Expedited Articles

Structure-Based Design of Cathepsin K Inhibitors Containing a Benzyloxy-Substituted Benzoyl Peptidomimetic†

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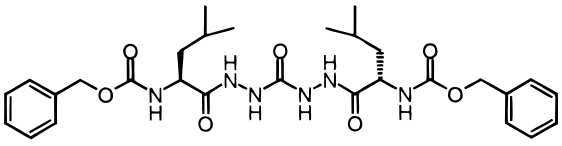
Peptidomimetic cathepsin K inhibitors have been designed using binding models which were based on the X-ray crystal structure of an amino acid-based, active site-spanning inhibitor complexed with cathepsin K. These inhibitors, which contain a benzyloxybenzoyl group in place of a Cbz-leucine moiety, maintained good inhibitory potency relative to the amino acid-based inhibitor, and the binding models were found to be very predictive of relative inhibitor potency. The binding mode of one of the inhibitors was confirmed by X-ray crystallography, and the crystallographically determined structure is in close qualitative agreement with the initial binding model. These results strengthen the validity of a strategy involving iterative cycles of structure-based design, inhibitor synthesis and evaluation, and crystallographic structure determination for the discovery of peptidomimetic inhibitors.

Cysteine proteases have been broadly implicated as targets for therapeutic intervention (e.g., cancer, arthritis, viral and parasitic diseases).[1] Cathepsin K, a cysteine protease of the papain superfamily, has been implicated in the process of bone resorption.[2,3] Selective inhibitors of cathepsin K could therefore be promising therapeutic agents for the treatment of diseases characterized by excessive bone loss, such as osteoporosis.

The structure-based design and X-ray crystallographic analysis of a new class of active site-spanning cathepsin K inhibitors which contain a 1,5-diacylcarbohydrazide scaffold have recently been reported from these laboratories.[4] Compound **1**, for example, is a



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potent, selective, kinetically irreversible inhibitor of cathepsin K but contains many groups, such as amino acid residues, with hydrogen-bond potential thought to have a negative impact on oral bioavailability. A key aspect of the design of therapeutically useful enzyme inhibitors has been the replacement of amino acid residues by isosteric elements. The design of such elements has met with much success in the recent past, owing greatly to the availability of crystallographic data from enzyme/inhibitor complexes. The present report describes the structure-based design and synthesis of potent 1,5-diacylcarbohydrazides which contain a benzyloxy-substituted benzoyl group as a mimic for a benzyloxycarbonyl (Cbz) leucine residue.

Compound **1** exhibits time-dependent kinetics consistent with an essentially irreversible mechanism of inhibition. Mass spectral and NMR analysis of the complex of **1** with cathepsin K give results consistent with formation of an acyl enzyme intermediate with cleavage of one of the acylhydrazine bonds and loss of Cbz-leucinylhydrazine as reported previously.[4] Nonetheless, X-ray crystallographic studies of the complex of **1** with cathepsin K show both halves of the inhibitor present in the active site with the inhibitor bound across both S and S′ sides (nomenclature of Schechter and Berger).[5] Examination of the X-ray crystal structure of **1** bound to cathepsin K revealed a nearly coplanar arrangement between the leucine carbonyl and the Cα−N bond in the half of the inhibitor bound on the S′ side of the active site. This particular arrangement of bonds suggested that a benzoyl group could function effectively as an achiral replacement for the leucine residue. Furthermore, the aromatic portion of the Cbz group, which appears to be involved in a $\pi-\pi$ interaction with the aromatic side chain of Trp-184, a residue that is conserved throughout this family of proteases, was in a position such that attachment of a benzyloxy substituent in either the ortho- or meta-position of the

† The refined coordinates for the complexes of compounds **1** and **3** with cathepsin K have been deposited at the Protein Data Bank under the filenames 1AYU and 1AYW, respectively.

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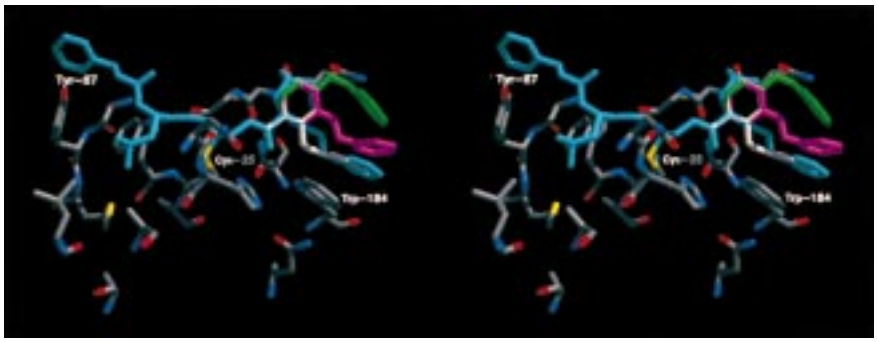
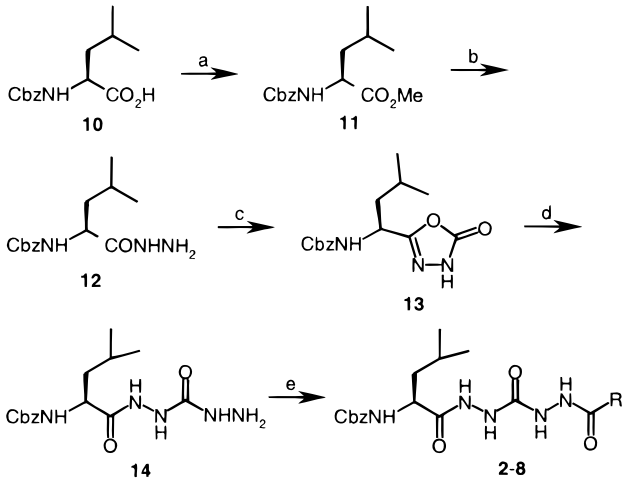


Figure 1. Stereoviews of binding models for ortho-substituted (white), meta-substituted (magenta), and para-substituted (green) benzoyl peptidomimetics superimposed with inhibitor **1** (cyan). Selected active site residues are colored by atom (C = gray, O = red, N = blue, S = yellow).

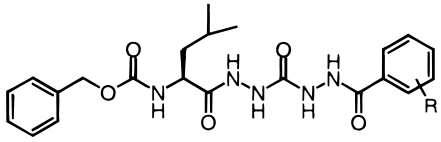
Scheme 1[a]



[a] (a) $PhCH_2OCOCl$, Na_2CO_3, 1,4-dioxane; (b) $H_2NNH_2 \cdot H_2O$, methanol; (c) $COCl_2$, toluene, reflux; (d) $H_2NNH_2 \cdot H_2O$, ethanol; (e) RCO_2H, EDC·HCl, 1-HOBT, DMF.

benzoyl group could result in effective mimicry of the entire Cbz-leucine moiety. Indeed, binding models of *o*-benzyloxy-substituted (white) and *m*-benzyloxy-substituted (magenta) benzoyl groups show very good overlap with the Cbz-leucine moiety when superimposed with inhibitor **1**, while para-substitution (green) does not allow for effective overlap of the benzyloxy groups (Figure 1). Note, however, that the flat nature of the benzoyl group precludes mimicry of both of the γ-methyl groups of the leucine side chain. These models therefore predict that compounds **2** and **3** will be more potent inhibitors of cathepsin K than is compound **4** or the unsubstituted benzoyl analogue **5** but may be less potent than the Cbz-leucine analogue **1** because of the failure to fully mimic the leucine side chain.

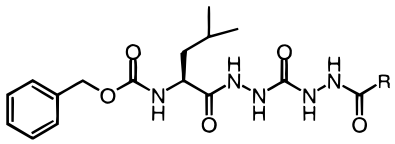
Compounds **2**−**8** were prepared as shown in Scheme 1. Compounds **2**−**5** are all time-dependent inhibitors of cathepsin K, exhibiting apparent second-order rate constants (k_{obs}/[I]) in the range of 38 000−318 000 M^{-1} s^{-1} (Table 1). The ortho- and meta-substituted analogues **2** and **3** are about 1 order of magnitude less potent than **1**, while the para-substituted analogue **4** is about 2 orders of magnitude less potent. The equivalent potency of compounds **2** and **3** suggests that phenyl portions of the benzyloxy groups in the two inhibitors are likely occupying similar positions in the cathepsin K active site, and this is easily accommodated by rotation about the C−O−C−C bonds of the benzyloxy



2: R = *o*-benzyloxy
3: R = *m*-benzyloxy
4: R = *p*-benzyloxy
5: R = H

group. A benzyloxy group in the para-position cannot occupy this space due to geometric constraints. Indeed, the unsubstituted benzoyl analogue **5** is equivalent in potency to **4**, suggesting that substitution of the benzyloxy group in the para-position does not allow for any interaction of this group with the enzyme. This result is in contrast to that reported for a structurally similar class of active site-spanning 1,3-diamino ketone cathepsin K inhibitors in which an inhibitor containing a *p*-phenoxybenzoyl peptidomimetic retained the potency of the Cbz-leucine analogue,[7] but this may be a consequence of the inherent differences in the positions that can be occupied by the terminal phenyl group in a phenoxy substituent relative to a benzyloxy substituent. The decrease in potency observed for compounds **2** and **3** relative to the Cbz-leucine analogue **1** appears to be a result of the inability of the benzoyl group to mimic the γ-methyl groups of the leucine side chain. Indeed, a similar decrease in potency is observed when the leucine residue in **1** is replaced with alanine (compound **6**) or (*S*)-2-aminobutyric acid (compound **7**), while replacement with L-norvaline did not lead to a decrease in potency (compound **8**), indicating that only one of the γ-methyl groups of the leucine side chain is necessary to achieve the high potency of compound **1**. The relative binding affinities of compounds **1**−**8** as measured by $K_{i,app}$, determined from initial rates of substrate hydrolysis (Table 1), indicate that the differences in potency are largely a result of binding interactions but appear to be influenced to some degree by rate differences in the time-dependent step in the inhibition mechanism. For example, the 10-fold decrease in apparent second-order rate constant for inhibition by compound **7** relative to compound **8** appears to be governed by a difference in the rate of the time-dependent step in the inhibition mechanism as well as binding affinity ($K_{i,app}$) since the difference between $K_{i,app}$ values for compounds **7** and **8** is only about 4-fold. Nonetheless, these data are wholly consistent with predictions based on the binding models.

Table 1. Inhibition of Cathepsin K by Compounds **1**−**6**[a]



compd	RCO	formula	k_{obs}/[I] (M^{-1} s^{-1})[b]	$K_{i,app}$ (nM)[b]
1	Cbz-L-leucine	$C_{29}H_{40}N_6O_7$	3 100 000	0.7
2	*o*-benzyloxybenzoyl	$C_{29}H_{33}N_5O_6$	318 000	12
3	*m*-benzyloxybenzoyl	$C_{29}H_{33}N_5O_6$	224 000	27
4	*p*-benzyloxybenzoyl	$C_{29}H_{33}N_5O_6$	44 000	100
5	benzoyl	$C_{22}H_{27}N_5O_5$	38 000	120
6	Cbz-L-alanine	$C_{26}H_{34}N_6O_7$	685 000	1.0
7	Cbz-(*S*)-2-aminobutyric acid	$C_{27}H_{36}N_6O_7 \cdot 0.5H_2O$	490 000	3.4
8	Cbz-L-norvaline	$C_{28}H_{38}N_6O_7 \cdot 0.5H_2O$	4 000 000	0.8

[a] Assays were conducted as previously described.[6] [b] Average standard errors for the k_{obs}/[I] and $K_{i,app}$ values were ±3−10%.

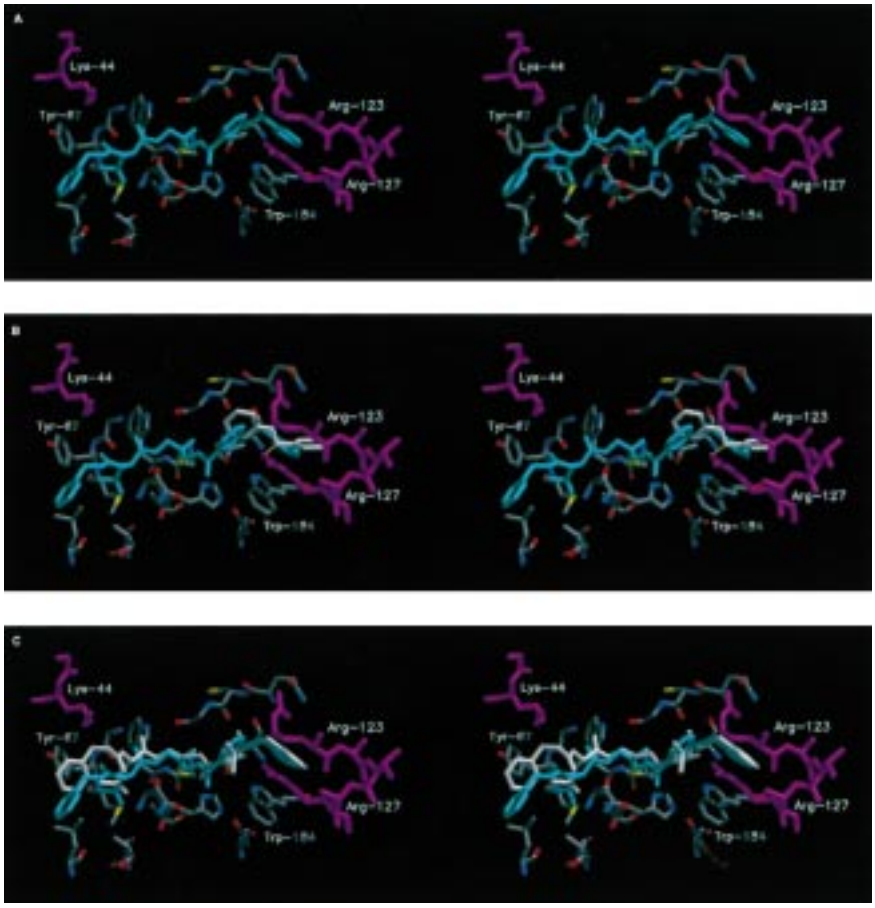
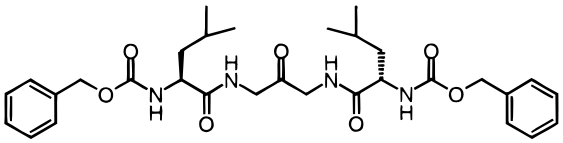


Figure 2. (A) Stereoview of **3** (cyan) bound in the active site of cathepsin K. Side chains from neighboring cathepsin K molecules in the crystal lattice are shown in magenta. Selected active site residues are colored by atom (C = gray, O = red, N = blue, S = yellow). (B) Overlay of the bound conformation of **3** (cyan) with the binding model for its peptidomimetic portion (white) shown in stereoview with selected active site residues. (C) Overlay of the bound conformations of **3** (cyan) and **9** (white) shown in stereoview with selected active site residues.

Because of the equivalent potency and the likelihood of similar binding modes of compounds **2** and **3**, only one of them, compound **3**, was selected initially for cocrystallization with cathepsin K. The complex of compound **3** with cathepsin K crystallized under conditions similar to that reported for the cathepsin K:**1** complex,[4] except in a different crystal form. X-ray analysis of this complex at 2.4 Å shows the inhibitor bound across both S and S′ sides of the active site with a mode of binding consistent with addition of the catalytic cysteine thiol to the central urea carbonyl (Figure 2A). On the S side of the active site, the leucine side chain is bound in the hydrophobic S2 pocket. The phenyl portion of the Cbz group appears to participate in an edge−face aromatic−aromatic interaction with Tyr-67, although from the opposite face of the tyrosine ring than is observed in the cathepsin K:**1** complex (Figure 1). The centroid separation and dihedral angle for this interaction are 6.9 Å and 66°, respectively, both within the range preferred for an aromatic−aromatic interaction as defined by Burley and Petsko.[8] In the cathepsin K:**3** complex, binding of the Cbz group in the same site occupied by the Cbz group in the cathepsin K:**1** complex is precluded by the presence of the side

chain of Lys-44 from a neighboring cathepsin K molecule in the crystal lattice. There are also differences on the S′ side of the active site where, in the complex of **3** with cathepsin K, the side chains of two arginine residues (Arg-123 and Arg-127), also from a neighboring cathepsin K molecule, occupy a portion of the active site. Nonetheless, an apparent $\pi-\pi$ interaction with a centroid separation and dihedral angle of 4.9 Å and 39°, respectively, is observed between the benzyloxy portion of the peptidomimetic and the side chain of Trp-184, as was qualitatively predicted from the binding model.

Superposition of the bound conformation of **3** (cyan) with the binding model of its peptidomimetic portion (white) shows a good, but not perfect, match between them (Figure 2B). In this overlay, a number of close contacts (1.9−3 Å carbon−carbon distance) are observed between the Arg-127 side chain and various points on the benzyloxy portion of the peptidomimetic model. One such contact is between N_ω of Arg-127 and the benzylic carbon of the benzyloxy group (1.9 Å) and is highlighted in Figure 2B (yellow dash). In the cathepsin K:**3** complex, the corresponding nonbonded distance is 3.4 Å, and this may be a result of a slight movement in the position and/or conformation of the inhibitor to accommodate the presence of the arginine side chains. Thus, the differences between the bound conformation of **3** as observed in its complex with cathepsin K and the binding model may be at least partially a consequence of the particular crystal form in which the complex crystallized. Indeed, superposition of the bound conformation of **3** with that of a structurally similar Cbz-leucine-containing active site-spanning inhibitor[7] (compound **9**) which crystallizes in the same crystal form shows a very good match between all elements of the two inhibitors (Figure 2C). The bound conformation of **3** in its complex with cathepsin K is nonetheless in close qualitative agreement with the initial binding model.



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In conclusion, we have used the crystal structure of a cathepsin K/inhibitor complex to effectively design peptidomimetic inhibitors containing a benzyloxybenzoyl mimic of Cbz-leucine through the use of binding models. The models were subsequently validated by SAR studies and crystallographic analysis of the modeled inhibitor complexed with cathepsin K. The strategy involving iterative cycles of structure-based design, inhibitor synthesis and evaluation, and crystallographic structure determination has thus proven to be a viable approach to obtain potent cathepsin K inhibitors wherein an amino acid element has been replaced with an achiral surrogate.

Experimental Section

***N*-Benzyloxycarbonyl-L-leucine Methyl Ester (11).** To a stirring solution of 2.0 g (11 mmol) of L-leucine methyl ester hydrochloride in 20 mL of 1,4-dioxane was added 12.1 mL (24.2 mmol) of a 2 M aqueous solution of Na_2CO_3 followed by 1.96 g (11.5 mmol, 1.64 mL) of benzyl chloroformate. The mixture was allowed to stir at room temperature for 4 h and then partitioned between ethyl acetate and water. The organic layer was washed with saturated brine, dried ($MgSO_4$), filtered, and concentrated to give 3.1 g (100%) of **11** as a colorless oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 (m, 5H), 5.27 (d, 1H), 5.12 (s, 2H), 4.41 (s, 2H), 3.75 (s, 3H), 1.65 (m, 3H), 0.96 (m, 6H); MS (ESI) 302.1 $(M + Na)^+$.

***N*-Benzyloxycarbonyl-L-leucinehydrazide (12).** To a stirring solution of 3.1 g (11.0 mmol) of **11** in 15 mL of methanol was added 5.9 g (118 mmol, 5.73 mL) of hydrazine hydrate. The solution was allowed to stir at room temperature for 16 h and then concentrated to give 3.1 g (100%) of **12** as an off-white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.36 (bs, 1H), 7.30 (m, 5H), 5.77 (d, 1H), 5.06 (dd, 2H), 4.19 (m, 1H), 3.80 (b s, 2H), 1.62 (m, 2H), 1.54 (m, 1H), 0.89 (m, 6H); MS (ESI) 280.2 $(M + H)^+$.

1-(*N*-Benzyloxycarbonylamino)-3-methyl-1-(5-oxo-1,3,4-oxadiazol-2-yl)butane (13). To a solution of 0.5 g (1.79 mmol) of **12** in 10 mL of toluene was added 9.27 mL (17.9 mmol) of a 1.93 M solution of phosgene in toluene. After heating for 4 h at reflux, the solution was concentrated to an oily residue which was purified by column chromatography (silica gel, ethyl acetate/hexane) to give 0.427 g (78%) of **13** as an off-white foam: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.18 (s, 1H), 7.38 (m, 5H), 5.13 (m, 3H), 4.79 (m, 1H), 1.71 (m, 3H), 0.98 (dd, 6H); MS (ESI) 306.1 $(M + H)^+$.

2-(*N*-Benzyloxycarbonyl-L-leucinyl)carbohydrazide (14). To a solution of 0.427 g (1.4 mmol) of **13** in 2 mL of ethanol was added 0.7 g (14 mmol, 0.68 mL) of hydrazine hydrate. After stirring for 24 h at room temperature, the solution was concentrated to give 0.472 g (100%) of **14** as an off-white foam: ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.25 (bs, 1H), 8.03 (bs, 1H), 7.29 (m, 6H), 6.12 (d, 1H), 5.13 (d, 1H), 4.97 (d, 1H), 4.33 (m, 1H), 3.87 (m, 2H), 1.61 (m, 3H), 0.89 (m, 6H); MS (ESI) 338.2 $(M + H)^+$.

General Procedure for the Preparation of Benzyloxybenzoate Esters. To a suspension of 395 mg (9.87 mmol) of NaH (60% in mineral oil) in 20 mL of DMF was added 1.0 g (6.58 mmol) of the hydroxybenzoate methyl ester. After stirring for 15 min at room temperature, 1.1 g (6.58 mmol, 0.76 mL) of benzyl bromide was added. After stirring at room temperature for 3 h, the solution was partitioned between ethyl acetate and water. The organic layer was washed with water (2 × 75 mL), saturated aqueous sodium bicarbonate, and saturated brine, then dried ($MgSO_4$), filtered, and concentrated to give the desired product.

General Procedure for the Preparation of Benzyloxybenzoic Acids. To a solution of 400 mg (1.65 mmol) of the benzoate methyl ester in 2 mL of THF and 2 mL of water was added 76 mg (1.82 mmol) of lithium hydroxide monohydrate. After stirring at reflux for 5 h, the solution was partitioned between ethyl acetate and 3 N HCl. The organic layer was washed with saturated brine, dried ($MgSO_4$), filtered, and concentrated to give the desired product.

General Procedure for the Coupling of 14 with Carboxylic Acids. To a stirring solution of 100 mg (0.3 mmol) of **14**, a carboxylic acid (0.315 mmol), and 8 mg (0.06 mmol) of 1-hydroxybenzotriazole in 3 mL of DMF was added 60 mg (0.312 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride. After stirring for 16 h at room temperature, the solution was poured into water and filtered to give the desired product.

Spectral data for compounds **2**−**8** are as follows.

2-(2-Benzyloxybenzoyl)-2′-(*N*-benzyloxycarbonyl-L-leucinyl)carbohydrazide (2): ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD) δ 7.89 (d, 1H), 7.34−7.16 (m, 11H), 6.94 (d, 2H), 5.14 (m, 2H), 4.95 (dd, 2H), 4.08 (m, 1H), 1.60−1.43 (m, 3H), 0.81 (m, 6H); MS (ESI) 548.1 $(M + H)^+$. Anal. Calcd for $C_{29}H_{33}N_5O_6 \cdot 0.5H_2O$: C, 62.58; H, 6.16; N, 12.58. Found: C, 62.41; H, 5.44; N, 13.29.

2-(3-Benzyloxybenzoyl)-2′-(*N*-benzyloxycarbonyl-L-leucinyl)carbohydrazide (3): ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD) δ 7.46 (s, 1H), 7.39−7.26 (m, 12H), 7.07 (dd, 1H), 5.02 (m, 4H), 4.14 (m, 1H), 1.61 (m, 2H), 1.50 (m, 1H), 0.86 (m,

6H); MS (ESI) 548.1 $(M + H)^+$. Anal. Calcd for $C_{29}H_{33}N_5O_6 \cdot 0.1H_2O$: C, 63.40; H, 6.09; N, 12.75. Found: C, 63.02; H, 5.37; N, 13.27.

2-(4-Benzyloxybenzoyl)-2′-(*N*-benzyloxycarbonyl-L-leucinyl)carbohydrazide (4): ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD) δ 7.77 (d, 2H), 7.37−7.23 (m, 10H), 6.95 (d, 2H), 5.03 (m, 4H), 4.17 (m, 1H), 1.61 (m, 2H), 1.50 (m, 1H), 0.88 (m, 6H); MS (ESI) 548.1 $(M + H)^+$. Anal. Calcd for $C_{29}H_{33}N_5O_6$: C, 63.60; H, 6.07; N, 12.79. Found: C, 63.45; H, 5.87; N, 13.37.

2-Benzoyl-2′-(*N*-benzyloxycarbonyl-L-leucinyl)carbohydrazide (5): ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ 7.75 (d, 2H), 7.48−7.17 (m, 8H), 5.00 (d, 2H), 4.12 (m, 1H), 1.54 (m, 3H), 0.86 (m, 6H); MS (ESI) 442.1 $(M + H)^+$. Anal. ($C_{22}H_{27}N_5O_5$) C, H, N.

2-(*N*-Benzyloxycarbonyl-L-alanyl)-2′-(*N*-benzyloxycarbonyl-L-leucinyl)carbohydrazide (6): ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ 7.22 (m, 10H), 5.00 (m, 4H), 4.13 (m, 2H), 1.54 (m, 3H), 1.31 (d, 3H), 0.87 (m, 6H); MS (ESI) 543.1 $(M + H)^+$. Anal. Calcd for $C_{26}H_{34}N_6O_7$: C, 57.55; H, 6.32; N, 15.49. Found: C, 57.48; H, 5.85; N, 15.04.

2-(*N*-Benzyloxycarbonyl-(*S*)-2-aminobutyroyl)-2′-(*N*-benzyloxycarbonyl-L-leucinyl)carbohydrazide (7): ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ 7.26 (m, 10H), 5.00 (q, 4H), 4.12 (m, 1H), 4.04 (m, 1H), 1.78 (m, 1H), 1.55 (m, 4H), 0.86 (m, 9H); MS (ESI) 579.3 $(M + Na)^+$. Anal. ($C_{27}H_{36}N_6O_7 \cdot 0.5H_2O$) C, H, N.

2-(*N*-Benzyloxycarbonyl-L-leucinyl)-2′-(*N*-benzyloxycarbonyl-L-norvalinyl)carbohydrazide (8): ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$) δ 7.24 (m, 10H), 5.00 (q, 4H), 4.12 (m, 2H), 1.68 (m, 1H), 1.55 (m, 4H), 1.31 (m, 2H), 0.87 (m, 9H); MS (ESI) 571.3 $(M + H)^+$. Anal. ($C_{28}H_{38}N_6O_7 \cdot 0.5H_2O$) C, H, N.

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