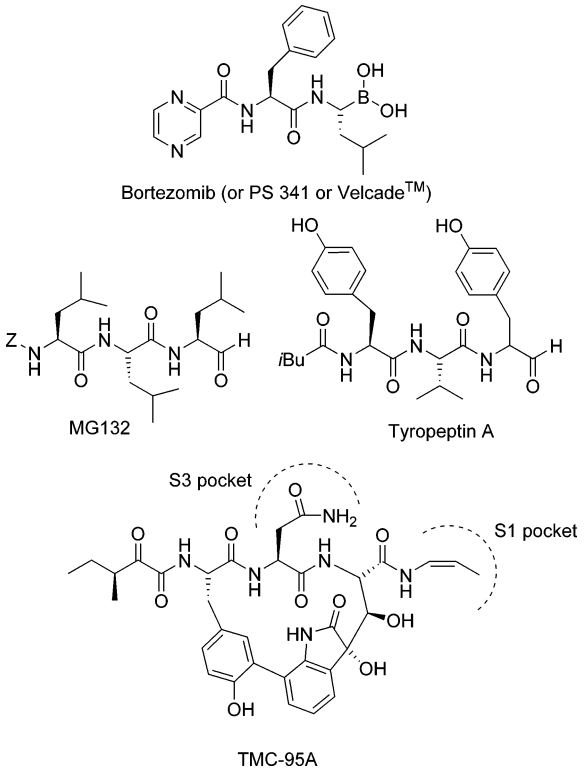

Figure 1. Some proteasome inhibitors. Binding of TMC-95A to proteasome S1 and S3 subsites is shown.[12]

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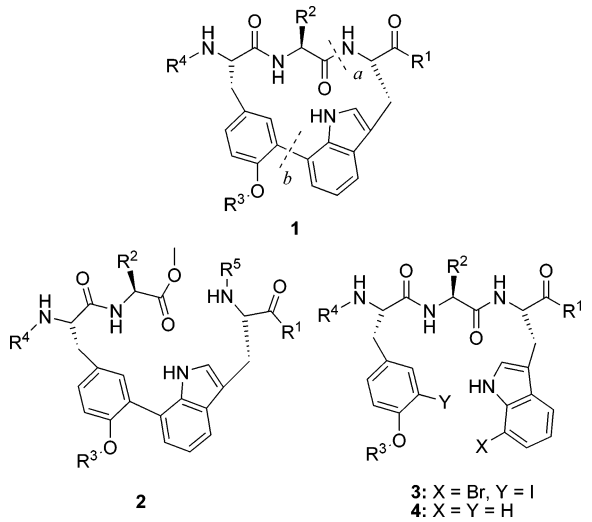
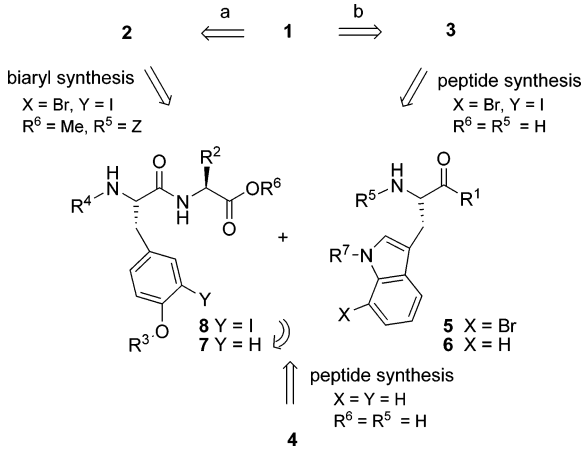


Figure 2. Structures of cyclic (**1**) and linear (**2**−**4**) compounds. The dotted lines a and b indicate the bonds chosen to be disrupted in order to generate (a) linear analogues **2** having a phenylindole group and (b) linear analogues **3** and **4** in which the tripeptide chain is retained.

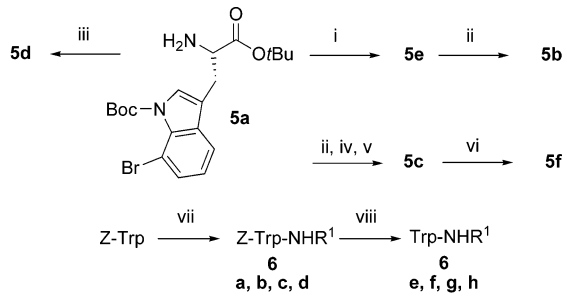
tanoyl group are exposed to the surface (Figure 1). The high-affinity binding of TMC-95A is mostly attributed to its constrained conformation mediated by the biaryl system. Total syntheses of this original 17-membered macrocycle have been achieved.[13−15] Although some cyclic synthesis precursors had very interesting enzymatic properties, the complexity of their preparation still hinders their accessibility.[16] Simpler cyclic rigid analogues of TMC-95A bearing a phenyloxindole linkage[17,18] or a biphenyl ether linkage[19] have also been described. Although their constrained conformation provides the driving force for entropically high-affinity binding, these derivatives are less strongly bound than TMC-95A.[20] We postulated that the structural elements of the natural product that are required for proteasome inhibition could be identified and used to design linear TMC-95A mimics that are less difficult to prepare than TMC-95A itself or its cyclic mimics. We have produced a library of linear mimics to selectively target one or more active sites.

We previously reported the synthesis of macrocycles **1** in which the peptide structure of TMC-95 was retained and the highly oxidized tryptophan was replaced by tryptophan (Figure 2).[21] Their poor inhibition of rabbit 20S proteasome reported here prompted us to design the linear mimics of TMC-95A **2**−**4**. The oxidized tryptophan of TMC-95A was replaced by tryptophan in all these molecules; they also lacked the synthetically challenging hydroxy groups of tryptophan, 3-methyl-2-oxopentanoyl group at the N-terminus, and (Z)-prop-1-enyl moiety at the C-terminus (Figure 2). The objective was to determine how the catalytic activities of the 20S proteasome were influenced by elements derived from TMC-95A. Consequently, compounds **2** contain the phenylindole group but not the tripeptide sequence; compounds **3** and **4** contain the tripeptide sequence but not the phenylindole group. The tryptophan and tyrosine residues were kept in all cases, and the substituents R^1, R^2, R^3, and R^4 and X and Y groups were varied. Compounds **3** contained nonproteogenic halogenated Trp and Tyr residues (X = Br, Y = I), while compounds **4** did not. This report describes the syntheses of three series of acyclic mimics of TMC-95A (compounds **2**−**4**), their activities and selectivities for 20S proteasome active sites, as well as that of cyclic mimics **1**, and their metabolic stability. The cytotoxic activities of the four most active inhibitors among the 45 newly synthesized compounds were assayed on several human tumor cell lines to evaluate their potentials as cytotoxic tumor agents.

Scheme 1. Convergent Retrosynthetic Pathway for Compounds **1**−**4**



Scheme 2. Syntheses of Tryptophan Derivatives **5** and **6**[a]



	R^1	R^5	R^7		R^1	R^5	R^7
5a	O*t*Bu	H	Boc	**6a**	$NHCH_2Ph$	Z	H
5b	OEt	Z	H	**6b**	NHPh	Z	H
5c	NHMe	Z	H	**6c**	NH-(4-OH)-C_6H_4	Z	H
5d	OMe	H, HCl	H	**6d**	$NH(CH_2)_4NHBoc$	Z	H
5e	OEt	H, HCl	H	**6e**	$NHCH_2Ph$	H	H
5f	NHMe	H, HBr	H	**6f**	NHPh	H	H
				6g	NH-(4-OH)-C_6H_4	H	H
				6h	$NH(CH_2)_4NHBoc$	H	H

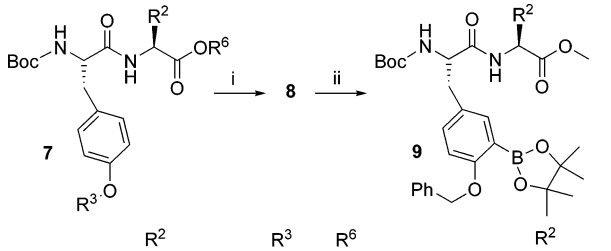
[a] Reagents: (i) HCl, EtOH, 100%; (ii) ZOSu, DMF, 70% (for **5b**); (iii) HCl, MeOH, 100%; (iv) HCl, AcOEt; (v) $ClCO_2Et$, Et_3N, THF, then $MeNH_2$, THF, 81% from **5a**; (vi) HBr, AcOH, 100%; (vii) DCC, R^1-NH_2, 31% (for **6a**), 69% (for **6b**), 77% (for **6c**), 82% (for **6d**); (viii) H_2, Pd/C, MeOH, DMF, 83% (for **6e**), 73% (for **6f**), 59% (for **6g**), 82% (for **6h**).

Results and Discussion

Chemistry. A convergent retrosynthetic scheme was used to prepare compounds **1**−**4** (Scheme 1). Two major disconnections were evident for macrocycles **1**: the first (path a) led to biaryl compounds **2**, while the second (path b) led to tripeptides **3**.[21] These synthons **2** and **3** required the same starting materials **5** and **8**, thereby making it possible to generate a library of analogues by varying R^1, R^2, R^3, or R^4. Tripeptides **4** were assembled in a similar convergent way from tryptophan derivatives **6** and dipeptides **7**, which were precursors of the iodized dipeptides **8**.

Derivative **5a** was the key compound for preparing unusual 7-bromotryptophans **5**, and gram quantities were synthesized as described previously,[21] with a very high ee and in six steps from 1-bromo-2-nitrobenzene (Scheme 2). The functional groups of **5a** were transformed using general methods of peptide synthesis.[22] The Boc protection in **5a** was removed with anhydrous HCl in alcohol (ethanol or methanol), and thus, transesterification led to amino esters **5d** or **5e** (R^1 = OEt or

Scheme 3. Syntheses of Dipeptides **8** and **9**[a]



	R^2	R^3	R^6
7a, 8a	$CH_2CH(CH_3)_2$	CH_2Ph	Me
7b, 8b	$CH_2CH(CH_3)_2$	Me	Me
7c, 8c	Me	CH_2Ph	Me
7d, 8d	Me	Me	Me
7e, 8e	CH_2CONH_2	CH_2Ph	Me
7f	$(CH_2)_4NHBoc$	CH_2Ph	H
7g	$(CH_2)_3NZC(NH)NHZ$	CH_2Ph	H

	R^2
9a	$CH_2CH(CH_3)_2$
9b	Me

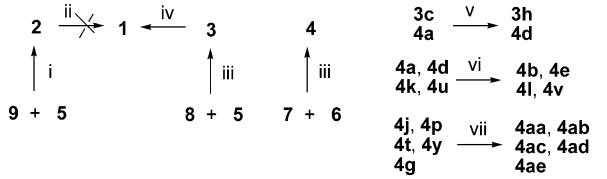
[a] Reagents and conditions: (i) I_2, Ag_2SO_4, EtOH, 72% (for **8a**), 85% (for **8b**), 85% (for **8c**), 72% (for **8d**), 69% (for **8e**); (ii) bis(pinacolato)diboron, $PdCl_2dppf{\cdot}CH_2Cl_2$, KOAc, DMSO, 80 °C, 16 h, unseparated mixture of **9** and **7** (4.2/1 ratio for **9a**/**7a**, 7.8/1 ratio for **9b**/**7c**). See Supporting Information for the preparation of **7**.

OMe) as their hydrochloride salts. **5e** or **5a** was Z-protected using *N*-(benzyloxycarbonyloxy)succinimide. In the latter case, this protection was necessary for the introduction of the R^1 methylamide of **5c**. Acidolysis of the Boc and ^{t}Bu group in AcOEt and coupling with methylamine by the mixed anhydride method led to the preparation of **5c** in 81% overall yield. The Z group in **5c** was removed to give **5f** under acid conditions (HBr/AcOH) because hydrogenation in the presence of palladium on charcoal also cleaved the carbon−bromine bond. The R^1 amide function in tryptophan derivatives **6a**−**d** was introduced by standard peptide coupling between Z-tryptophan and the corresponding amine in the presence of dicyclohexylcarbodiimide. Hydrogenolysis of the Z group in **6a**−**d** gave derivatives **6e**−**h** (Scheme 2).

Dipeptides **7** were prepared using conventional liquid-phase peptide synthesis[22] from the succinimidyl active ester of Boc-tyrosine (Scheme 3). Dipeptides **7** were converted to iododipeptides **8** with I_2/Ag_2SO_4[23] in good yields. Standard Miyaura conditions[24] transformed **8a** and **8c** to a mixture of the corresponding pinacol boronates **9a** and **9b** along with **7a** and **7c** derived from a protodeboronation reaction.[25] Yields were estimated to be 57% for **9a** and 62% for **9b** by NMR, since **9** and **7** could not be separated by column chromatography (Scheme 3).

The last step in the syntheses was the combination of tryptophan building blocks **5** or **6** with dipeptide building blocks **7**, **8**, or **9** to generate a library of target compounds **1**−**4** (Scheme 4). Because of its mild reaction conditions, the Suzuki coupling[26] of forming aryl−aryl bonds[27] was used to build the biaryl linkage in **2** from boronate **9** and 7-bromotryptophan **5**. The reaction parameters (source of palladium, solvent, and base) were varied to identify optimal conditions,[28] and the desired biaryl products were obtained in yields of 94% (**2a**), 72% (**2b**), and 60% (**2c**) from 7-bromotryptophan derivatives **5b** or **5c.** The macrolactamization of properly deprotected biaryl products **2** was then investigated as a route to the macrocycles **1**. The methyl ester in **2** was saponified with LiOH in THF, and the Z protective group was cleaved along with the Bn group using H_2/Pd. The resulting crude mixture was subjected to various common peptide-coupling conditions (EDC/HOAT in CH_2Cl_2 or DMF, HATU in DMF, PyBOP/HOBt), but no cyclic product **1** was detected. Only trace amounts of the corresponding cyclic dimer were isolated (HRMS (ESI) measurement: calcd for

Scheme 4. Syntheses of Target Compounds **1**−**4**[a]



[a] See Table 1 for definitions of R^1, R^2, R^3, and R^4 groups in **1**−**4**. Reagents and conditions: (i) 20% mol $Pd(OAc)_2$, 10% mol P(*o*-tolyl)$_3$, Na_2CO_3/water, dioxane, 80 °C, 5 h, 94% (for **2a**), 72% (for **2b**), 60% (for **2c**); (ii) LiOH, THF, water, then H_2, Pd/C, MeOH, then EDC, HOAt, CH_2Cl_2 or HATU, DMF or PyBop, HOBt; (iii) **8a**−**e** or **7a**−**e**, LiOH, THF, water, then aqueous HCl, then EDC, HOBt, NEt_3, CH_2Cl_2, DMF, **5b**−**f** or **6e**−**h**, 81% (for **3a**), 71% (for **3b**), 70% (for **3c**), 88% (for **3d**), 47% (for **3e**), 67% (for **3f**), 54% (for **3g**), 43% (for **3i**), 56% (for **4a**), 63% (for **4f**), 45% (for **4g**), 48% (for **4h**), 66% (for **4i**), 67% (for **4j**), 49% (for **4k**), 31% (for **4** m), 30% (for **4n**), 75% (for **4o**), 63% (for **4p**), 42% (for **4q**), 44% (for **4r**), 78% (for **4s**), 68% (for **4t**), 20% (for **4u**), 31% (for **4w**), 28% (for **4x**), 33% (for **4y**), 65% (for **4z**); (iv) $NiCl_2(PPh_3)_2$, Zn, PPh_3, DMF, 4% (for **1a**), 8% (for **1b**), 10% (for **1c**); (v) LiOH, THF, water, then HCl, water, 82% (for **3h**), 80% (for **4c**), 64% (for **4d**); (vi) H_2, Pd/C, DMF, MeOH, 76% (for **4b**), 76% (for **4e**), 81% (for **4l**), 86% (for **4v**); (vii) anhydrous HCl/MeOH, 24% (for **4aa**), 38% (for **4ab**), 86% (for **4ac**), 26% (for **4ad**), 64% (for **4ae**).

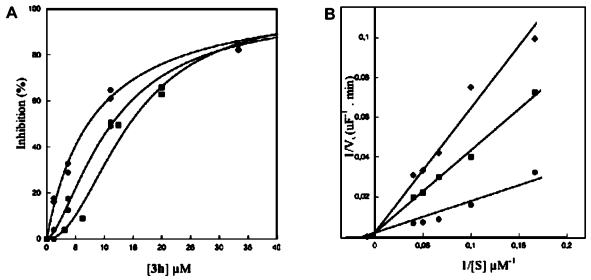


Figure 3. (A) Inhibition of CT-L (◆), PA (●), and T-L (■) activities of rabbit 20S proteasome by compound **3h**. The experimental data were fitted to equation % inhibition = $100[\mathbf{3h}]/(IC_{50} + [\mathbf{3h}]$ (◆) or % inhibition = $100[\mathbf{3h}]^{n_H}/(IC_{50}^{n_H} + [\mathbf{3h}]^{n_H}$ with $n_H = 1.5$ (●) and 2 (■). (B) Double reciprocal Lineweaver−Burk plot for the inhibition of CT-L activity by **3h** with [**3h**] = 0 (●), 10 (■), 20 (◆) μM. Experimental points were fitted to the equation $1/V_i = (1 + [\mathbf{3h}]/K_i)(K_m/V_m[S]) + 1/V_m$.

$[C_{58}H_{70}N_{10}O_{12} + Na]^+$ 1121.5072, found 1121.5065). Similar results were reported by Kaiser et al.[29]

Cyclization of the halogenated tripeptides **3** via formation of the aryl−aryl bond proved to be the most effective route to **1**, although yields were low (Scheme 4).[21] Quantitative saponification of esters **8** followed by coupling with 7-bromotryptophan derivatives **5** in the presence of EDC/HOBt gave tripeptides **3** in moderate to good yields.[21] Compounds **3a**−**c** were macrocyclized by Ni(0) mediated coupling of the aryl halides to form the biaryl linkage[30] of the corresponding constrained 17-membered macrocycles **1** in 4%, 8%, and 10% yields.[21]

Last, tripeptides **4** were readily obtained after saponification of dipeptides **7** and coupling with tryptophan derivatives **6** under low epimerizing conditions (EDC/HOBt). Tripeptides **3c** or **4** were deprotected under the usual conditions. Tripeptides **3c** or **4a** were saponified to give the corresponding acids **3h** or **4d**. The benzyl or Z groups were removed from **4a**, **4d**, **4k**, or **4u** by hydrogenolysis (H_2, Pd/C), and the Boc group in **4j**, **4p**, **4t,** **4y**, and **4g** was cleaved using anhydrous HCl.

Biology. The capacities of cyclic **1** and acyclic **2**−**4** analogues of TMC-95A to inhibit the three activities of rabbit 20S proteasome were assayed using appropriate fluorogenic substrates (Figure 3 and Table 1). The classical aldehyde proteasome inhibitor MG-132 (Z-Leu-Leu-Leu-H) (Figure 1) was used as standard. Cyclic analogues **1** that use the phenylindole as an

Table 1. Structures of Compounds **1**−**4** and Their Inhibition (or Activation) of Rabbit 20S Proteasome at pH 7.5 and 30 °C[a]

					K_i (μM) or activation factor x		
compd	R^1	R^2	R^3	R^4	CT-L	PA	T−L
1a	OMe	^{i}Bu	Bn	Boc	ni	>100	$x = 1.4$
1b	OMe	^{i}Bu	Me	Boc	ni	>100	$x = 1.8$
1c	OMe	Me	Me	Boc	ni	37.1	ni
2a	OEt	^{i}Bu	Bn	Boc	ni	ni	$x = 1.4$
2b	NHMe	^{i}Bu	Bn	Boc	ni	ni	69
2c	NHMe	Me	Bn	Boc	ni	>100	21.5
3a	OMe	^{i}Bu	Bn	Boc	ni	ni	$x = 2$
3b	OMe	^{i}Bu	Me	Boc	ni	>100	$x = 1.9$
3c	OMe	Me	Bn	Boc	ni	96.4	ni
3d	OMe	Me	Me	Boc	59.3	74.0	ni
3e	OMe	CH_2CONH_2	Bn	Boc	14.1	55.4	ni
3f	OEt	^{i}Bu	Bn	Boc	ni	ni	ni
3g	OEt	^{i}Bu	Me	Boc	ni	>100	ni
3h	OH	Me	Bn	Boc	1.6	2.8	3.0
3i	NHMe	Me	Bn	Boc	59.3	30.2	ni
4a	OMe	Me	Bn	Boc	44.8	>100	$x = 9.8$
4b	OMe	Me	H	Boc	9.2	8.7	$x = 6$
4c	OH	H	Bn	Boc	ni	ni	$x = 1.8$
4d	OH	Me	Bn	Boc	ni	ni	$x = 1.5$
4e	OH	Me	H	Boc	ni	ni	$x = 6.8$
4f	NHBn	H	Bn	Boc	ni	31.5	$x = 1.7$
4g	NHBn	Me	Bn	Boc	ni	55.4	$x = 1.7$
4h	NHBn	CH_2CONH_2	Bn	Boc	59.3	84.2	$x = 2.2$
4i	NHBn	^{i}Bu	Bn	Boc	$x = 1.3$	ni	$x = 1.9$
4j	NHBn	$(CH_2)_4NHBoc$	Bn	Boc	$x = 1.3$	ni	$x = 1.8$
4k	NHBn	$(CH_2)_3NZC(NH)NHZ$	Bn	Boc	ni	ni	$x = 1.9$
4l	NHBn	$(CH_2)_3NHC(NH)NH_2$	H	Boc	1.2	0.6	3.9
4m	NHPh	Me	Bn	Boc	$x = 1.4$	ni	$x = 1.6$
4n	NHPh	CH_2CONH_2	Bn	Boc	$x = 1.5$	ni	$x = 2.5$
4o	NHPh	^{i}Bu	Bn	Boc	ni	ni	$x = 1.9$
4p	NHPh	$(CH_2)_4NHBoc$	Bn	Boc	ni	ni	$x = 1.6$
4q	NHC_6H_4OH	Me	Bn	Boc	65.7	1	ni
4r	NHC_6H_4OH	CH_2CONH_2	Bn	Boc	>100	23.4	ni
4s	NHC_6H_4OH	^{i}Bu	Bn	Boc	ni	96.4	$x = 1.6$
4t	NHC_6H_4OH	$(CH_2)_4NHBoc$	Bn	Boc	65.7	ni	>100
4u	NHC_6H_4OH	$(CH_2)_3NZC(NH)NHZ$	Bn	Boc	ni	ni	ni
4v	NHC_6H_4OH	$(CH_2)_3NHC(NH)NH_2$	H	Boc	2.1	0.7	4.3
4w	$NH(CH_2)_4NHBoc$	Me	Bn	Boc	$x = 1.5$	ni	$x = 3.2$
4x	$NH(CH_2)_4NHBoc$	CH_2CONH_2	Bn	Boc	$x = 1.6$	ni	$x = 2.2$
4y	$NH(CH_2)_4NHBoc$	^{i}Bu	Bn	Boc	81.8	31.5	$x = 2.3$
4z	$NH(CH_2)_4NHBoc$	$(CH_2)_4NHBoc$	Bn	Boc	$x = 1.6$	ni	$x = 1.9$
4aa	NHBn	$(CH_2)_4NH_2$	Bn	H	1.07	ni	0.32
4ab	NHPh	$(CH_2)_4NH_2$	Bn	H	2.22	ni	1.12
4ac	NHC_6H_4OH	$(CH_2)_4NH_2$	Bn	H	0.85	ni	0.98
4ad	$NH(CH_2)_4NH_2$	^{i}Bu	Bn	H	3.69	22.5	0.33
4ae	NHBn	Me	Bn	H	0.48	>100	>100
MG132					0.075[b]	0.67[b]	4.5[b]

[a] ni: no inhibition at 100 μM. K_i > 100 μM: <20% inhibition at 100 μM. [b] IC_{50} values obtained for MG132 under our experimental conditions.

endocyclic clamp were very poor inhibitors of PA activity and did not inhibit the CT-L and T-L activities. This confirms how difficult it is to obtain simplified cyclic TMC-95A analogues without any loss of inhibitory capacity.[12b,16−19] Since the synthesis of the macrocycle is expensive and lengthy, we concentrated on acyclic compounds **2** and **3** in order to determine the influence of the phenylindole group (**2**) and the tripeptide moiety (**3**) on inhibition. Analogues **2**, which are generated by virtual disruption of the tripeptide chain of the cyclic compounds **1** (Figure 2), had no action on the CT-L and PA activities, but the amide derivatives **2b**,**c** (R^1 = NHMe) inhibited T-L activity. Because the peptide backbone of TMC-95A forms an antiparallel β sheet with residues of the S1 and S3 pockets of the active site clefts[12] (Figure 1), linear TMC-95A mimics that may fit into the individual clefts should be good inhibitors. We therefore synthesized the tripeptide compounds **3** and **4**. Several compounds **3** and **4** gave better inhibition of the three catalytic activities than did cyclic (**1**) and linear (**2**) compounds, depending on the structures of the R^1, R^2, R^3, and R^4 groups. In the yeast proteasome−TMC-95A complex, the side chain of asparagine is deeply inserted in the S3 pocket. This lateral chain was replaced by the ^{i}Bu group of leucine in compounds **3** (R^2 group) in order to increase specificity for the CT-L active site. Leucine is present in the calpain I inhibitor Ac-Leu-Leu-Ile-H. This substitution clearly abolished the inhibition of the three activities (**3e** versus **3a**, **3b**, **3f**, and **3g**), whereas the less bulky methyl was favorable (**3d**, **3h**, and **3i**; **3c** inhibits PA activity). This suggests that there must not be much bulk at this position if compounds **3** are to bind to the three catalytic sites. The nature of R^3 (Me or Bn) had no apparent influence on inhibition, but changing the OMe (**3a**) R^1 group to NHMe (**3i**) showed the beneficial effect of the amide group. The competitive inhibitor **3h** was obtained by inserting the negatively charged carboxylate group at this position (R^1 = OH) (Figure 3); it had K_i values of 1.6 (CT-L), 2.8 (PA), and 3.0 (T-L) μM. The P1 residue (here, the R^1 substituent), together with potential P3 residue (here, the R^2 substituent), is an important modulator of the specific inhibition of CT-L, T-L, and PA activities. Compounds **4**, which have the same framework as compounds **3** with no halogen substitution on the indole and phenyl groups (Figure 2, X = Y = H), were studied because they are more readily prepared than **3**.

The negatively charged compounds **4c**−**e** (R^1 = OH) were not inhibitors (**4d** versus **3h**), whereas the amides (R^1 = NH−) were inhibitors (as in series **3**), as were the esters (R^1 = OMe) (unlike in series **3**). Aromatic amides (R^1 = NHBn or NHC_6H_4OH) favored inhibition of PA activity alone (**4f**−**h**, **4r**, **4s**) when the R^2 groups were H, Me, or $CONH_2$, but this effect was lost when the R^2 group was bulky (**4i**−**k**,**m**−**p**,**t**,**u**). But this result was modulated by the nature of the group R^1 since inhibition of PA activity was obtained with R^1 = NHC_6H_4OH and R^2 = ^{i}Bu (**4r**, **4s**). The NHPh group prevented inhibition of all three proteasome activities (**4m**−**p**). A tyrosine at P1 favored inhibition by tyropeptin A aldehyde derivatives.[31] The importance of an optimal fit into the S1 pocket of the three catalytic sites has been pointed out for endocyclic biphenyl ether compounds derived from TMC-95A, but binding to the S3 pocket is also essential.[20] The asparagine side chain at position R^2, which may bind to the S3 subsite, favored the inhibition of CT-L activity because compounds containing H, Me, and ^{i}Bu were not inhibitors (**4h** versus **4f**, **4g**, and **4i**). We then varied the R^2 position in order to orientate the inhibition toward the T-L activity. Molecular modeling studies suggested that an R^2 group with chains of arginine or lysine would stimulate binding to the T-L active site. Thus, molecules bearing protected (**4k**, **4u**) or unprotected (**4l**, **4v**) arginine and protected (**4j**, **4p**, **4t**, **4z**) or unprotected (**4aa**−**c**) lysine lateral chains were evaluated. Molecules with protected lysine or arginine residues (R^2 group) did not inhibit T-L activity, although compound **4t**, with R^1 = NHC_6H_4OH, was a very poor inhibitor of T-L (K_i = 126 μM). All the molecules with positively charged lateral chains (**4l**, **4v**, **4aa**−**c**) inhibited the T-L active site (K_i = 0.32 μM for **4aa** and 0.98 μM for **4ac**). Compound **4ac** inhibited with equal potency T-L (K_i = 0.98 μM) and CT-L activities (K_i = 0.85 μM), whereas ($K_{i,CT-L}/K_{i,T-L}$ = 2 for **4ab** and 3 for **4aa**); PA activity was not inhibited (**4aa**−**c**). Compound **4ad**, with an $NH(CH_2)_4NH_2$ group at R^1 and not at R^2, inhibited all three activities ($K_{i,CT-L}/K_{i,T-L}$ = 10, $K_{i,PA}$ = 22.5 μM). The protected lysine lateral chain $NH(CH_2)_4$NH-Boc at position R^1 is unfavorable for inhibition (**4w**−**z**) except when R^2 = ^{i}Bu, since **4y** inhibited both CT-L and PA activities. The lateral chain of arginine was also recently introduced into endocyclic biphenyl ether compounds as a ligand of the S1 and S3 subsites.[20] The tyrosine hydroxyl group was protected in most compounds to facilitate their synthesis (R^3 = Me or Bn). Its deprotection led to inhibitory activity (**4b** versus **4a**). Finally, the nature of the R^4 substituent may influence selectivity (**4ae** with R^4 = H inhibited CT-L activity, while **4g** with R^4 = Boc inhibited PA activity). Several of our linear mimics were moderate activators of proteasomes (over 1.2-fold).

We have designed and synthesized effective small proteasome inhibitors whose structures can be rationally modulated to act on all three active sites (**3h**, **4l**, **4v**, **4ad**), two active sites (**4aa**−**c** for CT-L and T-L; **3d**, **3e**, **3i**, **4b**, **4h**, **4q** for CT-L and PA), or only one active site (**1c**, **4f**, and **4g** for PA; **2b** for T-L) with K_i values of 0.32−84 μM. Several compounds were more effective inhibitors of T-L than MG-132 (**3h**, **4v**, **4aa**−**d**) or equally inhibited PA activity (**4l**, **4v**) under our experimental conditions. Nevertheless, MG-132 is a better inhibitor of CT-L activity (12-fold better than **4aa**). The linear mimics **3** and **4** compare favorably with arecoline tripeptides,[32] lipotripeptides having a large alkyl chain linked to the peptide N-terminus,[33] 5-methoxy-1-indanone dipeptide benzamides,[34] and the calpain I inhibitor Ac-LLN-H.[12b] Moderate changes in the substituents of the TMC-95A macrocycle produced inhibitors with nanomolar K_i,[16] whereas greater alterations of the macrocycle structure to facilitate synthesis gave inhibitors with activities similar to those of our linear molecules.[17−19] Finally, we studied the metabolic stability of some linear mimics in RPMI culture medium containing fetal calf serum. The half-life of compound **3h** was 37 h and that of **4h** was 6.5 h, indicating reasonable stability.

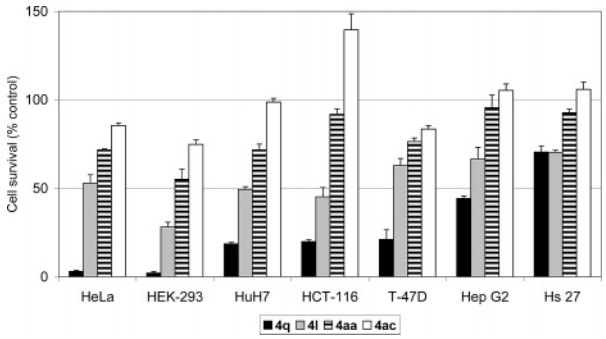


Figure 4. Cytotoxicity of some linear mimics for human cell lines. Cells were incubated with 50 μM **4q**, **4l**, **4aa**, or **4ac** for 48 h. Cell survival was then measured using an MTT assay.

The proteasome is an interesting new target for products designed to combat many diseases, particularly cancers.[4,6] Targeting the T-L activity may be useful, since it is reported to be the rational choice for antitrypanosoma drugs.[35] Compounds **4l** and **4aa**−**d**, which inhibit T-L activity, may be molecules from which clinically useful noncovalent inhibitors of proteasomes can be developed. We tested the most potent in vitro inhibitors (**4l**, **4q**, **4aa**, and **4ac**) in six human tumor cell lines and in primary fibroblasts to determine their effects on cancer cells. The effects of these inhibitors increased in the order **4ac** < **4aa** < **4l** < **4q** (Figure 4). Compound **4ac** had negligible inhibitory effects and even slightly stimulated HCT-116 cells. But all the cell lines were sensitive to incubation with 50 μM **4q** for 48 h. Two of them (HeLa and HEK-293) were killed, while the growth of three others was severely inhibited (HuH7, HCT 116, T-47D). One cell line (Hep G2) was significantly resistant to **4q**. The less sensitive cells (primary fibroblasts Hs 27) were the only nontumoral cells tested. Compound **4q** had an IC_{50} of 15 ± 5 μM on HeLa cells, about 500-fold higher than that of MG132 tested under the same conditions (data not shown) and close to that of TMC-95A for human tumor cell lines (HCT-116, IC_{50} = 4.4 μM, and HL-60, IC_{50} = 9.8 μM).[11] Cell cycle analysis of synchronized HeLa cells showed that progression through the cell cycle phases was slower in cells incubated with **4q** than in control untreated cells (data not shown).

Conclusion

We have prepared and tested new reversible noncovalent inhibitors of proteasomes using TMC-95A as template. They differ dramatically from the parent molecule TMC-95A, since they lack the biaryl linkage responsible for a constrained conformation. They also differ from the traditional electrophilic peptide inhibitors of proteasome (peptide boronates, peptide aldehydes, α,β-epoxyketones) in that they have no reactive group ("warhead") that could react with the catalytic O^γ-Thr1 of the proteasome. Hence, they are all readily synthesized and their skeleton can be used to introduce structural variations to orientate selectivity for one or more of the three catalytic sites. The linear scaffold is suitable for an optimized display of groups interacting with each binding subsite. Analysis of the roles of R^1 and R^2 substituents susceptible to act as P1 and P3 residues indicates (a) the favorable effect of a electron-rich aromatic

substituent at R^1 position (NHBn, NHC_6H_4OH) or its total absence with a free carboxylic C-end, (b) an orientation toward T-L activity with lysine and arginine lateral chains at position R^2 or even at position R^1, (c) a possible orientation toward specific inhibition of PA activity. They are significant inhibitors in vitro and in cell tumor lines and are metabolically stable. These versatile compounds open a new avenue for the development of new low molecular weight and noncovalently binding proteasome inhibitors that act selectively on one or more of the three proteasome activities.

Experimental Section

Chemistry. The syntheses of the more active compounds are indicated below. For description of general methods and preparation of the other compounds, see Supporting Information.

***N*-Boc-Tyr(Bn,3-iodo)-Ala-Trp(7-bromo)-OH (3h).** A solution of *N*-Boc-Tyr(Bn,3-iodo)-Ala-Trp(7-bromo)-OMe **3c** (85.5 mg, 0.100 mmol) in THF (0.4 mL) was treated at 0 °C by 1 M aqueous NaOH (0.12 mL, 0.12 mmol). After 5 h at room temperature, 1 M aqueous HCl was added (0.36 mL, 0.36 mmol). The resulting mixture was diluted by water and extracted by CH_2Cl_2 (3 × 10 mL). After drying of the organic phase over Na_2SO_4 and evaporation of the solvent, the residue was subjected to flash chromatography on silica gel (2% MeOH/CH_2Cl_2) to afford remaining *N*-Boc-Tyr-(Bn, 3-iodo)-Ala-Trp(7-bromo)-OMe **3c** (10.13 mg, 12%) and *N*-Boc-Tyr(Bn, 3-iodo)-Ala-Trp(7-bromo)-OH **3h** (58.3 mg, 70%, corrected yield 82%) as a white amorphous solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.9 (d, J = 6.4 Hz, 3H, CH_3), 1.42 (s, 9H, $(CH_3)_3$), 2.87 (m, CH_2 Tyr), 3.32 (m, 2H, CH_2 Trp), 4.40 (m, 1H, CH Tyr), 4.52 (m, 1H CH Ala), 4.85 (m, 1H, CH Trp), 5.06 (s, 2H, CH_2O), 5.16 (broad d, J = 7.6 Hz, 1H, NHBoc), 6.71 (d, J = 8 Hz, 1H, H5 Tyr), 6.91 − 7.58 (m, 14 H, aromatic H and NH), 8.68 (s, 1H, NHind). ^{13}C NMR (75 MHz, acetone-d_6) δ 19.4 (CH_3 Ala), 29.1 (CH_2 Trp), 29.5 ($(CH_3)_3$), 38.3 (CH_2 Tyr), 50.4 (CH Ala), 54.7 (CH Trp), 57.4 (CH Tyr), 72.3 (CH_2O), 80.5 ($C(CH_3)_3$), 87.6 (C3 Tyr), 106.1 (C7 Trp), 114.3 (CH ar), 120.0 (CH ar), 120.7 (Car), 122.0 (CH ar), 125.6 (CH ar), 126.7 (Car), 129.0 (CH ar), 129.5 (CH ar), 130.2 (CH ar), 131.3 (Car), 132.7 (CH ar), 134.2 (Car), 136.5 (Car), 138.9 (Car), 142.0 (CH ar), 157.3, 157.8 (C4 Tyr or CO Boc), 172.9, 173.7, 174.1 (2 CO amide, CO acid). HRMS (ESI) calcd for $C_{35}H_{38}{}^{79}BrIN_4O_7Na$ [$(M + Na)^+$] 855.0866, found 855.0896. Anal. ($C_{35}H_{38}BrIN_4O_7 \cdot 2H_2O$) C, H. N: calcd, 6.44; found, 5.73. HPLC homogeneity: >97% (system A, t_R =13); >99% (system B, t_R = 17.8).

***N*-Boc-Tyr-Ala-Trp-OMe (4b).** *N*-Boc-Tyr(Bn)-Ala-Trp-OMe **4a** (119 mg, 0.185 mmol) in solution in MeOH (4 mL) was hydrogenated overnight at atmospheric pressure in the presence of 10% Pd on charcoal (30 mg). After filtration, evaporation of the solvent, and trituration with ether, *N*-Boc-Tyr-Ala-Trp-OMe (77 mg, 76%) was obtained as a solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.19 (d, J = 7 Hz, 3H, CH_3), 1.29 (s, 9H, $(CH_3)_3$), 2.55 and 2.80 (ABX system, 2H, $CH_2\beta$), 3.13 and 3.16 (ABX system, 2H, CH_2 β), 3.55 (s, 3H, OCH_3), 4.05 (m, 1H, CHα), 4.35 (m, 1H, CHα), 4.50 (m, 1H, CHα), 6.63 (d, J = 8.3 Hz, 2H), 6.83 (d, J = 8.6 Hz, 1H), 7.03 (m, 4H), 7.16 (d, J = 2.1 Hz, 1H), 7.34 (d, J = 7.9 Hz, 1H), 7.47 (d, J = 7.8 Hz, 1H), 7.92 (d, J = 7.4 Hz, 1H, NH), 8.32 (d, J = 7.3 Hz, 1H, NH), 9.14 (s, 1H, OH), 10.86 (s, 1NH). ^{13}C NMR (75 MHz, DMSO-d_6) δ 18.4 (CH_3), 26.9 (CH_2 Trp), 28.1 ($(CH_3)_3$), 36.4 (CH_2 Tyr), 47.8, 51.7, 53.0, 55.9 (CHα Ala, Tyr, Trp or OCH_3), 77.9 ($C(CH_3)_3$), 109.1 (aromatic C), 111.4, 114.8, 117.9, 118.4, 120.9, 123.7 (aromatic CH), 127.0, 128.2 (aromatic C), 130.0 (aromatic CH), 136.0 (aromatic C), 155.2, 156.6 (Car-O or CO carbamate), 171.4, 172.0, 172.2 (2 CO amide or CO ester). HRMS (ESI) calcd for $C_{29}H_{36}N_4O_7Na$ [$(M + Na)^+$] 575.2482, found 575.2480. Anal. ($C_{29}H_{36}N_4O_7 \cdot H_2O$) C, H, N.

***N*-Boc-Tyr-Arg-Trp-NHCH$_2$Ph (4l).** A solution of *N*-Boc-Tyr-(Bn)-Arg(Z_2)-Trp-$NHCH_2Ph$ **4k** (86.15 mg, 0.0804 mmol) in MeOH/DMF (1.4 mL, 1/0.4) was hydrogenated at atmospheric pressure over 10% Pd on charcoal (9.14 mg) for 18 h. The mixture was filtered over a pad of Celite and concentrated. After the mixture was washed with ether, the product was afforded as a white solid (46.55 mg, 81%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.09−1.63 (m, 4H, $(CH_2)_2$ Arg), 1.30 (s, 9 H, $(CH_3)_3$), 2.62−3.14 (m, 6H), 4.05 (m, 1H, CHα), 4.23 (m, 3H, CH_2 Ph and CHα), 4.56 (m, 1H, CHα), 6.60 (m, 2H, aromatic CH), 6.96−7.58 (m, 11H, aromatic H), 7.96 (m, 1H, aromatic H). ^{13}C NMR (75 MHz, CD_3OD) δ 25.9 (CH_2 γ Lys), 28.7 (CH_3 Boc), 28.9, 29.9(CH_2 Trp or CH_2 β Lys), 38.1 (CH_2 Tyr), 41.9 (CH_2N Lys), 44.2 (CH_2NPh), 54.3, 55.9, 57.9 (CH α Lys or Trp or Tyr), 80.9 (C Boc), 110.7 (Car), 112.4, 118.0, 119.4, 119.9, 122.5, 124.6 (CH ar), 126.0 (Car), 128.1, 128.4 (CH ar), 128.8 (Car), 129.4, 131.3 (CH ar), 138.0, 139.4 (Car), 157.9, 158.6, 161.5 (C═N or CarO or NCOO), 173.3, 173.8, 175.0 (CONH). HRMS (ESI) calcd for $C_{38}H_{49}N_8O_6$ [$(M + H)^+$] 713.3775, found 713.3778. HPLC homogeneity: >95% (system A, t_R = 10.1); >95% (system B, t_R = 13.8).

***N*-Boc-Tyr(Bn)-Ala-Trp-NH(4-OH)Ph (4q).** To a solution of *N*-Boc-Tyr(Bn)-Ala-OH (106.4 mg, 0.24 mmol) in CH_2Cl_2/DMF (1.5 mL, 1/1) at 0 °C were successively added EDC (53.8 mg, 0.28 mmol), HOBt (37.9 mg, 0.28 mmol), and Trp-NHPhOH **6g** (75 mg, 0.254 mmol). The resulting mixture was allowed to warm to room temperature overnight. The solvent was evaporated, and the crude was triturated with water. After filtration, the solid was collected and washed with Et_2O to afford a pale-brown solid (72.8 mg, 42%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.21 (d, J = 6.7 Hz, 3H, CH_3), 1.28 (s, 9H, $(CH_3)_3$), 2.62−3.21 (m, 4H, CH_2 Tyr, CH_2 Trp), 4.1 (m, 1H, CHα), 4.32 (m, 1H, CHα), 4.64 (m, 1H, CHα), 5.02 (s, 2H, CH_2 (Bn)), 6.66 (d, J = 8.4 Hz, 1H), 6.87−7.39 (m, 17H), 7.6 (d, J = 7 Hz, 1H), 8 (d, J = 6.9 Hz, 1H), 8.1 (d, J = 7.1 Hz, 1H), 9.18 (s, 1 NH), 9.74 (s, 1 NH), 10.8 (s, 1H, NH). ^{13}C NMR (75 MHz, DMSO-d_6) δ 18.2 (CH_3), 27.8 (CH_2 Trp), 28 ($(CH_3)_3$), 36.2 (CH_2 Tyr), 48.1, 53.9, 55.7 (CHα Ala, Tyr, Trp), 69 (CH_2 (Bn)), 78 ($C(CH_3)_3$), 109.6, 111.1, 114.2, 114.9, 118.1, 118.4, 120.7, 121, 123.4, 127.2, 127.5, 127.6, 128.3, 130.1, 130.2, 130.3, 135.9, 137.1 (24 aromatic C), 153.3, 155.2, 156.7 (2 Car-O, CO carbamate), 169.2, 171.4, 171.9 (3 CO amide). HRMS (ESI) calcd for $C_{41}H_{45}N_5O_7Na$ [$(M + Na)^+$] 742.3217, found 742.3223. Anal. ($C_{41}H_{45}N_5O_7 \cdot 2H_2O$) C, H, N.

***N*-Boc-Tyr-Arg-Trp-NH(4-OH)Ph (4v).** The same procedure as for **4l** was used, using *N*-Boc-Tyr(Bn)-Arg(Z_2)-Trp-NH(4-OH)-Ph **4u** (60.81 mg, 0.0567 mmol) and 10% Pd on charcoal (6.43 mg) in MeOH/DMF (0.8 mL, 7/1) and affording after washing with ether *N*-Boc-Tyr-Arg-Trp-NH(4-OH)Ph as a white solid (34.89 mg, 86%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.23−1.65 (m, 4H, $(CH_2)_2$ Arg), 1.31 (s, 9 H, $(CH_3)_3$), 2.73−3.17 (m, 6H), 4.09 (m, 1H, CHα), 4.28 (m, 1H, CHα), 4.63 (m, 1H, CHα), 6.59 (m, 3H, aromatic CH), 6.92−7.31 (m, 7H, aromatic H), 7.57 (d, J = 7.6 Hz, 1H, aromatic H). ^{13}C NMR (75 MHz, CD_3OD) δ 26.1 (CH_2 γ Lys), 28.7 (CH_3 Boc), 26.7, 28.9 (CH_2 Trp or CH_2 β Lys), 34.8 (CH_2 Tyr), 41.9 (CH_2N Lys), 53.7, 56.4, 57.6 (CH α Lys or Trp or Tyr), 80.8 (C Boc), 110.6 (Car), 112.3, 116.9 (CH ar), 117.2 (C ar), 119.4, 119.9, 122.4, 124.1, 124.6 (CH ar), 128.7 (Car), 131.3 (CH ar), 138.0 (Car), 157.8, 158.4 (C═N or CarO or NCOO), 172.2, 173.3, 174.7 (CONH). HRMS (ESI) calcd for $C_{37}H_{47}N_8O_7$ [$(M + H)^+$] 715.3568, found 715.3572. HPLC homogeneity: >95% (system A, t_R = 9.8); >95% (system B, t_R = 13.2).

Tyr(Bn)-Lys-Trp-NHCH$_2$Ph (4aa). *N*-Boc-Tyr(Bn)-Lys(Boc)-Trp-$NHCH_2Ph \cdot 2H_2O$ (80.23 mg, 0.0881 mmol) was reacted with 3 M anhydrous HCl in MeOH (1.5 m L) overnight. After solvent evaporation and washing of the residue with ether, a solid was obtained (33.3 mg). Purification of this crude product by MPLC over RP silica (C18, 3 g) afforded after lyophilization Tyr(Bn)-Lys-Trp-$NHCH_2Ph \cdot$ $3HCl \cdot 2H_2O$ as a white solid (17 mg, 24%). ^{1}H NMR (300 MHz, CD_3OD) δ 1.30 (m, 2H, CH_2 Lys), 1.49−1.72 (m, 4H, CH_2 Lys), 2.73−2.83 (m, 3H, CH of ABX system and CH_2 Lys), 2.95−3.21 (m, 3H, CH of ABX system and CH_2), 4.00 (dd, J = 7.5 Hz, J = 6.0 Hz, 1H, CHα), 4.11 and 4.20 (AB system, J = 15 Hz, 2H, NCH_2Ph), 4.29 (t, J = 7 Hz, 1H, CHα Lys), 4.58 (t, J = 7.2 Hz, 1H, CHα), 4.89 (m, 2H, CH_2 (Bn)), 6.80 (d, J = 8.6 Hz, 2H), 6.89−7.30 (m, 15H), 7.54 (d, J = 7.6 Hz,

1H). ^{13}C NMR (75 MHz, CD_3OD) δ 23.5 (CH_2), 28.1 (CH_2), 29.3 (CH_2), 32.5 (CH_2), 37.8 (CH_2), 40.4 (CH_2), 44.1 (NCH_2Ph), 54.7 (CH), 55.6 (CH), 55.9 (CH), 70.9 (CH_2), 110.6 (C), 112.4 (CH), 116.4 (CH), 119.4 (CH), 120.0 (CH), 122.5 (CH), 124.8 (CH), 127.5 (C), 128.1 (CH), 128.4 (C), 128.5 (CH), 128.8 (C), 128.9 (CH), 129.4 (CH), 129.5 (CH), 131.7 (CH), 138.1 (C), 138.6 (C), 139.4 (C), 159.8 (C), 169.9 (CO), 172.9 (CO), 173.7 (CO). HRMS (LSIMS with Cs^+) calcd for $C_{40}H_{47}N_6O_4$ [M + H^+] 675.3659, found 675.3659. Anal. ($C_{40}H_{46}N_6O_4 \cdot 1.5H_2O \cdot 3HCl$) C, H, N.

Tyr(Bn)-Lys-Trp-NHPh (4ab). *N*-Boc-Tyr(Bn)-Lys(Boc)-Trp-NHPh·1.5H_2O (91.88 mg, 0.103 mmol) was reacted with 3 M anhydrous HCl in MeOH (1.5 mL) for 3 h. After solvent evaporation and washing of the residue with ether, a solid was obtained (68.3 mg). Purification of a portion of this crude product (37.8 mg) by MPLC over RP silica (C18, 3 g) afforded after lyophilization Tyr(Bn)-Lys-Trp-NHPh·3HCl·2H_2O as a white solid (17.2 mg, 38%). ^{1}H NMR (300 MHz, D_2O) δ 1.20 (m, 2H, CH_2 Lys), 1.53 (m, 4H, CH_2 Lys), 2.78 (t, J = 7.8 Hz, 2H, CH_2 Lys), 2.85 (dd, J = 13.5 Hz, J = 6.8 Hz, 1H), 2.98 (dd, J = 13.5 Hz, J = 6.8 Hz, 1H), 3.42 (m, 2H, CH_2), 4.08 (t, J = 6.8 Hz, 1H, CHα), 4.27 (t, J = 7 Hz, 1H, CHα Lys), 4.56 (t, J = 6.4 Hz, 1H, CHα), 4.84 (s, 2H, CH_2 (Bn)), 6.75 (d, J = 8.45 Hz, 2H), 6.92 (d, J = 8.45 Hz, 2H), 7.01−7.34 (m, 14H), 7.58 (d, J = 7.3 Hz, 1H). ^{13}C NMR (75 MHz, CD_3OD) δ 23.5 (CH_2), 28.1 (CH_2), 29.3 (CH_2), 32.5 (CH_2), 37.8 (CH_2), 40.4 (CH_2), 54.7 (CH), 55.6 (CH), 56.3 (CH), 70.9 (CH_2), 110.6 (C), 112.4 (CH), 116.5 (CH), 119.5 (CH), 120.0 (CH), 121.7 (CH), 122.5 (CH), 124.8 (CH), 125.5 (C), 127.5 (CH), 128.5 (C), 128.8 (CH), 128.9 (CH), 129.5 (CH), 129.8 (CH), 131.73 (CH), 138.0 (C), 138.6 (C), 139.2 (C), 159.8 (C), 169.9 (CO), 172.2 (CO), 173.0 (CO). HRMS (LSIMS with Cs^+) calcd for $C_{39}H_{45}N_6O_4$ [M + H^+] 661.3502, found 661.3503. Anal. ($C_{39}H_{44}N_6O_4 \cdot 2H_2O \cdot 3HCl$) C, H, N.

Tyr(Bn)-Lys-Trp-NH(4-OH)Ph (4ac). *N*-Boc-Tyr(Bn)-Lys-(Boc)-Trp-NH(4-OH)Ph·3H_2O (80.04 mg, 0.0859 mmol) was reacted with 3 M anhydrous HCl in MeOH (1.5 m L) overnight. After solvent evaporation and washing of the residue with ether, a solid was obtained (61.5 mg). Purification of this crude product by MPLC over RP silica (C18, 6 g) afforded after lyophilization Tyr-(Bn)-Lys-Trp-NH(4-OH)Ph·3HCl·2H_2O as a white solid (60.7 mg, 86%). ^{1}H NMR (300 MHz, D_2O) δ 1.20 (m, 2H, CH_2 Lys), 1.52 (m, 4H, CH_2 Lys), 2.78 (t, J = 7.5 Hz, 2H, NCH_2 Lys), 2.84 (dd, J = 14.5 Hz, J = 7.5 Hz, 1H), 2.97 (dd, J = 14.5 Hz, J = 5 Hz, 1H), 3.21 (m, 2H, CH_2), 4.08 (t, J = 6.5 Hz, 1H, CHα), 4.25 (t, J = 7 Hz, 1H, CHα), 4.50 (t, J = 8 Hz, 1H, CHα), 4.80 (s, 2H, CH_2 (Bn)), 6.67−7.32 (m, 17H), 7.56 (d, J = 8 Hz, 1H). ^{13}C NMR (50 MHz, CD_3OD) δ 23.4 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 32.5 (CH_2), 37.8 (CH_2), 40.4 (CH_2), 54.7 (CH), 55.5 (CH), 56.1 (CH), 70.9 (CH_2), 110.6, 112.3, 116.1, 116.4, 119.4, 119.9, 122.5, 123.8, 124.8, 127.4, 128.5, 128.7, 128.8, 129.5, 130.9, 131.7, 138.0, 138.5, 155.7, 159.8, 169.8 (CO), 171.9 (CO), 172.9 (CO). HRMS (LSIMS with Cs^+) calcd for $C_{39}H_{45}N_6O_5$ [M + H^+]677.3451, found 677.3440. Anal. ($C_{39}H_{44}N_6O_5 \cdot 2H_2O \cdot 3HCl$) C, H, N.

Tyr(Bn)-Leu-Trp-NH(CH_2)$_4$$NH_2$ (4ad). *N*-Boc-Tyr(Bn)-Leu-Trp-NH(CH_2)$_4$-NHBoc·0.5H_2O (85.52 mg, 0.100 mmol) was reacted with 3 M anhydrous HCl in MeOH (1.5 mL) overnight. After solvent evaporation and washing of the residue with ether, a solid was obtained (28 mg). Purification of a portion of this crude product (14 mg) by MPLC over RP silica (C18, 3 g) afforded after lyophilization Tyr(Bn)-Leu-Trp-NH(CH_2)$_4$$NH_2$·3HCl·2$H_2O$ as a white solid (10.3 mg, 26%). ^{1}H NMR (300 MHz, D_2O) δ 0.75 (d, J = 5.7 Hz, 3H, CH_3 Leu), 0.80 (d, J = 5.7 Hz, 3H, CH_3 Leu), 1.20 (m, 4H, CH_2), 1.38 (m, 3H, CH_2 and CH), 2.70 (t, J = 6.7 Hz, 2H, CH_2), 2.81−3.11 (m, 6H), 4.06 (t, J = 7.5 Hz, 1H, CHα), 4.27 (t, J = 7.2 Hz, 1H, CHα Lys), 4.40 (t, J = 7.2 Hz, 1H, CHα), 4.81 (m, 2H, OCH_2), 6.73 (d, J = 8.5 Hz, 2H), 6.90 (d, J = 8.5 Hz, 2H), 7.01−7.35 (m, 8H), 7.56 (d, J = 7.1 Hz, 2H). ^{13}C NMR (75 MHz, D_2O) δ 22.0 (CH_3), 22.5 (CH_3), 24.5 (CH_2), 24.9 (CH), 25.8 (CH_2), 28.0 (CH_2), 36.6 (CH_2), 39.1 (CH_2), 39.6 (CH_2), 40.7 (CH_2), 52.8 (CH), 54.8 (CH), 55.6 (CH), 70.9 (CH_2), 109.6 (C), 112.6 (CH), 116.2 (CH), 119.1 (CH), 120.1 (CH), 122.6 (CH), 125.1 (CH), 126.9 (C), 127.6 (C), 128.6 (CH), 129.1 (CH), 129.5 (CH), 131.3 (CH), 136.9 (C), 137.0 (C), 158.1 (C), 169.2 (CO), 173.3 (CO), 173.4 (CO). HRMS (LSIMS with Cs^+) calcd for $C_{37}H_{49}N_6O_4$ [M + H^+] 641.3815, found 641.3806. Anal. ($C_{37}H_{48}N_6O_4 \cdot 2.5H_2O \cdot 3HCl$) C, H, N.

Tyr(Bn)-Ala-Trp-NHCH_2Ph (4ae). *N*-Boc-Tyr(Bn)-Ala-Trp-NHCH_2Ph **4g** (77.55 mg, 0.108 mmol) in THF (0.8 mL) was reacted with 3 M anhydrous HCl in MeOH (0.2 mL) overnight. After solvent evaporation, purification of the product by MPLC over RP silica (C18, 6 g) afforded after lyophilization Tyr(Bn)-Ala-Trp-NHCH_2Ph·2HCl·1.5H_2O as a white powder (49.3 mg, 64%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.23 (d, J = 6.9 Hz, 3H, CH_3), 2.80 (m, 1H, CH_2), 3.01 (m, 2H, CH_2), 3.14 (dd, J = 14.4 Hz, 6.9 Hz, 1H, CH_2), 3.96 (m, 1H, CHα), 4.23 (m, 2H, NCH_2), 4.43 (quint, J = 7.1 Hz, 1H, CHα), 4.61 (q, J = 7.5 Hz 1H, CHα), 4.96 (s, 2H, CH_2 (Bn)), 6.89−7.40 (m, 19 aromatic H), 7.63 (d, J = 7.8 Hz, 1H), 7.97 (m, 3H), 8.25 (d, J = 7.8 Hz, 1H), 8.47 (t, J = 6 Hz, 1H), 8.66 (d, J = 7.0 Hz,, 1H), 10.86 (s, 1NH). ^{13}C NMR (75 MHz, CD_3OD) δ 17.9 (CH_3), 29.2 (CH_2), 37.5 (CH_2), 44.0 (NCH_2-Ph), 50.5 (CH), 55.4 (CH), 55.7 (CH), 70.7 (CH_2), 110.5 (C), 112.3 (CH), 116.3 (CH), 119.3 (CH), 119.8 (CH), 122.4 (CH), 124.7 (C), 127.3 (C), 127.9 (CH), 128.2 (CH), 128.3 (CH), 128.7 (CH), 129.3 (CH), 129.3 (CH), 131.6 (CH), 137.9 (C), 138.4 (C), 139.2 (C), 159.7 (C), 169.4 (CO), 173.3 (CO), 173.7 (CO). HRMS (ESI) calcd for $C_{37}H_{40}N_5O_4$ [M + H^+] 618.3080, found 618.3106. Anal. Calcd for $C_{37}H_{39}N_5O_4 \cdot 1.5H_2O \cdot 2HCl$: C, 61.92; H, 6.18; N, 9.76. Found: C, 62.14; H, 6.17; N, 9.80.

Enzyme Studies. Rabbit reticulocyte 20S proteasome was obtained from Boston Biochem, Cambridge, MA. The fluorogenic substrates Suc-LLVY-AMC, Boc-LRR-AMC, and Z-LLE-βNA used to measure the proteasome CT-L, T-L, and PA activities were purchased from Bachem (France). Other reagents and solvents were purchased from commercial sources. Fluorescence was measured using a BMG Fluostar microplate reader.

Enzyme and Inhibition Assays. Enzyme activities were determined by monitoring the hydrolysis of the appropriate fluorogenic substrate (λ_{exc} = 360 and λ_{em} = 465 nm for AMC substrates; λ_{exc} = 340 and λ_{em} = 405 nm for the βNA substrate) for 1 h at 37 °C in the presence of untreated (control) or proteasome that had been incubated with 0.1−100 μM test compounds. Substrates and compounds were previously dissolved in DMSO, with the final solvent concentration kept constant at 3% (v/v). The buffers were (pH 7.5) 20 mM Tris, 1 mM DTT, 10% glycerol, 0.02% (w/v) SDS for CT-L and PA activities and 20 mM Tris, 1 mM DTT, 10% glycerol for T-L activity. Initial rates determined in control experiments (V_0) were considered to be 100% of the peptidase activity; initial rates (V_i) that were above 100% in the presence of a test compound were considered to be activations (expressed as activation factor), while initial rates below 100% were considered to be inhibitions. The inhibitory activities of compounds are expressed as K_i. The inhibition constants K_i were determined using the equation $V_i/V_0 = 1/(1+ [I]/K_{i(app)})$ with $K_{i(app)} = K_i(1 + [S]/K_m)$ or using the equation $IC_{50} = K_i(1 + [S]/K_m)$. The IC_{50} values (inhibitor concentrations giving 50% inhibition) were obtained by plotting the percent inhibition against inhibitor concentration and fitting the experimental data to the equation % inhibition = 100-$[I]_0/(IC_{50} + [I]_0)$ or, in few cases (compounds **3h** for PA and T-L activities and **4b**), to the equation % inhibition = 100 $[I]_0^{n_H}/(IC_{50}^{n_H} + [I]_0^{n_H})$ where n_H is the Hill number.

Evaluation of Metabolic Stability. The breakdown of the inhibitors was studied by incubating them in RPMI culture medium containing 20% fetal calf serum, at 37 °C for up to 2 days. The reaction was terminated by adding ethanol, and the mixture was separated by centrifugation at 4 °C (10 000 rpm for 10 min). Aliquots (20 μL) of the supernatant were injected onto the RP-HPLC column. The breakdown half-life was obtained by a least-squares linear regression analysis of a plot of the log [I] versus time using a minimum of five points.

Cell Culture and Cytotoxicity Assays. HeLa (cervical carcinoma), T-47D (mammary carcinoma), HEK-293 (epithelial kidney), HuH7 (hepatocarcinoma), Hep G2 (hepatoblastoma), HCT-116 (colorectal carcinoma) human cell lines, and Hs 27 primary

fibroblasts were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in DMEM (HeLa, T-47D, Hs 27), MEM (HEK-293, HuH7, Hep G2), or McCoy (HCT-116) media supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air.

The cells were incubated with varying concentrations of proteasome inhibitors, and cell survival was assessed at 37 °C by measuring the ability of the various cell lines to metabolize 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The 5×10^3 cells in 100 μL of culture medium were grown in triplicate for 48 h in 96-well plates, with and without 50 μM inhibitors. MTT solution (15 μL) was then added to each well, and the samples were treated according to the manufacturer's protocol (Promega). The results are expressed relative to the control values of cells treated with the DMSO alone.

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Supporting Information Available: General and experimental chemical procedures, characterization data for all new compounds, and elemental analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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