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Studies of the Synthesis of All Stereoisomers of MG-132 Proteasome Inhibitors in the Tumor Targeting Approach

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MG-132 is a tripeptide aldehyde (Z-L-leu-L-leu-L-leu-H, **2**) proteasome inhibitor that exerts antitumor activity and enhances cytostatic/cytotoxic effects of chemo- and radiotherapy. Because of a troublesome synthesis of tripeptides with a non-natural configuration and modified side chains of amino acids, only two stereoisomers of MG-132 have been reported. Here, we propose a new approach to the synthesis of tripeptide aldehydes based on the Ugi reaction. Chiral, enantiomerically stable 2-isocyano-4-methylpentyl acetates were used as substrates for Ugi reaction resulting in a formation of tripeptide skeletons. Further functionalization of the obtained products led to a synthesis of tripeptide aldehydes. All stereoisomers of MG-132 were synthesized and studied as potential inhibitors of chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing activities of proteasome. These studies demonstrated the influence of absolute configuration of chiral aldehydes on the cytostatic/cytotoxic effects of the synthesized compounds and revealed that only (*S,R,S*)-(−)-**2** stereoisomer is a more potent proteasome inhibitor than MG-132.

1. Introduction

Recent progress in biomedical research is focused, among others, on small peptides that reveal wide spectrum of biological activities.[1−3] Peptides with a C-terminal aldehyde group have been mostly reported as proteolytic enzyme inhibitors. Thus, tripeptide aldehydes (TPAs[a]) are known as inhibitors of IκB degradation,[4] human rhinovirus protease,[5] human calpain,[6] and proteasome.[7,8]

A ubiquitin−proteasome system (UPS) is responsible for the nonlysosomal ATP-dependent turnover and degradation of the majority of eukaryotic intracellular proteins. It participates in the degradation of normal and abnormal proteins involved in cell cycle control, apoptosis, and tumor growth.[9,10] Dysregulation of the system results in the development of multiple diseases such as malignancy,[11] neurodegenerative,[12] cardiovascular,[13] inflammatory, and autoimmune diseases.[10] Proteasome inhibitors represent a novel class of anticancer drugs that induce apoptosis and cell cycle arrest in tumor cells. The majority of proteasome inhibitors have a structure of small cyclic[14] and linear[15−17] peptides. The first inhibitor of proteasome that has been approved for clinical use in the treatment of drug-resistant multiple myeloma and recently also for mantle lymphoma was bortezomib (**1**, Figure 1). It is a dipeptidyl boronic acid, which decreases proliferation, induces apoptosis, enhances the activity of chemotherapy and radiation, and reverses chemoresistance in a variety of hematologic and solid malignancy models both in vitro and in vivo.[18]

A special attention in proteasome inhibition studies is paid to tripeptide aldehydes with the best characterized representative being Z-L-leu-L-leu-L-leu-H known as MG-132 (**2**). MG-132 exerts both direct antiproliferative and cytotoxic effects toward tumor cells and increases apoptosis induced by other agents. It also potentiates antitumor effects of chemo- and radiotherapy. Tripeptide **2** has been reported a: (a) an effective inducer of apoptosis in human osteosarcoma which is the most common malignant bone tumor, mainly occurring in children and adolescents,[19] (b) a potentiator of the cancer cells death induced by the histone deacetylase inhibitors,[20] and (c) an agent sensitizing prostate cancer cells to ionizing radiation.[21] MG-132 also exerts activities not associated with anticancer effects such as interference with proteasome-dependent proteolysis in skeletal muscle and increase of whole-body protein turnover.[22] Recent studies have revealed a new application of MG-132, which effectively prevents the development of morphine tolerance in rats.[23] Tripeptide aldehydes similar to MG-132 also reveal wide spectrum of activities. They quite often possess the activity of proteasome inhibitors, and sometimes they are more potent than aldehyde **2**. It has been confirmed that the presence of N-terminal benzyloxycarbonyl group and C-terminal leucine residue in TPAs is crucial for the achievement of high proteasome inhibition activity. The most active inhibitor of 20S (catalytic particle) proteasome having structure of TPA is Z-(2-naphthyl)alanyl-(1-naphthyl)alanylleucinal (**3**) with K_i = 0.015 nM against 4.0 nM for MG-132 and 0.62 nM for bortezomib.

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[a]Abbreviations: TPA, tripeptide aldehyde; UPS, ubiquitin−proteasome system; MG-132, Z-L-leu-L-leu-L-leu-H; DMB-NH_2, 2,4-dimethoxybenzylamine; HPLC, high performance liquid chromatography, DMF, dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; WGL, wheat germ lipase; ChTL, chymotrypsin-like; TL, trypsin-like; PGPH peptidylglutamyl peptide hydrolysis; DCM, methylene chloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EDTA, ethylenediaminetetraacetic acid.

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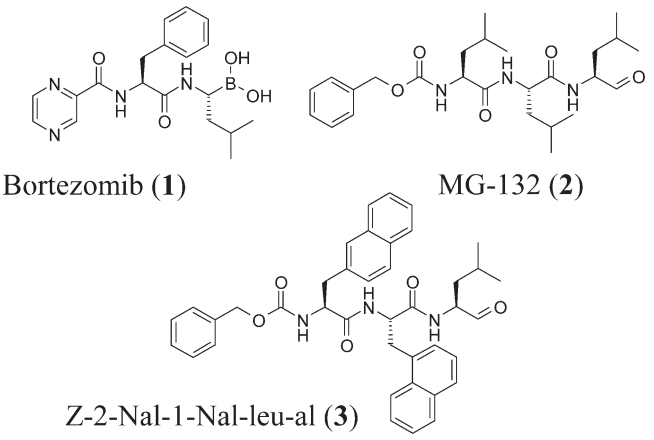


Figure 1. Examples of proteasome inhibitors.

This indicates that aldehyde **3** is about 270 and 40 times more potent than MG-132 and bortezomib, respectively.[15,24] It was found that more hydrophobic and hindered groups as side chains at P2 and P3 positions (counting from the C end) of tripeptides result in enhanced of proteasome inhibitory activity.[15] A great number of studied TPAs as well as **3** contain side chains of noncoded amino acids,[25] but they very rarely have the opposite to natural absolute configuration at any of α-carbon.[4,26,27] It is caused by the troublesome synthesis and very narrow access to commercially available precursors of peptides with non-natural configuration.

Generally, *N*-benzyloxycarbonyl tripeptide aldehydes, including MG-132, are synthesized by the classical coupling of N-protected amino acid with a dipeptide containing a C-terminal Weinreb amide group. In most cases, an aldehyde group is introduced to the structure of tripeptides by the reduction of the Weinreb amide group with lithium aluminum hydride.[4,24,25,28] However, this methodology is strongly limited by the availability of amino acids with non-natural side chains or/and configuration at α-carbon as precursors for the peptide synthesis. This results in very narrow access to other stereoisomers of small peptides, as MG-132 and restricted biological activity data of these compounds.[24]

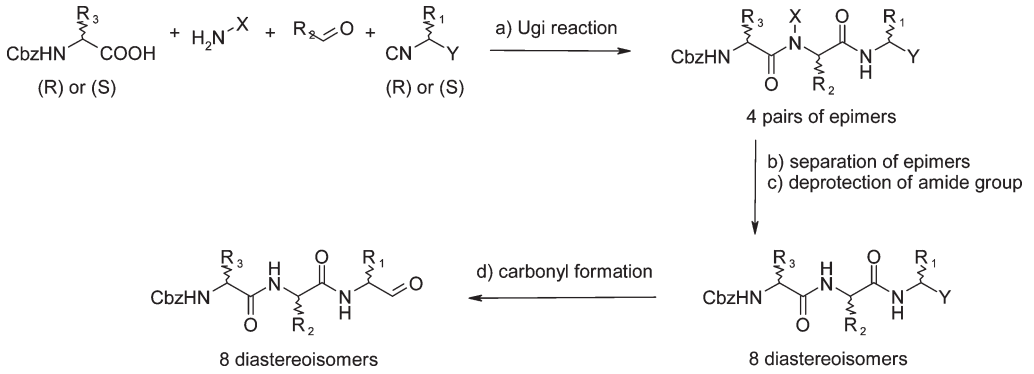
Recently, multicomponent reactions as Ugi and Passerini reactions create a new tool for the small peptides synthesis. However, these reactions have only been used for the synthesis of simple peptides. Application of the Passerini reaction followed by the enzymatic resolution of enantiomers and amino acid coupling led to tripeptides in a six-step procedure,[29] whereas use of Ugi reaction resulted in tripeptides directly. However, the application of Ugi reaction in the previous reports was limited to the synthesis of simple tripeptides containing glycine residues.[30,31] According to the mechanism of Ugi reaction when chiral, nonracemic substrates are used, products form as a mixture of two epimers that differ in configuration at the α-carbon of P2. However, synthesis of enantiomerically pure α-isocyanoacetates is problematic because of their tendency to racemization during the step of formamide group dehydrationp; this is why special conditions have to be used.[32] Moreover, although no significant racemization of chiral isocyanides is observed during Passerini reaction,[33] the same substrates are enantiomerically unstable under conditions of Ugi condensation.[34] In these cases Ugi reaction gives a mixture of more than two diastereoisomers. Therefore, studies on the preparation of new chiral nonracemizable isocyanides are also required to develop new strategies for the synthesis of tripeptide aldehydes.

2. Results and Discussion

Here, we present extension and application of the previously described general methodology for the synthesis of tripeptide aldehydes[35] together with the evaluation of their activity of proteasome inhibition. We have obtained a series of all eight diastereoisomers of tripeptide Z-leu-leu-leu-H (MG-132) by the application of chiral, enantiomerically pure carboxylic acids and isocyanides as substrates for the Ugi reaction. A general approach is based on (a) formation of a tripeptide scaffold by Ugi reaction, (b) separation of epimers, (c) deprotection of the amide function, and (d) formation of the C-terminal aldehyde group (Scheme 1).

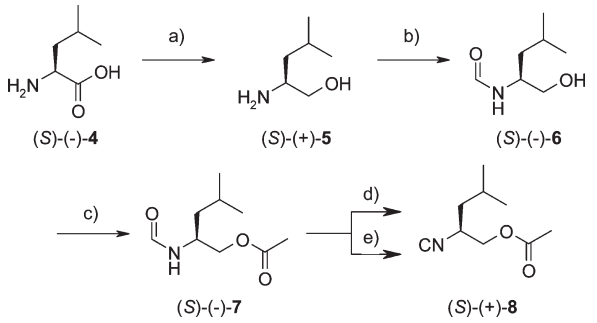
2.1. Synthesis of Isocyanides. At first, a new chiral nonracemizable isocyanide derived from leucine should be designed and synthesized. Application of this isocyanide for the Ugi reaction should avoid the formation of mixtures composed of more than two diastereoisomeric products. Because of the possibility of racemization, we rejected the use of isocyanides derived from amino esters as (*S*)- and (*R*)-2-isocyano-4-methylpentanoates,[36] the analogue of the best known group representative (*S*)-2-isocyano-3-phenylpropionate.[32,33] We decided to use, deriving from β-amino alcohol, both enantiomers of 2-isocyano-4-methylpentyl acetate ((*S*)-(+)-**8** and (*R*)-(−)-**8**). A replacement of electron withdrawing carboxylic ester group with a distant electron donating acetoxy moiety should avoid commonly observed racemization. The isocyanides were obtained by four-step procedure from (*S*)-(−)- and (*R*)-(+)-leucine (**4**) (Scheme 2). Reduction of the carboxylic group with borane generated in situ from sodium borohydride with iodine gave corresponding leucinols (**5**). (*S*)-(+)- and (*R*)-(−)-leucinols (**5**) have been N-formylated with ethyl formate in quantitative yields to (*S*)-(−)-**6** and (*R*)-(+)-**6** and then directly O-acetylated with acetic anhydride catalyzed by pyridine. (*S*)-(−)- and (*R*)-(+)-*N*-formylo-*O*-acetyloleucinol (**7**) have been obtained as white crystals in good 73% and 74% yields, respectively. We have found in aging tests that these precursors of isocyanides are almost completely enantiomerically stable under storage conditions (4 °C). Optical rotations for the fresh obtained compounds were −47.3 and +46.8 (*c* 1.0, $CHCl_3$), respectively, and after 4 months of storage of these samples the values have almost not decreased, −46.2 and +44.9, respectively. This indicates that progress of racemization is about 0.5−1.0% per month. Next, the dehydratation of formamide to isocyanide group was investigated. In most cases chiral isocyanides are synthesized from amino esters by the application of diphosgene or triphosgene as dehydrating agent in the presence of *N*-methylmorpholine as a base. Such conditions together with the lower temperature (−78 °C) enables isocyanide synthesis without racemization.[32,36] The standard procedure of formamide deprotection with phosphoryl oxychloride and triethylamine results in total racemization of isocyanides derived from amino esters.[32] In our methodology we decided to try both deprotection procedures. At first, reaction of formyl derivatives (*S*)-(−)-**7** and (*R*)-(+)-**7** with phosphorus oxychloride and triethylamine at −60 to −30 °C resulted in isocyanides (*S*)-(+)-**8** and (*R*)-(−)-**8** formation in good to excellent yields, 100% and 75%, respectively. The $[\alpha]_D$ for both enantiomers amounted to +2.66 and −2.75, respectively. After 4 months of storage at −20 °C the $[\alpha]_D$ amounted to +2.60 and −2.70, respectively. Thus, it confirms that the isocyanides are almost nonracemizable under storage conditions (∼0.5% per month). Next,

Scheme 1. General Methodology for the Synthesis of Tripeptide Aldehydes[a]



[a] R^1, R^3 = H, alkyl; X = *N*-amide bond protection; Y = aldehyde group equivalent.

Scheme 2. Synthesis of Isocyanide (*S*)-(−)-**8**[a]

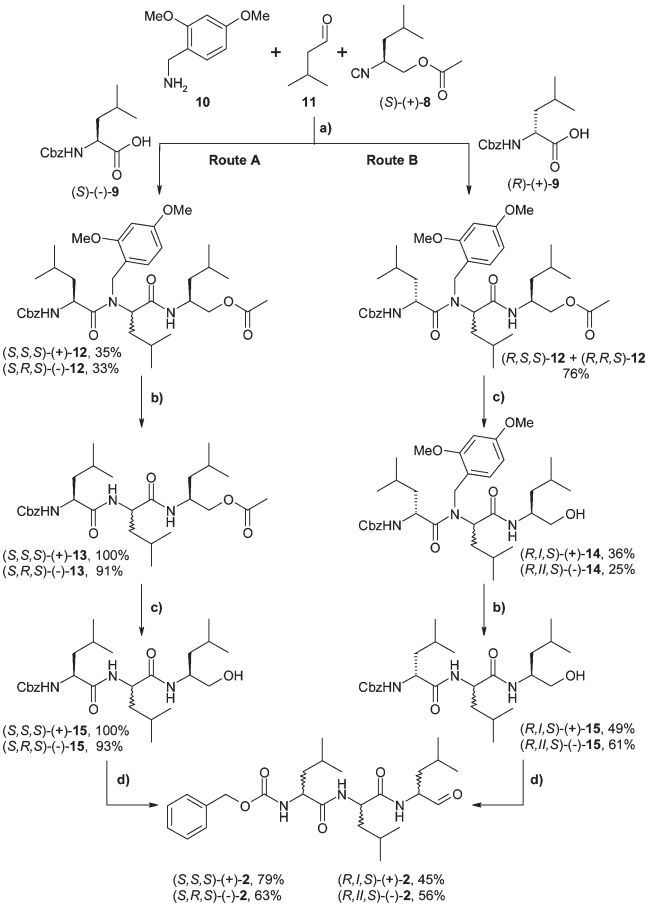


[a] Reagents and conditions: (a) $NaBH_4$, I_2, THF, reflux, 16 h, 73%; (b) HCOOEt, reflux, 4 h, 100%; (c) Ac_2O, pyridine, DMAP, DCM, room temp, 2 h, 73%; (d) $POCl_3$, Et_3N, DCM, −70 to −40 °C, 2.5 h; 100%; (e) $ClCOOCCl_3$, NMM, DCM, −40 °C, 1 h, 84%. Enantiomer (*R*)-(+)-**8** was obtained by the same methodology.

milder conditions like diphosgene as dehydrating agent and *N*-methylmorpholine have been applied. Herein, the (*S*)-(+)-2-isocyano-4-methylpentyl acetate has been obtained in 84% and the $[\alpha]_D$ amounted to +2.87. The difference between the results of the methods is negligible, which indicates that both dehydration conditions do not induce the racemization effect. The next experiments also indicate that the isocyanides were obtained as enantiomerically pure compounds.

2.2. Synthesis of Tripeptide Aldehydes. For Ugi reactions we have applied the isocyanides obtained by the phosphorus oxychloride dehydration process, according to the previous reported methodology, as the other components *N*-benzyloxycarbonylleucines (*S*)-(−)-**9** and (*R*)-(+)-**9**, 2,4-dimethoxybenzylamine (DMB-NH_2) (**10**) and isovaleraldehyde (**11**) have been used.[35] Cbz-leucines (**9**) enable the insertion of leucine residue at P3 position without the risk of racemization under the Ugi reaction conditions. Application of isovaleraldehyde as a carbonyl compound gives an opportunity to insert the isobutyl group as leucine residue at the P2 position of tripeptide. According to the mechanism of the Ugi reaction, products are formed as a mixture of two epimers differing in the absolute configuration at the α-carbon of the P2 position. Application of 2,4-dimethoxybenzylamine enables further easy cleavage of amide bond protection formed during the Ugi reaction.[35] At first, (*S*)-(+)-2-isocyano-4-methylpentyl acetate ((*S*)-(+)-**8**) was used together with *N*-Cbz-leucines (*S*)-(−)-**9** and (*R*)-(+)-**9** in two reactions. As a result, two pairs of epimers (*S,S,S*)-(+)-**12**/(*S,R,S*)-(−)-**12** and (*R,S,S*)-**12**/(*R,R,S*)-**12** were

Scheme 3. Synthesis of Tripeptide Aldehydes **2**[a]



[a] (a) MeOH, room temp, 48 h; (b) TFA, DCM, 50 °C, 1 h; c) $NaOH_{aq.}$, MeOH, room temp, 30 min; (d) Dess−Martin reagent, DCM, room temp, 1.5 h. Enantiomers of presented aldehydes were obtained by the same methodology.

obtained (Scheme 3). HPLC analyses confirmed no racemization appearance during the multicomponent condensation and revealed only two signals of main products in each of reaction mixtures. Products have been obtained as almost equimolar mixtures, both in 51:49 ratio and in good 82% and 76% yields, respectively. Moreover, each signal was derived from a different diastereoisomer, which indicates that none of them was the enantiomer of the other one. The corresponding Ugi reactions of (*R*)-(−)-**8** with (*S*)-(−)-**9** and (*R*)-(+)-**9**, **10**, and **11** resulted in the formation of the rest of the epimer pairs

in 80% and 76% yields, respectively. The results demonstrate that the 2-isocyano-4-methylpentyl acetates (*S*)-(+)-**8** and (*R*)-(−)-**8** are nonracemizable both under Ugi reaction and under storage conditions. To our knowledge, this is the first example of isocyanides derived from amino acids that are not susceptible to racemization during the Ugi reaction.

Next, we focused on a methodology for the separation of the epimers mixtures. First, because of the large experience of our group in the enzymatic resolution of stereoisomeric mixtures of compounds with the acetoxyl group,[29,37,38] we have examined an enzymatic approach with the application of lipases to a diastereoselective hydrolysis of the mixture of epimers (*S,S,S*)-(+)-**12** and (*S,R,S*)-(−)-**12**. We have examined 18 commercially available lipases and 12 enzyme preparations as acetone powders from animal tissues (livers and kidneys). The products were observed in the reactions catalyzed by acetone powders from pig, rabbit, and turkey livers and lipases from wheat germ, *C. cylindracea*, *P. roqueforti*, and recombinant *R. miehei*. However, in standard screening procedure (phosphate buffer (pH 7.0)/acetone, 9:1) the best results have been obtained for the application of wheat germ lipase (WGL); therefore, for the next experiments we have used WGL and we performed a screening of nonaqueous cosolvents as additives to the reaction medium in different ratios. Thus, such solvents as acetone, *i*-Pr_2O, Et_2O, *tert*-butyl methyl ether, cyclohexane, DMF, DMSO, THF, toluene, methanol, and ethanol were examined. Herein, products were observed in the mixtures with acetone, DMSO, and methanol additives. We repeated these reactions in preparative scale and products ratios were examined by HPLC. The best diastereoisomeric excess was obtained with the acetone and then with DMSO and methanol: 90%, 76%, and 60%, respectively. However, the yields were inversely proportional to the values of 17%, 52%, and 83%, respectively. So the results were not satisfactory because of low yield for the best diastereoisomerically enriched product.

Because the application of enzymes did not give the expected results, we decided to apply the physical methods for the separation of epimers. The TLC's analysis revealed that epimers (*S,S,S*)-(+)-**12** and (*S,R,S*)-(−)-**12** are characterized by the significant difference in polarities.

Thus, we tried to separate the epimers by column chromatography on silica gel. Triple repetition of the process allowed us to obtain pure diastereoisomers of (*S,S,S*)-(+)-**12** and (*S,R,S*)-(−)-**12** in 35% and 33% yields, respectively. The purities of the products were higher than 95% (HPLC). However, a similar separation of epimers (*R,S,S*)-**12** and (*R,R,S*)-**12** was not possible because of small difference between polarities of these compounds, but the problem of separation was resolved in the next step of the studies. According to our previously reported procedures,[35] separated epimers of (*S,S,S*)-(+)-**12** and (*S,R,S*)-(−)-**12** were subjected to deprotection of the amide bond by cleavage of the DMB group with TFA, resulting in (*S,S,S*)-(+)-**13** and (*S,R,S*)-(−)-**13** in excellent 100% and 91% yields, respectively. Next, basic hydrolysis of the acetoxy group gave (*S,S,S*)-(+)-**15** and (*S,R,S*)-(−)-**15** in 100% and 93% yields, respectively. Finally, selective oxidation of alcohol groups with Dess−Martin reagent gave aldehydes (*S,S,S*)-(+)-**2** and (*S,R,S*)-(−)-**2** in 79% and 63% yields, respectively (Scheme 3, route A). Because no racemization was observed during the whole process, the products were obtained as single diastereoisomers of tripeptide aldehydes. We assigned the absolute configurations of tripeptide aldehydes and their precursors by a comparison of commercially available MG-132 properties with obtained final products. The enantiomers (*R,R,R*)-(−)-**2** and (*R,S,R*)-(+)-**2** were obtained in an analogous manner in route A (Scheme 3) with similar results.

Table 1. Optical Activities of Tripeptide Aldehydes **2**

tripeptide aldehyde **2**	enantiomer	$[\alpha]_D^{25}$
(*S,S,S*)-(+)	A	+37.1
(*S,R,S*)-(−)	B	−57.3
(*S,I,R*)-(−)	C	−47.1
(*S,II,R*)-(+)	D	+29.0
(*R,I,S*)-(+)	C′	+45.9
(*R,II,S*)-(−)	D′	−27.1
(*R,R,R*)-(−)	A′	−36.5
(*R,S,R*)-(+)	B′	+55.5

Because the separation of epimers (*R,S,S*)-**12** and (*R,R,S*)-**12** was not possible, we subjected them to a deprotection process directly, but we have realized that it did not change the difference between the polarities of compounds. The separation was also not possible after the hydrolysis stage. Thus, we tried to change the order of the reactions and to perform hydrolysis of the ester group (Scheme 3, route B). Herein, we obtained *N*-DMB protected alcohols (*R,I,S*)-(+)-**14** and (*R,II,S*)-(−)-**14**, which were characterized by considerable difference in polarity, which allowed us to separate the epimers by triple column chromatography. The products have been obtained in good purities (>95%, HPLC) and 36% and 25% yields, respectively, but at this time we are not able to determine the absolute configurations at α-carbon of P2 residues; therefore, we have described the less polar compounds as (*R,I,S*) and more polar compounds as (*R,II,S*). Next, we subjected the alcohols to a deprotection stage with TFA. We have obtained deprotected tripeptide alcohols (*R,I,S*)-(+)-**15** and (*R,II,S*)-(−)-**15** in 49% and 61% yields, respectively. The lower yields are the result of byproducts formation which were not observed during the deprotection of *N*-DNB protected esters. And finally the alcohol was oxidized with Dess−Martin reagent to aldehydes (*R, I, S*)-(+)-**2** and (*R,II,S*)-(−)- **2** in 45% and 56% yields, respectively. For the synthesis of enantiomers (*S,I, R*)-(−)- **2** and (*S,II,R*)-(+)-**2** the same methodology (route B) was applied and similar results were obtained. Thus, the presented methodology allowed synthesis of all eight diastereoisomers of tripeptide aldehyde Z-leu-leu-leu-H (Table 1) which were then analyzed as inhibitors of chymotrypsin-like (ChTL) proteasome activity.

2.3. Inhibition of Proteasome Activity. In a preliminary experiment MG-132 ((*S,S,S*)-(+)-**2**) and its stereoisomers were investigated for inhibition of proteasome activity in lysates of J558L multiple myeloma cells or EMT6 breast cancer cells. Only MG-132 and (*S,R,S*)-(−)- **2** were capable of inhibiting proteasome activity at 100 nM in lysates obtained from both J558L and EMT6 cells. At 1000 nM a partial proteasome inhibition was also observed with (*S, I,R*)-(−)-**2**, (*R,R,R*)-(−)- **2**, and (*R,II, S*)-(−)-**2** (Tables 2 and 3).

Although proteasomes are responsible for most intracellular proteolysis, other cytosolic (tripeptidyl peptidases) and lysosomal (cathepsins) enzymes are present in tumor cell lysates and might influence the processing of tripeptide aldehydes used as fluorogenic substrates.[39] Therefore, MG-132 ((*S,S,S*)-(+)-**2**) and its stereoisomers were investigated for inhibition of ChTL, trypsin-like (TL) and peptidylglutamyl peptide hydrolyzing (PGPH) activities of purified 20S proteasomes isolated from human erythrocytes. These experiments revealed that only two stereoisomers ((*S,R,S*)-(−)-**2** and (*S,I,R*)-(−)-**2**) as well as MG-132 itself inhibit ChTL

Table 2. Influence of MG-132 and Its Stereoisomers on the Cleavage of Suc-leu-leu-Val-Tyr-AMC in J558L Cells

	proteasome activity at 100 nM[a]			proteasome activity at 1000 nM[a]		
compd	15 min	30 min	60 min	15 min	30 min	60 min
DMSO	100 ± 5	100 ± 3	100 ± 2	100 ± 5	100 ± 3	100 ± 2
(*R*,*I*,*S*)-(+)-**2**	115 ± 3	118 ± 2	123 ± 2	114 ± 3	117 ± 2	121 ± 3
(*S*,*II*,*R*)-(+)-**2**	104 ± 2	105 ± 3	107 ± 5	107 ± 3	106 ± 3	106 ± 3
(*R*,*S*,*R*)-(+)-**2**	100 ± 5	110 ± 2	112 ± 1	102 ± 2	96 ± 8	99 ± 3
(*S*,*I*,*R*)-(−)-**2**	92 ± 3	93 ± 1	94 ± 2	55 ± 3	64 ± 3	77 ± 4
(*R*,*R*,*R*)-(−)-**2**	87 ± 2	89 ± 2	89 ± 1	58 ± 2	66 ± 2	71 ± 3
(*S*,*S*,*S*)-(+)-**2**	84 ± 3	91 ± 3	95 ± 5	71 ± 4	72 ± 2	74 ± 3
(*R*,*II*,*S*)-(−)-**2**	71 ± 2	77 ± 2	82 ± 2	30 ± 1	40 ± 2	53 ± 4
(*S*,*R*,*S*)-(−)-**2**	25 ± 2	36 ± 2	50 ± 2	11 ± 0	21 ± 1	38 ± 2
MG-132	32 ± 3	44 ± 4	60 ± 5	15 ± 0	25 ± 1	44 ± 3

[a] Data refer to % of control activity ± SD measured after 15, 30, or 60 min.

Table 3. Influence of MG-132 and Its Stereoisomers on the Cleavage of Suc-leu-leu-Val-Tyr-AMC in EMT6 cells

	proteasome activity at 100 nM[a]			proteasome activity at 1000 nM[a]		
compd	15 min	30 min	60 min	15 min	30 min	60 min
DMSO	100 ± 5	100 ± 4	100 ± 4	100 ± 5	100 ± 4	100 ± 4
(*R*,*I*,*S*)-(+)-**2**	97 ± 1	95 ± 1	95 ± 1	104 ± 6	100 ± 5	99 ± 3
(*S*,*II*,*R*)-(+)-**2**	110 ± 2	103 ± 1	99 ± 2	118 ± 10	111 ± 8	106 ± 7
(*S*,*I*,*R*)-(−)-**2**	95 ± 1	91 ± 2	91 ± 2	44 ± 4	46 ± 4	53 ± 4
(*R*,*R*,*R*)-(−)-**2**	90 ± 4	90 ± 3	91 ± 2	62 ± 3	70 ± 4	78 ± 6
(*S*,*S*,*S*)-(+)-**2**	71 ± 3	77 ± 4	86 ± 5	29 ± 3	37 ± 2	50 ± 2
(*R*,*II*,*S*)-(−)-**2**	87 ± 4	85 ± 5	84 ± 7	61 ± 3	61 ± 3	67 ± 3
(*S*,*R*,*S*)-(−)-**2**	17 ± 4	22 ± 4	31 ± 4	5 ± 0	7 ± 0	13 ± 1
MG-132	20 ± 3	25 ± 3	35 ± 2	12 ± 1	14 ± 1	19 ± 1

[a] Data refer to % of control activity ± SD measured after 15, 30, or 60 min.

Table 4. Influence of MG-132 and Its Stereoisomers on the ChTL, TL, and PGPH Activities of Purified Human Erythrocyte 20S Proteasomes

	IC_{50} (μM)		
compd	ChTL	TL	PGPH
(*R*,*I*,*S*)-(+)-**2**	> 100	> 100	> 100
(*S*,*II*,*R*)-(+)-**2**	> 100	> 100	> 100
(*R*,*S*,*R*)-(+)-**2**	> 100	> 100	> 100
(*S*,*I*,*R*)-(−)-**2**	0.97	91.33	22.82
(*R*,*R*,*R*)-(−)-**2**	9.81	> 100	11.29
(*S*,*S*,*S*)-(+)-**2**	5.46	> 100	> 100
(*R*,*II*,*S*)-(−)-**2**	1.79	> 100	17.25
(*S*,*R*,*S*)-(−)-**2**	0.22	34.4	2.95
MG-132	0.89	104.43	5.70

proteasome activity with an IC_{50} of less than 1 μM (Table 4). TL and PGPH activities were inhibited at markedly higher concentrations, but again (*S*,*R*,*S*)-(−)-**2** was more effective than MG-132. Significant cytostatic/cytotoxic effects against J588L and EMT6 tumor cells were observed only with the compounds that blocked ChTL activity of 20S proteasomes with IC_{50} below 1 μM (Figures 1 and 2 of Supporting Information). However, (*S*,*R*,*S*)-(−)-**2**, despite being the most effective inhibitor, having a 5-fold lower IC_{50} than MG-132 (0.22 μM versus 0.89 μM) did not exert stronger cytostatic/cytotoxic effects against tumor cells. Interestingly, (*R*,*II*,*S*)-(−)-**2** did not exert any cytostatic/cytotoxic effects against tumor cells despite being able to inhibit the ChTL activity of purified 20S proteasomes with an IC_{50} of 1.79 μM.

3. Conclusions

Application of Ugi reaction gives the opportunity for a quick and easy synthesis of small peptides with non-natural configuration at α-carbon. The presented methodology allowed the synthesis of all stereoisomers of tripeptide aldehyde Z-leu-leu-leu-H (MG-132, **2**) with the application of isocyanides (**8**) obtained as enantiomerically stable under storage and Ugi reaction conditions. This is the first attempt to synthesize the complete group of eight possible diastereoisomers of such compounds. This is also the first attempt at full analytical and biological characterization of MG-132 and its stereoisomers; however, assignment of the absolute configuration at all α-carbons of all compounds based on magnetic resonance spectra was not possible because of the affinity of three equal isobutyl side chains and their vicinity on the tripeptide structure. All stereoisomers of **2** were studied as inhibitors of ChTL, TL, and PGPH activities of purified human 20S proteasomes, and intriguingly stereoisomers (*S*,*R*,*S*)-(−)-**2** and (*S*,*I*,*R*)-(−)-**2**, having non-natural absolute configuration, were capable of inhibiting the activity of purified 20S proteasomes with (*S*,*R*,*S*)-(−)-**2** being 5-fold more effective than MG-132 in terms of inhibiting ChTL activity. Cytostatic/cytotoxic effects exerted by the stereoisomers against tumor cells correlated with the ability to inhibit ChTL activity of the proteasome.

4. Experimental Section

4.1. General. NMR spectra were measured with Varian 200 GEMINI, Varian 400 GEMINI, and Bruker AM 500 spectrometers, with TMS used as an internal standard. TLCs were performed with silica gel 60 (230−400 mesh, Merck) and silica gel 60 PF254 (Merck). CHN analysis was performed on a Perkin-Elmer 240 elemental analyzer. MS spectra were recorded on an API-365 (SCIEX) apparatus. IR spectra were recorded on a Perkin-Elmer FT-IR Spectrum 2000 spectrometer. Optical rotations were measured with a JASCO P-2000 polarimeter. HPLC experiments were carried out on a KROMASIL 100 C-18 column, λ = 230 nm: method 1 (eluent methanol/water 85:15 (v/v), flow of 1 mL/min); method 2 (eluent methanol/water 80:20 (v/v), flow of 1.2 mL/min); method 3 (eluent methanol/water 83:17 (v/v), flow of 1.0 mL/min). All key compounds were proven by these methods to show > 95% purity.

4.2. Synthesis of Isocyanides. 4.2.1. Synthesis of (*S*)-(+)-2-Isocyano-4-methylpentyl Acetate (*S*)-(+)-8. 4.2.1.a. (*S*)-(+)-2-Amino-4-methylpentan-1-ol (*S*)-(+)-Leucinol (*S*)-(+)-5. To a suspension of sodium borohydride (5.77 g, 152.4 mmol) in THF (100 mL), L-leucine (10.0 g, 76.2 mmol) was added under nitrogen atmosphere. The reaction mixture was cooled to 0 °C, and a solution of iodine (19.3 g, 76.2 mmol) in THF (50 mL) was added for 1 h. Then the reaction mixture was stirred until the gas bubbles stopped evolving and was refluxed for 16 h and cooled to room temperature, and methanol was added until the mixture became clear. After 30 min, the volatiles were evaporated. The residue was dissolved in aqueous solution of potassium hydroxide (100 mL, 20%), stirred for 3 h, and extracted with DCM (4 × 75 mL). The combined organic layers were dried ($MgSO_4$), and the solvent was evaporated. The residual dense oil was distilled under reduced pressure (57 °C, 1.0 mmHg). Yield: 73%, 6.49 g (55.3 mmol) of a colorless oil. R_f = 0.42 ($CHCl_3$/MeOH/NH_4OH, 10:2:0.25, v/v/v). ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.89 (t, J = 6.4 Hz, 6H), 1.17 (t, J = 7.0 Hz, 2H), 1.64−1.70 (m, 1H), 2.10 (bs, 3H), 2.82−2.96 (m, 1H), 3.21 (dd, J = 10.6 Hz, J = 7.8 Hz, 1H), 3.55 (dd, J = 10.6 Hz, J = 3.8 Hz, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 22.5, 23.7, 25.0, 44.0, 50.9, 67.4. $[\alpha]_D^{25}$ +4.1 (*c* 4.0, EtOH) (lit.[40] +4.2 (*c* 0.9, EtOH)).

4.2.1.b. (S)-(−)-*N*-[-1-(Hydroxymethyl)-3-methylbutyl]formamide (*S*)-(−)-6. A solution of (*S*)-(+)-**5** (6.3 g, 53.8 mmol) in ethyl formate (60 mL) was refluxed for 3 h. Then the reaction mixture was cooled, and volatiles were evaporated to give 7.68 g (53.7 mmol) of crude product as slightly yellow oil. Yield 100%. Product was used without further purification. R_f = 0.69 ($CHCl_3$/MeOH/NH_4OH, 10:2:0.25, v/v/v). ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.93 (d, J = 6.6 Hz, 6H), 1.20−1.48 (m, 2H), 1.50−1.66 (m, 1 H), 3.40−3.78 (m, 3H), 4.12 (bs, 1H), 6.11 (bs, 1H), 8.18 (s, 1H) . ^{13}C NMR (50 MHz, $CDCl_3$) δ 22.4, 23.4, 25.2, 40.4, 49.2, 65.8, 162.3. IR (film) ν_{max}: 3280 (O−H), 2958 (CH_3), 1660 (C═O) cm^{-1}. $[\alpha]_D^{25}$ −32.5 (*c* 4.0, $CHCl_3$).

4.2.1.c. (*S*)-(−)-2-Formamido-4-methylpentyl Acetate (*S*)-(−)-7. To a solution of (*S*)-(−)-**6** (7.66 g, 52.8 mmol) in DCM (100 mL), pyridine (8.5 mL, 105.6 mmol), acetic anhydride (15.0 mL, 158.4 mmol), and *N,N*-dimethylaminopyridine (20 mg) were added under nitrogen atmosphere. The reaction mixture was stirred for 2 h at room temperature and washed with an aqueous solution of copper sulfate (3 × 25 mL, 5%), a saturated aqueous solution of sodium bicarbonate (2 × 25 mL), and water (2 × 25 mL). The organic layer was dried ($MgSO_4$) and the solvent was evaporated to leave a crude product that was crystallized from diethyl eter. Yield: 73%, 7.25 g (38.8 mmol) of white crystals. Mp 45−48 °C. R_f = 0.50 (hexane/EtOAc, 5:5, v/v). ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.80−1.00 (m, 6H), 1.28−1.48 (m, 2H), 1.50−1.75 (m, 1H), 2.08 (s, 3H), 3.90−4.20 (m, 2H), 4.30−4.50 (m, 1H), 5.72 (bs, 1H), 8.19 (s, 1H). IR (film) ν_{max}: 2960 (CH_3), 1740 (NC═O), 1660 (OC═O), 1242 (CH_2−O) cm^{-1}. $[\alpha]_D^{25}$ −47.3 (*c* 1.5, $CHCl_3$).

4.2.1.d. (*S*)-(+)-2-Isocyano-4-methylpentyl Acetate (*S*)-(+)-8. 4.2.1.d.1. Method 1. To a solution of (*S*)-(−)-**7** (2.0 g, 10.7 mmol) in DCM (30 mL) cooled to −40 °C triethylamine (7.44 mL, 53.4 mmol) was added. The mixture was cooled to −70 °C, and a solution of phosphorus oxychloride (1.08 mL, 11.7 mmol) in DCM (5 mL) was added dropwise for 5 min. The reaction mixture was stirred for 2.5 h, when the temperature was allowed to increase to −30 °C. Then the reaction mixture was poured to an ice cooled solution of sodium bicarbonate (100 mL, 1 M). Layers were separated, and the aqueous layer was extracted with DCM (3 × 50 mL). Organic layers were combined, dried ($MgSO_4$), and concentrated. The residual dark oil was purified by column chromatography (silica gel, 70−230 mesh, 37 g, DCM). Yield: 100%, 1.80 g (10.6 mmol) of yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.89 (dd, J = 8.8 Hz, J = 6.6 Hz, 6H), 1.14−1.35 (m, 1H), 1.50−1.70 (m, 1H), 1.71−1.90 (s, 1H), 2.06 (s, 3H), 3.70−3.90 (m, 1H), 3.94−4.20 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 20.9, 21.4, 23.2, 24.9, 40.3, 52.3, 65.6, 170.7. IR (film) ν_{max}: 2960 (CH_3), 2142 (N≡C), 1745 (C═O), 1235 (CH_2−O) cm^{-1}. Anal. Calcd for $C_9H_{15}NO_2$: C, 63.86; H, 8.94; N, 8.28. Found: C, 63.91; H, 8.82; N, 8.24. $[\alpha]_D^{25}$ 2.66 (*c* 1.0, $CHCl_3$).

4.2.1.d.2. Method 2. To a solution of (*S*)-(−)-**7** (1.0 g, 5.34 mmol) in DCM (15 mL) cooled to −40 °C *N*-methylmorpholine (1.76 mL, 16.0 mmol) was added. Diphosgene (0.322 mL, 2.67 mmol) in DCM (3 mL) was added dropwise for 5 min. The reaction mixture was stirred for 2.5 h, when the temperature was allowed to increase to −30 °C. Then the reaction mixture was poured to an ice cooled water (50 mL, 1 M). Layers were separated, and the aqueous layer was extracted with DCM (3 × 30 mL). Organic layers were combined, dried ($MgSO_4$), and concentrated. The residual brownish oil was purified by column chromatography (silica gel, 70−230 mesh, 19 g, DCM). Yield: 84%, 0.76 g (4.50 mmol) of colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.90 (dd, J = 8.7 Hz, J = 6.6 Hz, 6H), 1.12−1.32 (m, 1H), 1.48−1.70 (m, 1H), 1.71−1.92 (s, 1H), 2.08 (s, 3H), 3.70−3.88 (m, 1H), 3.94−4.18 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 20.9, 21.5, 23.2, 24.9, 40.2, 52.3, 65.5, 170.7. IR (film) ν_{max}: 2962 (CH_3), 2142 (N≡C), 1744 (C═O), 1236 (CH_2−O) cm^{-1}. Anal. Calcd for $C_9H_{15}NO_2$: C, 63.86; H, 8.94; N, 8.28. Found: C, 63.94; H, 8.80; N, 8.30. $[\alpha]_D^{25}$ +2.87 (*c* 1.0, $CHCl_3$).

4.2.2. Synthesis of (*R*)-(−)-2-Isocyano-4-methylpentyl Acetate (*R*)-(−)-8. Product has been obtained by method 1 for the synthesis of (*S*)-(+)-**8**. Yield: 75%, 1.35 g (7.95 mmol) of yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.90 (dd, J = 8.8 Hz, J = 6.7 Hz, 6H), 1.12−1.34 (m, 1H), 1.48−1.70 (m, 1H), 1.70−1.92 (s, 1H), 2.08 (s, 3H), 3.70−3.92 (m, 1H), 3.92−4.20 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 20.8, 21.4, 23.3, 25.0, 40.3, 52.3, 65.7, 170.6. IR (film) ν_{max}: 2960 (CH_3), 2140 (N≡C), 1745 (C═O), 1236 (CH_2−O) cm^{-1}. Anal. Calcd for $C_9H_{15}NO_2$: C, 63.86; H, 8.94; N, 8.28. Found: C, 63.98; H, 8.75; N, 8.15. $[\alpha]_D^{25}$ −2.75 (*c* 1.0, $CHCl_3$).

4.3. General Procedures for Tripeptides Synthesis. 4.3.1. General Procedure 1 for the Ugi Reaction. A solution of an amine (1 equiv) and an aldehyde (1 equiv) in methanol (2 mL/1 mmol) was stirred for 15 min at room temperature. Then an acid (1 equiv) in methanol (2 mL/1 mmol) was added. After an additional 15 min an isocyanide (1 equiv) was added. The reaction mixture was stirred at room temperature for 2 days. The solvent was evaporated, and the products were purified by gradient flash column chromatography (silica gel, 230−400 mesh, hexane/EtOAc).

4.3.2. General Procedure 2 for the Deprotection of Amide Bond. To a solution of Ugi reaction product (1.0 mmol) in DCM (5 mL), trifluoroacetic acid (0.5 mL) was added. The reaction mixture was stirred at 50 °C for 1 h and then cooled in an ice−water bath, diluted with DCM (5 mL), and neutralized with an aqueous saturated solution of sodium bicarbonate until the violet color disappeared. Layers were separated, and the aqueous layer was extracted with DCM (3 × 15 mL). The combined organic layers were washed with water (20 mL) and brine (20 mL), dried ($MgSO_4$) and evaporated. Product(s) was-(were) purified by gradient flash column chromatography (silica gel, 230−400 mesh, hexane/EtOAc).

4.3.3. General Procedure 3 for the Hydrolysis of Acetic Ester Group. To a solution of an ester (1 equiv) in MeOH (5 mL/1 mmol), an aqueous solution of sodium hydroxide (2.5 equiv, 4 M) was added. The reaction mixture was stirred at room temperature for 30 min, and then the solvent was evaporated. The residue was diluted with EtOAc (15 mL) and washed with an aqueous solution of hydrochloric acid (15 mL, 1 M). Layers were separated, and the aqueous layer was extracted with EtOAc (2 × 15 mL). The combined organic layers were dried ($MgSO_4$), and solvent was evaporated. Product were purified by gradient flash column chromatography (silica gel, 70−230 mesh, hexane/EtOAc).

4.3.4. General Procedure 4 for the Oxidation of Alcohol Group. To a solution of alcohol (1 equiv) in DCM (2 mL), Dess−Martin reagent (2 equiv) was added. The reaction mixture was stirred at

room temperature for 1.5 h. An aqueous solution of sodium hydroxide (2 mL, 5%) was added, and the mixture was stirred for an additional 10 min. Then water (10 mL) and DCM (10 mL) were added, layers were separated, and the aqueous layer was extracted with DCM (3 × 10 mL). The combined organic layers were dried ($MgSO_4$), and solvent was evaporated. Product was purified by column chromatography (silica gel, 70−230 mesh, hexane/EtOAc).

4.4. Synthesis of Aldehydes (*S*,*S*,*S*)-(+)-2 (Cbz-L-leu-L-leu-L-leu-CHO) and (*S*,*R*,*S*)-(−)-2 (Cbz-L-leu-D-leu-L-leu-CHO). 4.4.1. Synthesis of Compounds (*S*,*S*,*S*)-(+)-12 (Cbz-L-leu-n(DMB)-L-leu-L-leu-CH_2OAc) and (*S*,*R*,*S*)-(−)-12 (Cbz-L-leu-n(DMB)-D-leu-L-leu-CH_2OAc). General Procedure 1. Products (*S*,*S*,*S*)-(+)-**12** and (*S*,*R*,*S*)-(−)-**12** were separated by triple flash column chromatography.

Compound (*S*,*S*,*S*)-(+)-12. Yield: 35%, 459 mg (0.69 mmol) of colorless oil. R_f = 0.38 (hexane/AcOEt, 7:3, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.69 (d, J = 6.3 Hz, 3H), 0.80−0.96 (m, 15H), 1.20−1.40 (m, 3H), 1.45−1.68 (m, 6H), 2.04 (s, 3H), 3.78 (s, 3H), 3.79 (s, 3H), 3.88 (dd, J = 6.0 Hz, J = 11.1 Hz, 1H), 3.96 (dd, J = 4.4 Hz, J = 11.1 Hz, 1H), 4.16 (octet, J = 4.5 Hz, 1H), 4.34 (d, J = 17.2 Hz, 1H), 4.62 (t, J = 7.7 Hz, 1H), 4.71 (d, J = 17.0 Hz, 1H), 5.08 (q, J = 11.9 Hz, 2H), 5.37 (d, J = 7.6 Hz, 1H), 6.43 (d, J = 3.9 Hz, 1H), 6.44 (d, J = 4.1 Hz, 1H), 6.76 (d, J = 8.8 Hz, 1H), 7.04 (d, J = 9.0 Hz, 1H), 7.30−7.40 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.7, 20.8, 22.0, 22.3, 22.6, 23.1, 23.5, 24.4, 24.7, 25.3, 36.9, 40.5, 42.3, 46.1, 50.2, 55.3, 55.4, 66.3, 66.9, 98.6, 103.8, 116.9, 128.0, 128.2, 128.5, 129.1, 136.2, 156.1, 158.0, 160.8, 170.2, 171.0, 175.3. HRMS calcd for $C_{37}H_{55}N_3O_8$Na [M + Na]$^+$: 692.3885. Found: 692.3862. [α] $^{25}_{D}$ +6.1 (*c* 1.0, $CHCl_3$). Retention time (method 1) t_R = 17.42 min.

Compound (*S*,*R*,*S*)-(−)- 12. Yield: 33%, 438 mg (0.65 mmol) of colorless oil. R_f = 0.30 (hexane/AcOEt, 7:3, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.80−0.96 (m, 18H), 1.12−1.40 (m, 3H), 1.44−1.63 (m, 4H), 1.65 (m, 2H), 1.99 (s, 3H), 3.77 (d, J = 4.3 Hz, 1H), 3.80 (s. 3H), 3.81 (s, 3H), 4.06−4.14 (m, 1H), 4.14−4.24 (m, 1H), 4.34 (d, J = 16.0 Hz, 1H), 4.62 (d, J = 16.2 Hz, 1H), 4.88 (t, J = 8.5 Hz, 1H), 5.04−5.16 (m, 2H), 5.53 (d, J = 8.6 Hz, 1H), 6.03 (d, J = 8.8 Hz, 1H), 6.43−6.49 (m, 2H), 7.11 (d, J = 8.8 Hz, 1H), 7.30−7.38 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.7, 21.3, 22.3, 22.6, 22.7, 23.3, 23.5, 23.6, 24.3, 24.6, 25.3, 29.7, 37.0, 40.5, 43.1, 45.9, 50.4, 55.3, 55.4, 66.3, 66.7, 98.7, 104.4, 116.2, 127.9, 128.0, 128.1, 128.3, 128.5, 130.0, 136.4, 155.9, 158.2, 161.1, 170.3, 170.9, 173.4. HRMS calcd for $C_{37}H_{55}N_3O_8$Na [M + Na]$^+$: 692.3881. Found: 692.3885. [α]$^{25}_{D}$ −54.2 (*c* 1.0, $CHCl_3$). Retention time (method 1) t_R = 15.39 min.

4.4.2. Synthesis of Compound (*S*,*S*,*S*)-(+)-13 (Cbz-L-leu-L-leu-L-leu-CH_2OAc). General Procedure 2. Yield: 91%, 265 mg (0.51 mmol) of white crystals. Mp 128−131 °C. R_f = 0.25 (hexane/AcOEt, 7:3, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87−0.97 (m, 18H), 1.26−1.33 (m, 1H), 1.37−1.45 (m, 1H), 1.45−1.55 (m, 2H), 1.56−1.71 (m, 4H), 1.74−1.85 (m, 1H), 2.04 (s, 3H), 4.00 (dd, J = 5.7 Hz, J = 11.1 Hz, 1H), 4.04−4.16 (m, 2H), 4.18−4.28 (m, 1H), 4.38−4.49 (m, 1H), 5.05 (d, J = 12.3 Hz, 1H), 5.14 (d, J = 12.1 Hz, 1H), 5.30 (d, J = 6.9 Hz, 1H), 6.37 (d, J = 6.7 Hz, 1H), 6.55 (br s, 1H), 7.30−7.39 (m, 5H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.9, 21.7, 22.0, 22.1, 22.8, 23.0, 24.7, 24.7, 24.9, 40.1, 40.3, 40.7, 46.8, 51.7, 54.1, 66.3, 67.2, 128.0, 128.3, 128.6, 135.9, 156.4, 171.2, 171.4, 172.2. HRMS calcd for $C_{28}H_{45}N_3O_6$Na [M + Na]$^+$: 542.3201. Found: 542.3193. [α]$^{25}_{D}$ +0.27 (*c* 1.0, $CHCl_3$). Retention time (method 3) t_R = 6.31 min.

4.4.3. Synthesis of Compound (*S*,*R*,*S*)-(−)-13 (Cbz-L-leu-D-leu-L-leu-CH_2OAc). General Procedure 2. Yield: 82%, 245 mg (0.47 mmol) of white crystals. Mp 144−146 °C. R_f = 0.24 (hexane/AcOEt, 7:3, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85−0.97 (m, 18H), 1.22−1.31 (m, 1H), 1.32−1.42 (m, 1H), 1.44−1.54 (m, 3H), 1.54−1.66 (m, 3H), 1.66−1.77 (m, 1H), 2.05 (s, 3H), 3.98−4.10 (m, 2H), 4.16−4.29 (m, 2H), 4.38 (q, J = 7.6 Hz, 1H), 5.04−5.18 (m, 2H), 5.39 (d, J = 7.6 Hz, 1H), 6.35 (d, J = 8.4 Hz, 1H), 6.61 (d, J = 7.8 Hz, 1H), 7.29−7.38 (m, 5H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.8, 22.0, 22.2, 22.6, 22.9, 23.0, 24.6, 24.8, 40.4, 40.6, 41.3, 46.4, 51.9, 53.6, 66.2, 67.1, 128.0, 128.3, 128.5, 136.0, 156.2, 171.0, 171.2, 172.2. HRMS calcd for $C_{28}H_{45}N_3O_6$Na [M + Na]$^+$: 542.3201. Found: 542.3175. [α]$^{25}_{D}$ −63.1 (*c* 1.0, $CHCl_3$). Retention time (method 3) t_R = 5.99 min.

4.4.4. Synthesis of Compound (*S*,*S*,*S*)-(+)-15 (Cbz-L-leu-L-leu-L-leu-CH_2OH). General Procedure 3. Yield: 95%, 204 mg (0.43 mmol) of white crystals. Mp 111−113 °C. R_f = 0.20 ($CHCl_3$/MeOH, 95:5, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86−0.96 (m, 18H), 1.18−1.30 (m, 1H), 1.32−1.43 (m, 1H), 1.44−1.57 (m, 2H), 1.57−1.68 (m, 4H), 1.68−1.80 (m, 1H), 3.26 (br s, 1H), 3.38 (dd, J = 6.4 Hz, J = 11.5 Hz, 1H), 3.61 (dd, J = 3.4 Hz, J = 11.4 Hz, 1H), 3.96−4.09 (m, 1H), 4.10−4.18 (m, 1H), 4.42−4.52 (m, 1H), 5.02 (d, J = 12.3 Hz, 1H), 5.11 (d, J = 12.2 Hz, 1H), 5.68 (d, J = 7.0 Hz, 1H), 6.85 (d, J = 8.3 Hz, 1H), 7.25 (br s, 1H), 7.29−7.36 (m, 5H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.9, 22.1, 22.8, 22.9, 23.1, 24.7, 24.8, 24.8, 39.9, 40.1, 41.0, 49.9, 52.0, 54.0, 65.2, 67.2, 128.0, 128.2, 128.5, 135.9, 156.6, 172.4, 173.1. HRMS calcd for $C_{26}H_{43}N_3O_5$Na [M + Na]$^+$: 500.3095. Found: 500.3093. [α]$^{25}_{D}$ 10.3 (*c* 1.0, $CHCl_3$). Retention time (method 2) t_R = 4.73 min.

4.4.5. Synthesis of Compound (*S*,*R*,*S*)-(−)-15 (Cbz-L-leu-D-leu-L-leu-CH_2OH). General Procedure 3. Yield: 84%, 148 mg (0.31 mmol) of white crystals. Mp 161−163 °C. R_f = 0.19 ($CHCl_3$/MeOH, 95:5, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85−0.96 (m, 18H), 1.27−1.36 (m, 1H), 1.36−1.46 (m, 1H), 1.46−1.56 (m, 2H), 1.56−1.67 (m, 4H), 1.67−1.78 (m, 1H), 2.76 (br s, 1H), 3.52 (dd, J = 5.7 Hz, J = 11.3 Hz, 1H), 3.65 (dd, J = 3.5 Hz, J = 11.3 Hz, 1H), 3.95−4.07 (m, 1H), 4.12−4.22 (m, 1H), 4.35−4.44 (m, 1H), 5.10 (s, 2H), 5.47 (d, J = 5.9 Hz, 1H), 6.72 (d, J = 6.3 Hz, 1H), 6.78 (d, J = 6.5 Hz, 1H), 7.30−7.38 (m, 5H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.0, 22.1, 22.9, 23.0, 24.7, 24.8, 24.9, 39.8, 40.4, 41.1, 50.2, 52.4, 54.0, 65.6, 67.3, 128.1, 128.3, 128.6, 135.9, 156.5, 172.1, 172.7. HRMS calcd for $C_{26}H_{43}N_3O_5$Na [M + Na]$^+$: 500.3095. Found: 500.3087. [α]$^{25}_{D}$ −63.6 (*c* 1.0, $CHCl_3$). Retention time (method 2) t_R = 4.47 min.

4.4.6. Synthesis of (*S*,*S*,*S*)-(+)-2 (Cbz-L-leu-L-leu-L-leu-CHO). General Procedure 4. Yield: 79%, 55 mg (0.12 mmol) of colorless oil. R_f = 0.22 ($CHCl_3$/MeOH, 95:5, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88−1.00 (m, 18H, CH(C**H**$_3$)$_2$), 1.38−1.48 (m, 1H, C**H**(CH$_3$)$_2$), 1.49−1.58 (m, 1H, C**H**(CH$_3$)$_2$), 1.60−1.74 (m, 6H, C**H**$_2$CH(CH$_3$)$_2$), 1.74−1.86 (m, 1H, C**H**(CH$_3$)$_2$), 4.04−4.15 (m, 1H, C**H**CHO), 4.40−4.49 (m, 1H, NHC**H**CO), 4.50−4.58 (m, 1H, NHC**H**CO), 5.00−5.14 (m, 2H, OC**H**$_2$Ph), 5.29 (d, J = 6.4 Hz, 1H, N**H**C(O)O), 6.59 (d, J = 8.3 Hz, 1H, N**H**CHCHO), 7.06 (d, J = 7.1 Hz, 1H, N**H**), 7.29−7.38 (m, 5H, Ar**H**), 9.49 (s, 1H, C**H**O). ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.6, 21.7, 22.1, 22.7, 23.0, 23.1, 24.7, 24.7, 24.9, 37.4, 40.1, 40.6, 51.6, 54.1, 57.2, 67.3, 128.0, 128.3, 128.6, 135.8, 156.5, 172.2, 172.6, 199.8. HRMS calcd for $C_{26}H_{41}N_3O_5$Na [M + Na]$^+$: 498.2938. Found: 498.2943. [α]$^{25}_{D}$ 37.1 (*c* 0.5, $CHCl_3$).

4.4.7. Synthesis of Compound (*R*,*R*,*R*)-(−)-2 (Cbz-D-leu-D-leu-D-leu-CHO). General Procedure 4. Yield: 76%, 35 mg (0.073 mmol) of colorless oil. R_f = 0.23 ($CHCl_3$/MeOH, 95:5, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88−0.96 (m, 18H, CH(C**H**$_3$)$_2$), 1.35−1.47 (m, 1H, C**H**(CH$_3$)$_2$), 1.47−1.57 (m, 1H, C**H**(CH$_3$)$_2$), 1.58−1.74 (m, 6H, C**H**$_2$CH(CH$_3$)$_2$), 1.74−1.86 (m, 1H, C**H**-(CH$_3$)$_2$), 4.05−4.15 (m, 1H, C**H**CHO), 4.38−4.49 (m, 1H, NHC**H**CO), 4.49−4.57 (m, 1H, NHC**H**CO), 4.98−5.15 (m, 2H, OC**H**$_2$Ph), 5.29 (d, J = 6.5 Hz, 1H, N**H**C(O)O), 6.56 (d, J = 8.8 Hz, 1H, N**H**CHCHO), 7.07 (d, J = 6.3 Hz, 1H, N**H**), 7.29−7.38 (m, 5H, Ar**H**), 9.49 (s, 1H, C**H**O). ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.6, 21.7, 22.1, 22.7, 23.0, 23.1, 24.7, 24.7, 24.9, 26.9, 37.4, 40.1, 40.7, 41.5, 51.6, 54.1, 57.2, 67.2, 128.0, 128.3, 128.6, 135.9, 156.5, 172.2, 172.6, 199.7. HRMS calcd for $C_{26}H_{41}N_3O_5$Na [M + Na]$^+$: 498.2938. Found: 498.2928. [α]$^{25}_{D}$ −36.5 (*c* 0.5, $CHCl_3$).

4.4.8. Synthesis of Compound (*S*,*R*,*S*)-(−)-2 (Cbz-L-leu-D-leu-L-leu-CHO). General Procedure 4. Yield: 63%, 39 mg (0.082 mmol) of colorless oil. R_f = 0.22 ($CHCl_3$/MeOH, 95:5, v/v).

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88−0.98 (m, 18H, CH(C**H**$_3$)$_2$), 1.40−1.58 (m, 3H, C**H**(CH$_3$)$_2$), 1.59−1.79 (m, 6H, C**H**$_2$CH-(CH$_3$)$_2$), 4.12−4.22 (m, 1H, C**H**CHO), 4.40−4.54 (m, 2H, NHC**H**CO), 5.10 (s, 2H, OC**H**$_2$Ph), 5.24 (d, J = 7.5 Hz, 1H, N**H**C(O)O), 6.50 (d, J = 7.9 Hz, 1H, N**H**CHCHO), 6.77 (d, J = 7.0 Hz, 1H, N**H**), 7.32−7.39 (m, 5H, Ar**H**), 9.53 (s, 1H, C**H**O). ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.7, 21.9, 22.0, 22.8, 22.9, 23.1, 24.6, 24.7, 24.8, 37.6, 40.3, 41.1, 51.7, 53.7, 57.2, 67.3, 128.0, 128.3, 128.6, 135.9, 156.4, 171.9, 172.3, 199.6. HRMS calcd for $C_{26}H_{41}N_3O_5Na$ [M + Na]$^+$: 498.2938. Found: 498.2951. $[\alpha]_D^{25}$ −57.3 (*c* 0.5, $CHCl_3$).

4.4.9. Synthesis of Compound (*R*,*S*,*R*)-(+)-2 (Cbz-D-leu-L-leu-D-leu-CHO). General Procedure 4. Yield: 25%, 15 mg (0.032 mmol) of colorless oil. R_f = 0.22 ($CHCl_3$/MeOH, 95:5, v/v). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88−0.98 (m, 18H, CH(C**H**$_3$)$_2$), 1.40−1.57 (m, 3H, C**H**(CH$_3$)$_2$), 1.58−1.79 (m, 6H, C**H**$_2$CH-(CH$_3$)$_2$), 4.12−4.22 (m, 1H, C**H**CHO), 4.40−4.52 (m, 2H, NHC**H**CO), 5.10 (s, 2H, OC**H**$_2$Ph), 5.28 (d, J = 7.6 Hz, 1H, N**H**C(O)O), 6.53 (d, J = 6.2 Hz, 1H, N**H**CHCHO), 6.77 (d, J = 7.0 Hz, 1H, N**H**), 7.30−7.38 (m, 5H, Ar**H**), 9.52 (s, 1H, C**H**O). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.8, 21.9, 22.0, 22.8, 22.9, 23.1, 24.7, 24.7, 24.8, 37.6, 40.4, 41.1, 51.8, 53.8, 57.3, 67.2, 128.0, 128.1, 128.3, 128.6, 128.6, 136.0, 156.4, 172.0, 172.4, 199.3. HRMS calcd for $C_{27}H_{45}N_3O_6Na$ [M + CH_3OH + Na]$^+$: 530.3201. Found: 530.3177. $[\alpha]_D^{25}$ 55.5 (*c* 0.5, $CHCl_3$).

4.5. Synthesis of Aldehydes (*R*,*I*,*S*)-2 (Cbz-D-leu-I-leu-L-leu-CHO) and (*R*,*II*,*S*)-2 (Cbz-D-leu-II-leu-L-leu-CHO). 4.5.1. Synthesis of Compounds (*R*,*I*,*S*)-12 (Cbz-D-leu-n(DMB)-I-leu-L-leu-CH$_2$OAc) and (*R*,*II*,*S*)-12 (Cbz-D-leu-n(DMB)-II-leu-L-leu-CH$_2$OAc). General Procedure 1. Products (*R*,*I*,*S*)-**12** and (*R*,*II*,*S*)-**12** were obtained as a mixture in 51:49 ratio. Yield: 82%, 1.098 g (1.64 mmol) of colorless oil. R_f = 0.65 (hexane/AcOEt, 7:3, v/v). ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.70 (d, J = 6.4 Hz, 2H), 0.74−1.06 (m, 16H), 1.06−1.40 (m, 3H), 1.40−1.70 (m, 6H), 2.03 (s, 3H), 3.77 (s, 3H), 3.79 (s, 3H), 3.85−4.45 (m, 3H), 4.50−5.0 (m, 3H), 5.02−5.15 (m, 2H), 5.34 (d, J = 6.9 Hz, 0.5H), 5.53 (d, J = 6.8 Hz, 0.5H), 6.02 (d, J = 7.2 Hz, 0.5H), 6.35−6.50 (m, 2H), 6.71 (d, J = 7.0 Hz, 0.5H), 7.04 (d, J = 7.4 Hz, 0.5H), 7.12 (d, J = 7.5 Hz, 0.5H), 7.35 (s, 6H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 21.2, 22.2, 22.5, 23.0, 23.4, 23.9, 25.0, 25.6, 37.3, 40.6, 42.7, 43.3, 45.8, 46.7, 50.5, 55.7, 58.6, 66.6, 67.1, 67.3, 99.0, 104.1, 117.2, 128.3, 128.8, 129.6, 156.4, 170.6, 171.2, 175.4. Anal. Calcd for $C_{37}H_{55}N_3O_8$: C, 66.34; H, 8.28; N, 6.27. Found: C, 66.18; H, 8.34; N, 6.12. Retention time of epimers (method 1): $t_{R(R,I,S)\text{-}12}$ = 19.48 min; $t_{R(R,II,S)\text{-}12}$ = 16.17 min.

4.5.2. Synthesis of Compounds (*R*,*I*,*S*)-(+)-14 (Cbz-D-leu-n-(DMB)-I-leu-L-leu-CH$_2$OH) and (*R*,*II*,*S*)-(−)-14 (Cbz-D-leu-n-(DMB)-II-leu-L-leu-CH$_2$OH). General Procedure 3. Products (*R*,*I*,*S*)-(+)-**14** and (*R*,*II*,*S*)-(−)-**14** were separated by flash column chromatography.

Compound (*R*, *I*,*S*)-(+)-14. Yield: 36%, 328 mg (0,52 mmol) of white crystals. Mp 80−83 °C. R_f = 0.46 (hexane/AcOEt, 7:3, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.76 (d, J = 6.2 Hz, 6H), 0.82−1.04 (m, 15H), 1.04−1.15 (m, 1H), 1.38−1.58 (m, 2H), 1.60−1.70 (m, 1H), 1.70−1.90 (m, 2H), 2.73 (br s, 1H), 3.13 (dd, J = 5.8, J = 11.5, 1H), 3.52 (dd, J = 2.6 Hz, J = 11.5 Hz, 1H), 3.81 (s, 6H), 3.86−3.95 (m, 1H), 4.07 (d, J = 14.9 Hz, 1H), 4.84 (d, J = 14.7 Hz, 1H), 5.02−5.11 (m, 1H), 5.12 (s, 2H), 5.41 (d, J = 8.6 Hz, 1H), 5.54 (d, J = 8.8 Hz, 1H), 6.50 (s, 2H), 7.24 (d, J = 7.9 Hz, 1H), 7.28−7.41 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.9, 21.0, 21.3, 21.9, 22.0, 22.3, 23.0, 23.3, 23.5, 24.4, 24.8, 25.3, 37.4, 39.9, 41.9, 48.3, 49.3, 50.7, 55.4, 55.5, 58.4, 65.4, 66.9, 99.2, 104.6, 115.1, 128.0, 128.4, 131.8, 136.4, 156.7, 158.8, 161.7, 170.3, 172.9. Anal. Calcd for $C_{35}H_{53}N_3O_7$: C, 66.96; H, 8.51; N, 6.69. Found: C, 66.84; H, 8.44; N, 6.48. $[\alpha]_D^{25}$ 29.4 (*c* 1.0, $CHCl_3$). Retention time (method 2) t_R = 14.18 min.

Compound (*R*,*II*,*S*)-(−)-14. Yield: 25%, 225 mg (0.36 mmol) of white crystals. Mp 55−58 °C. R_f = 0.34 (hexane/AcOEt, 7:3, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.73 (d, J = 6.4 Hz, 3H), 0.81−0.94 (m, 15H), 1.20−1.44 (m, 3H), 1.46−1.68 (m, 6H), 2.42 (br s, 1H), 3.43 (dd, J = 6.1 Hz, J = 11.3 Hz, 1H), 3.59 (dd, J = 3.1 Hz, J = 11.4 Hz, 1H), 3.79 (s, 3H), 3.80 (s, 3H), 3.82−3.89 (m, 1H), 4.39 (d, J = 16.5 Hz, 1H), 4.62−4.72 (m, 1H), 4.74 (d, J = 16.5 Hz, 1H), 5.04−5.16 (m, 2H), 5.40 (d, J = 7.7 Hz, 1H), 6.44 (s, 2H), 6.64 (d, J = 6.6 Hz, 1H), 7.11 (d, J = 8.6 Hz, 1H), 7.35 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.9, 22.2, 22.2, 22.7, 23.0, 23.5, 24.5, 24.8, 25.3, 37.0, 39.7, 42.0, 46.0, 50.2, 50.7, 55.3, 55.4, 58.3, 65.9, 67.0, 98.7, 103.9, 116.5, 128.0, 128.1, 128.5, 129.7, 136.2, 156.3, 158.2, 161.0, 171.5, 175.1. Anal. Calcd for $C_{35}H_{53}N_3O_7$: C, 66.96; H, 8.51; N, 6.69. Found: C, 66.63; H, 8.44; N, 6.45. $[\alpha]_D^{25}$ −29.7 (*c* 1.0, $CHCl_3$). Retention time (method 2) t_R = 11.42 min.

4.5.3. Synthesis of Compound (*R*,*I*,*S*)-(+)-15 (Cbz-D-leu-I-leu-L-leu-CH$_2$OH). General Procedure 2. Yield: 49%, 73 mg (0.152 mmol) of white crystals. Mp 124−127 °C. R_f = 0.21 ($CHCl_3$/MeOH, 95:5, v/v). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88−0.96 (m, 18H), 1.24−1.34 (m, 1H), 1.38−1.46 (m, 1H), 1.46−1.56 (m, 2H), 1.56−1.69 (m, 4H), 1.70−1.79 (m, 1H), 2.77 (br s, 1H), 3.40−3.47 (m, 1H), 3.58−3.66 (m, 1H), 3.97−4.06 (m, 1H), 4.11−4.19 (m, 1H), 7.38−4.46 (m, 1H), 5.05 (d, J = 12.1 Hz, 1H), 5.12 (d, J = 12.0 Hz, 1H), 5.40 (d, J = 5.2 Hz, 1H), 6.57 (d, J = 6.9 Hz, 1H), 6.73 (d, J = 7.2 Hz, 1H), 7.30−7.37 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.8, 22.1, 22.8, 22.9, 23.0, 24.8, 24.9, 39.9, 40.3, 41.0, 50.1, 52.1, 54.2, 65.5, 67.4, 128.1, 128.3, 128.6, 135.9, 156.5, 172.2, 172.8. HRMS calcd for $C_{26}H_{43}N_3O_5$-Na [M + Na]$^+$: 500.3095. Found: 500.3079. $[\alpha]_D^{25}$ 38.4 (*c* 1.0, $CHCl_3$). Retention time (method 2) t_R = 4.73 min.

4.5.4. Synthesis of Compound (*R*,*II*,*S*)-(−)-15 (Cbz-D-leu-II-leu-L-leu-CH$_2$OH). General Procedure 2. Yield: 61%, 88 mg (0.184 mmol) of white crystals. Mp 107−109 °C. R_f = 0.19 ($CHCl_3$/MeOH, 95:5, v/v). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87−0.97 (m, 18H), 1.29−1.36 (m, 1H), 1.38−1.47 (m, 1H), 1.49−1.56 (m, 2H), 1.57−1.68 (m, 4H), 1.75−1.85 (m, 1H), 2.47 (br s, 1H), 3.44−3.51 (m, 1H), 3.62 (dd, J = 3.5 Hz, J = 11.3 Hz, 1H), 3.95−4.03 (m, 1H), 4.03−4.10 (m, 1H), 4.35−4.42 (m, 1H), 5.05 (d, J = 12.1 Hz, 1H), 5.14 (d, J = 12.0 Hz, 1H), 5.34 (br s, 1H), 6.43 (br s, 1H), 6.66 (br s, 1H), 7.30−7.38 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.6, 22.1, 22.2, 22.7, 23.0, 24.7, 24.8, 25.0, 29.7, 39.8, 40.4, 40.8, 50.2, 52.6, 54.3, 65.5, 67.4, 128.0, 128.3, 128.6, 135.8, 156.6, 172.0, 172.7. HRMS calcd for $C_{26}H_{43}N_3O_5Na$ [M + Na]$^+$: 500.3095. Found: 500.3090. $[\alpha]_D^{25}$ −24.0 (*c* 1.0, $CHCl_3$). Retention time (method 2) t_R = 4.87 min.

4.5.5. Synthesis of Compound (*R*,*I*,*S*)-(+)-2 (Cbz-D-leu-I-leu-L-leu-CHO). General Procedure 4. Yield: 45%, 33 mg (0.069 mmol) of colorless oil. R_f = 0.23 ($CHCl_3$/MeOH, 95:5, v/v). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87−0.98 (m, 18H), 1.40−1.57 (m, 2H), 1.57−1.69 (m, 6H), 1.69−1.83 (m, 1H), 4.11−4.21 (m, 1H), 4.40−4.55 (m, 2H), 5.09 (s, 2H), 5.24 (d, J = 5.9 Hz, 1H), 6.49 (d, J = 8.2 Hz, 1H), 6.86 (d, J = 6.5 Hz, 1H), 7.30−7.38 (m, 5H), 9.49 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.7, 21.8, 22.9, 22.9, 23.1, 24.7, 24.8, 24.9, 29.7, 37.4, 40.3, 41.0, 51.6, 54.2, 57.3, 67.4, 128.0, 128.0, 128.3, 128.6, 135.9, 156.6, 172.1, 172.3, 199.5. HRMS calcd for $C_{27}H_{45}N_3O_6Na$ [M + CH_3OH + Na]$^+$: 530.3201. Found: 530.3212. $[\alpha]_D^{25}$ 45.9 (*c* 0.5, $CHCl_3$).

4.5.6. Synthesis of Compound (*S*,*I*,*R*)-(−)-2 (Cbz-L-leu-I-leu-D-leu-CHO). General Procedure 4. Yield: 40%, 19 mg (0.041 mmol) of colorless oil. R_f = 0.23 ($CHCl_3$/MeOH, 95:5, v/v). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.80−1.02 (m, 18H), 1.42−1.57 (m, 2H), 1.58−1.69 (m, 6H), 1.69−1.82 (m, 1H), 4.13−4.20 (m, 1H), 4.40−4.56 (m, 2H), 5.09 (s, 2H), 5.30 (d, J = 5.9 Hz, 1H), 6.57 (d, J = 8.1 Hz, 1H), 6.91 (d, J = 6.8 Hz, 1H), 7.30−7.38 (m, 5H), 9.48 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.7, 21.8, 22.8, 22.9, 23.1, 24.7 24.8, 24.9, 29.7, 37.4, 40.3, 41.0, 51.6, 54.2, 57.3, 67.4, 128.0, 128.1, 128.4, 128.6, 135.9, 156.6, 172.1, 172.4, 199.6. HRMS calcd for $C_{26}H_{41}N_3O_5Na$ [M + Na]$^+$: 498.2938. Found: 498.2947. $[\alpha]_D^{25}$ −47.1 (*c* 0.5, $CHCl_3$).

4.5.7. Synthesis of Compound (*R*,*II*,*S*)-(−)-2 (Cbz-D-leu-II-leu-L-leu-CHO). General Procedure 4. Yield: 56%, 30 mg (0.063 mmol) of colorless oil. R_f = 0.21 ($CHCl_3$/MeOH, 95:5, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86−0.99 (m, 18H), 1.39−1.57 (m,

2H), 1.58−1.73 (m, 6H), 1.75−1.85 (m, 1H), 4.04−4.15 (m, 1H), 4.38−4.46 (m, 1H), 4.46−4.58 (m, 1H), 4.97−5.06 (m, 1H), 5.06−5.13 (m, 1H), 5.25 (d, J = 5.9 Hz, 1H), 6.41 (d, J = 6.5 Hz, 1H), 7.07 (br s, 1H), 7.30−7.36 (m, 5H), 9.45 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 21.6, 21.7, 22.1, 22.8, 23.0, 23.1, 23.1, 24.7, 24.7, 24.9, 40.3, 40.9, 51.8, 54.2, 57.2, 67.3, 128.0, 128.3, 128.6, 135.9, 156.5, 172.1, 172.5, 199.5. HRMS calcd for $C_{26}H_{41}N_3O_5$Na $[M + Na]^+$: 498.2938. Found: 498.2937. $[\alpha]_D^{25}$ −27.1 (c 0.5, $CHCl_3$).

4.5.8. Synthesis of Compound (*R*,*II*,*S*)-(+)-2 (Cbz-L-leu-II-leu-D-leu-CHO). General Procedure 4. Yield: 32%, 15 mg (0.031 mmol) of colorless oil. R_f = 0.22 ($CHCl_3$/MeOH, 95:5, v/v). ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.89−0.97 (m, 18H), 1.40−1.58 (m, 2H), 1.60−1.72 (m, 6H), 1.74−1.85 (m, 1H), 4.06−4.14 (m, 1H), 4.39−4.45 (m, 1H), 4.46−4.54 (m, 1H), 4.98−5.05 (m, 1H), 5.07−5.12 (m, 1H), 5.28 (d, J = 6.5 Hz, 1H), 6.46 (d, J = 7.3 Hz, 1H), 7.00 (br s, 1H), 7.29−7.38 (m, 5H), 9.49 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 21.7, 21.7, 22.1, 22.7, 23.0, 23.1, 24.7, 24.7, 24.9, 37.4, 40.4, 41.0, 51.9, 54.2, 57.2, 67.3, 128.0, 128.3, 128.6, 135.9, 156.5, 172.1, 172.5, 199.6. HRMS calcd for $C_{26}H_{41}N_3O_5$Na $[M + Na]^+$: 498.2938. Found: 498.2945. $[\alpha]_D^{25}$ 29.0 (c 0.5, $CHCl_3$).

4.6. Cell Lines and Reagents. Murine multiple myeloma (J588L) and murine breast cancer (EMT6) cell lines were purchased from ATCC (Manassas, VA). Cells were cultured in RPMI-1640 (J588L) or Dulbecco's modified Eagle's medium (EMT6) supplemented with 10% heat-inactivated fetal calf serum, antibiotics, 2-mercaptoethanol (50 mM), and L-glutamine (2 mM) (all from Invitrogen, Carlsbad, CA). MG132 was purchased from Calbiochem/EMD (San Diego, CA) and was dissolved in DMSO to 10 mM stock concentration.

4.7. Cytotoxic/Cytostatic Assays. The cytostatic/cytotoxic effects in EMT6 cells were measured using crystal violet staining. Briefly, tumor cells were dispensed into 96-well plates (Sarstedt, Numbrecht, Germany) at 1.5×10^3 cells per well and allowed to attach overnight. The following day the investigated agents were added at indicated concentrations. After 24 h of incubation the cells were rinsed with PBS and stained with 0.5% crystal violet in 2% ethanol for 10 min at room temperature. Next, plates were washed four times with tap water and cells were lysed with 1% SDS solution. Absorbance was measured at 595 nm using an ELISA reader (Bio-Rad, Hercules, CA).

The cytostatic/cytotoxic effects in J588L cells were measured using standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, tumor cells were dispensed into 96-well plates (Sarstedt) at 3×10^3 cells per well. The following day the investigated agents were added at indicated concentrations. MTT solution at a concentration of 1 mg/mL was added to each well for the last 4 h of incubation. Then the cells were lysed using resuspension buffer containing SDS (0.2 g/mL) and DMF (0.5 mL/mL) at pH 4.7 and left overnight in the incubator. On the following day the absorbance was measured at 570 nm using an ELISA reader (Bio-Rad).

Cytotoxicity was expressed as relative viability of cells (% of control cultures incubated with medium only) and was calculated as follows: relative viability = $(A_e - A_b) \times 100/(A_c - A_b)$, where A_b is the background absorbance, A_e is experimental absorbance, and A_c is the absorbance of untreated controls.

4.8. Proteasome Activity. To determine the inhibition of Suc-Leu-Leu-Val-Tyr-AMC cleavage in lysates of J588L and EMT6 cells, the cells were trypsinized (EMT6), washed with PBS, and lysed in 0.05 M Tris-HCl buffer (pH 7.6) without protease inhibitors using freeze−thaw cycles and 10 stokes through a 26G needle. Then the lysates were centrifuged for 10 min at 12 000 rpm at 4 °C and protein concentration in supernatants was estimated using Bio-Rad Bradford's protein assay (BioRad). Equal amounts of total protein (25 μg per well) were dispensed into a black 96-well plate (Fluotrac 200, Greiner Bio-One, Monroe, NC). After addition of tested compounds at 100 or 1000 nM, proteasome activity was determined using 25 μM fluorogenic substrate Suc-leu-leu-Val-Tyr-AMC (Suc = succinyl; AMC = 7-amido-4-methylcoumarin) (Bachem, Weil am Rhein, Germany). Purified 20S proteasomes isolated from human erythrocytes were purchased from Enzo Life Sciences (www.enzolifesciences.com). Purified 20S proteasomes (300 ng) were incubated with 20 μM fluorogenic peptide substrates for chymotrypsin-like, PGPH, and trypsin-like proteasomal activities (Suc-leu-leu-Val-Tyr-AMC, Z-leu-leu-Glu-βNA and Ac-Arg-leu-Arg-AMC, respectively, all purchased from Bachem, Weil am Rhein, Germany) in 100 μL assay buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM NaN_3, 1 mM DTT) in the presence of tested compounds for 30 min at 37 °C, followed by measurement of hydrolysis of the fluorogenic substrates using a Victor2 D fluorometer (Perkin-Elmer, Waltham, MA) equipped with 355 nm excitation and 460 nm emission filters. Corresponding solvent (DMSO) concentrations were used for the controls.

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Supporting Information Available: Cytostatic/cytotoxic effects exerted by MG-132 and its stereoisomers against J558L and EMT6 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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