

Selective, Tight-Binding Inhibitors of Integrin $\alpha 4\beta 1$ That Inhibit Allergic Airway Responses

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Integrin $\alpha 4\beta 1$ mediates leukocyte recruitment, activation, mediator release, and apoptosis inhibition, and it plays a central role in inflammatory pathophysiology. High-affinity, selective inhibitors of $\alpha 4\beta 1$, based on the Leu-Asp-Val (LDV) sequence from the alternatively spliced connecting segment-1 (CS-1) peptide of cellular fibronectin, are described that employ a novel N-terminal peptide "cap" strategy. One inhibitor, BIO-1211, was $\sim 10^6$ -fold more potent than the starting peptide and exhibited tight-binding properties ($k_{\text{off}} = 1.4 \times 10^{-4} \text{ s}^{-1}$, $K_{\text{D}} = 70 \text{ pM}$), a remarkable finding for a noncovalent, small-molecule inhibitor of a protein receptor. BIO-1211 was also 200-fold selective for the activated form of $\alpha 4\beta 1$, and it stimulated expression of ligand-induced epitopes on the integrin $\beta 1$ subunit, a property consistent with occupancy of the receptor's ligand-binding site. Pretreatment of allergic sheep with a 3-mg nebulized dose of BIO-1211 inhibited early and late airway responses following antigen challenge and prevented development of nonspecific airway hyperresponsiveness to carbachol. These results show that highly selective and potent small-molecule antagonists can be identified to integrins with primary specificity for peptide domains other than Arg-Gly-Asp (RGD); they confirm the generality of integrins as small molecule targets; and they validate $\alpha 4\beta 1$ as a therapeutic target for asthma.

Introduction

Integrin receptors mediate essential cell–cell and cell–matrix interactions in a diverse range of developmental, physiological, and pathological settings.^{1,2} The leukocyte cell adhesion receptor integrin $\alpha 4\beta 1$ plays a fundamental role in cellular recruitment processes.³ Additionally, $\alpha 4\beta 1$ provides a key costimulatory signal supporting cell activation leading to growth factor⁴ and cytokine⁵ production and mediator release;⁶ through interactions with extracellular matrix, it increases the survival of activated cells by inhibiting apoptosis.^{7,8} Animal model studies using blocking monoclonal antibodies (mAb)⁹ and inhibitor peptides^{10–12} demonstrate a critical role for $\alpha 4$ integrins in cellular recruitment and leukocyte-mediated inflammation and support the idea that inhibition of $\alpha 4$ integrins may provide clinical benefit in inflammatory diseases.

Integrin $\alpha 4\beta 1$ mediates cell adhesion by binding with either of two protein ligands: vascular cell adhesion molecule-1 (VCAM-1)^{13,14} or the alternatively spliced fibronectin variant containing the type III connecting segment (III-CS).^{15–17} In contrast to the prototypical integrins $\alpha 5\beta 1$, $\alpha \text{IIb}\beta 3$, and $\alpha \text{V}\beta 3$ that recognize the RGD peptide sequence in their respective ligands, $\alpha 4\beta 1$ binds to different primary sequence determinants. It recognizes Gln-Ile-Asp-Ser (QIDS) in VCAM-1¹⁸ and Ile-Leu-Asp-Val (ILDV) in fibronectin.¹⁶ These peptides share a conserved aspartic acid residue with RGD; however, they are otherwise unrelated.

The rich biology mediated by integrins in general, together with compelling evidence for the pathophysiologic role of $\alpha 4\beta 1$, prompted us to undertake the discovery of small-molecule inhibitors against this integrin. We reasoned that potent, highly selective inhibitors would allow for pharmacological investigations to complete the validation of $\alpha 4\beta 1$ as a drug discovery target. Moreover, an appropriate agent could also be employed in human clinical trials to establish proof-of-principle in chronic inflammatory disease. Early work from several laboratories, focusing largely on cyclic peptides, has begun to confirm these conclusions, and compounds from these studies have improved upon the potency and efficacy of initial peptide inhibitors.^{19–22}

Herein, we report the discovery of highly potent, selective inhibitors of $\alpha 4\beta 1$ based on the LDV peptide sequence in Fn recognized by $\alpha 4\beta 1$. Receptor binding studies reveal that effective inhibitors are selective for activated integrin and exhibit tight-binding kinetics with a dissociation rate remarkably long for small molecules. Moreover, these salutary properties translate to the in vivo setting in an $\alpha 4\beta 1$ -dependent model of allergic airways responses in sheep.

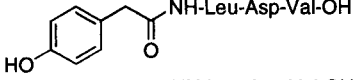
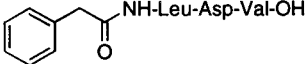
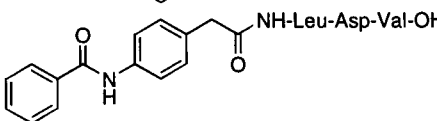
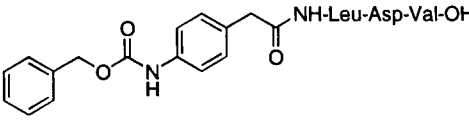
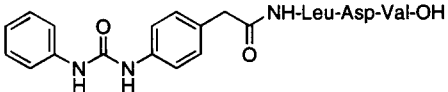
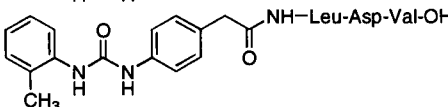
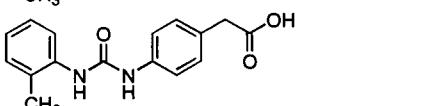
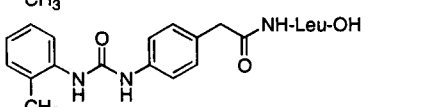
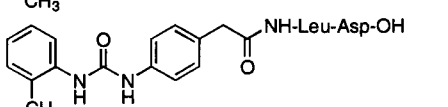
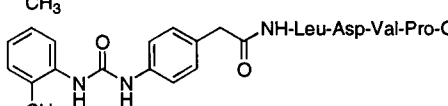
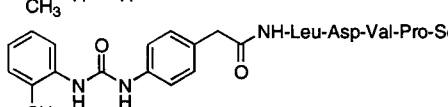
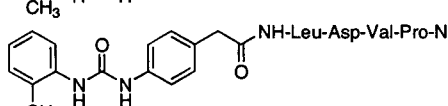
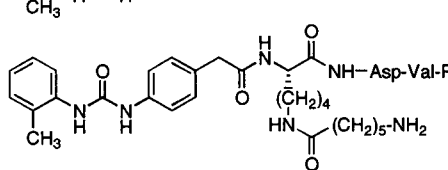
Results and Discussion

As a starting point for inhibitor design, we selected the tetrapeptide Ile-Leu-Asp-Val (ILDV) which comprises the primary binding site in the III-CS and falls within the CS-1 peptide.¹⁶ This sequence is homologous, and essentially isosteric, with the tetrapeptide Gln-Ile-Asp-Ser (QIDS), the $\alpha 4\beta 1$ binding site in VCAM-1.¹⁸ Both ILDV (**1**, Table 1) and QIDS¹⁸ weakly inhibited

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Table 1. Inhibition of $\alpha 4\beta 1$ -Mediated Ligand Binding and Cell Adhesion by Peptide Analogue Inhibitors^a

cmpd	Inhibitor Structure	IC ₅₀ (nM)	
		Binding	Adhesion
1	NH ₂ -Ile-Leu-Asp-Val-OH	66,000	175,000
2	NH ₂ -Tyr-Leu-Asp-Val-OH	12,600	51,000
3		430	5,000
4		1,000	40,000
5		295	3,250
6		30	200
7		3	30
8		0.6	6
9		NI	NI
10		NI	NI
11		18	68
12		1	4
13		1	5
14		1	8
15		4	20

^a Serial dilutions of the test compounds were evaluated for their ability to block binding or cell adhesion as described in the Experimental Section. IC₅₀ data were calculated from inhibition curves resulting from the concentration-dependent inhibition of VCAM-Ig binding by the $\alpha 4\beta 1$ inhibitors. Abbreviations: NI, no inhibition to 100 μ M.

ligand binding and cell adhesion, and by systematically replacing each amino acid in ILDV with alternative L- and D-amino acids, it was possible to improve peptide inhibitor activity (compound **2**, where Ile is substituted by Tyr). Deletion of the α -amino group on Tyr sustained activity, but the significantly improved analogue **3** was

obtained when, in addition, the methylene side chain was shortened by one carbon. Deletion of the aromatic hydroxyl group in **4** decreased activity suggesting this part of the inhibitor contributes to an important hydrogen bond donor-acceptor pair with the receptor, so we sought to optimize this interaction. Substitution of the

Table 2. Inhibition of Adhesion through Alternative Integrins by $\alpha 4\beta 1$ Inhibitors^a

cmpd	IC ₅₀ (μ M)						
	$\alpha 4\beta 1$	$\alpha 4\beta 7$	$\alpha 1\beta 1$	$\alpha 5\beta 1$	$\alpha 6\beta 1$	$\alpha L\beta 2$	$\alpha IIB\beta 3$
11	0.068	59	>100	>100	>100	>100	>100
8	0.006	8	>100	>100	>100	>50	>100
12	0.004	2	>100	>100	>100	>100	>100

^a The selectivity of compounds **11**, **8**, and **12** toward the seven integrins indicated were tested in adhesion formats as described in the Experimental Section. Serial dilution of each compound (from 1 nM to 100 μ M) was evaluated, and IC₅₀ values were calculated from the concentration dependence of the inhibition curves.

aromatic hydroxyl group with benzamido (**5**) did not improve activity; however, incorporation of benzyloxy-carboxamido (**6**), which extends the appended aromatic ring by two additional atoms, increased binding affinity 10-fold. A further 10-fold improvement was achieved upon incorporation of the more conformationally rigid phenylurea functionality at the hydroxyl position (**7**). And finally, by exploring a range of functional group substitutions on the terminal aromatic ring, the optimal inhibitor **8**, containing a 2-methylphenylurea group, was obtained.

The peptide domain of **8** targets the molecule to the receptor. Indeed, deletion of the peptide (**9**) eliminated receptor interaction, and significant activity returned only when the essential Asp was incorporated back into the peptide (**11**). Addition of Pro to the C-terminus in the inhibitor **12** (also referred to as BIO-1211), present at the same relative position in both the CS-1 and VCAM-1 sequences, supported high-affinity receptor binding and may impart enhanced stability to carboxypeptidase degradation. However, further extending the peptide with the Fn sequence (**13**) or terminating the Pro residue as the amide (**14**) failed to enhance activity. Importantly, replacement of Leu with the aminohexanoylamide of lysine (**15**) retained high-affinity binding and provides a reagent useful for further modification, for example, in receptor cross-linking studies.

Several representative compounds were examined for their integrin selectivity in cell-based adhesion assays involving different integrins. As noted in Table 2, high-affinity inhibitors were specific with regard to alternative $\beta 1$ integrins and to selected members of the $\beta 2$ and $\beta 3$ families. Only the homologous integrin $\alpha 4\beta 7$, which binds the endogenous ligands for $\alpha 4\beta 1$ in addition to MadCAM, exhibited significant activity; however, good selectivity was still observed. From these results, we conclude that it is possible to identify inhibitors of integrin $\alpha 4\beta 1$ that exhibit high affinity and selectivity.

Weak $\alpha 4\beta 1$ inhibitors were typically as much as 10-fold less potent at blocking cell adhesion to Fn (Table 1) and to VCAM-1 (data not shown) compared to direct binding experiments employing a bivalent VCAM-immunoglobulin fusion protein (VCAM-Ig).²³ Adhesion assay formats comprise highly avid cell-matrix interactions resulting from the simultaneous binding of multiple receptors on the cell surface. While the physiological relevance of these in vitro adhesion assays is not known at this point, it is striking that as the binding affinity of compounds increased, the IC₅₀s for cell adhesion experiments converged to the values obtained

in binding experiments. We hypothesized that these high-affinity inhibitors exhibit slow dissociation rates from the receptor and that high-affinity analogues such as **12** are actually tight-binding inhibitors that enjoy a kinetic advantage in competing highly avid cell adhesion interactions. To test this hypothesis, we synthesized a radiolabeled version of **12** to study its properties in direct binding experiments with $\alpha 4\beta 1$.

The inhibitor [³H]**12** bound to Jurkat cells in the presence of 2 mM Mn²⁺ (standard integrin-activating conditions) in a concentration-dependent fashion, achieving half-maximal saturation at 1–2 nM (Figure 1A). Both VCAM-Ig and CS-1 inhibited the binding of **12** (IC₅₀ = 20 nM and 10 μ M, respectively, data not shown) suggesting that these $\alpha 4\beta 1$ ligands occupy an identical or overlapping site on the integrin. Additionally, [³H]**12** binding to Jurkat cells was proportional to the number of cells in the incubation mixture and thus to receptor concentration and reflected an $\alpha 4\beta 1$ copy number of ~80 000/cell, consistent with results determined by FACS analysis using a monoclonal antibody specific for $\alpha 4\beta 1$ (data not shown).

The binding properties of [³H]**12** were further explored using K562 cells transfected with either the integrin $\alpha 2$ -subunit ($\alpha 2$ -K562) or $\alpha 4$ -subunit ($\alpha 4$ -K562) gene to probe binding-site selectivity. [³H]**12** bound, in a divalent cation-dependent manner, to $\alpha 4$ - but not to $\alpha 2$ -transfected cells (Figure 1B). This binding was >98% specific for the $\alpha 4$ binding site as determined by incubating [³H]**12** with $\alpha 4$ -K562 in the presence of a 100-fold molar excess of unlabeled **12**. Importantly, binding was unaffected by the presence of serum containing ~1 μ M plasma fibronectin, each dimeric molecule of which bears one copy of the alternatively spliced III-CS containing the ILDV sequence.²⁶ This result is consistent with the recent report that the $\alpha 4\beta 1$ binding site in soluble plasma fibronectin is cryptic²⁷ and unable to occupy $\alpha 4\beta 1$ on circulating cells even when activated, an important result if $\alpha 4\beta 1$ inhibitor drugs are to be effective in vivo.

The nonhyperbolic binding curves in these experiments suggested that the system was not at equilibrium, likely because the inhibitor dissociated slowly from the receptor. This observation is highlighted in the Figure 1A inset where [³H]**12** binding is plotted as a function of receptor occupancy at different receptor concentrations and where, if the system were at equilibrium, the two curves would be expected to align. Thus, the data obtained in this experiment underestimate the actual affinity of [³H]**12** for $\alpha 4\beta 1$.

The kinetic on- and off-rates were measured for [³H]**12** binding to $\alpha 4\beta 1$ to more accurately determine its dissociation constant. As noted in Figure 1C, the on-rate was fast with a second-order rate constant of $2.0 \times 10^6 \text{ M}^{-1}\cdot\text{s}^{-1}$ for two different drug concentrations at fixed receptor levels. In contrast, the first-order dissociation rate constant was $1.4 \times 10^{-4} \text{ s}^{-1}$ with a corresponding half-life of 70 min, remarkably slow for a small-molecule receptor ligand. From the rate constants, the dissociation constant at equilibrium, $K_D = 70 \text{ pM}$, can be calculated. The tight-binding properties of [³H]**12** were not specific to Jurkat cells; when the kinetic experiments were repeated with Mn²⁺-activated $\alpha 4$ -K562 and with freshly isolated peripheral blood

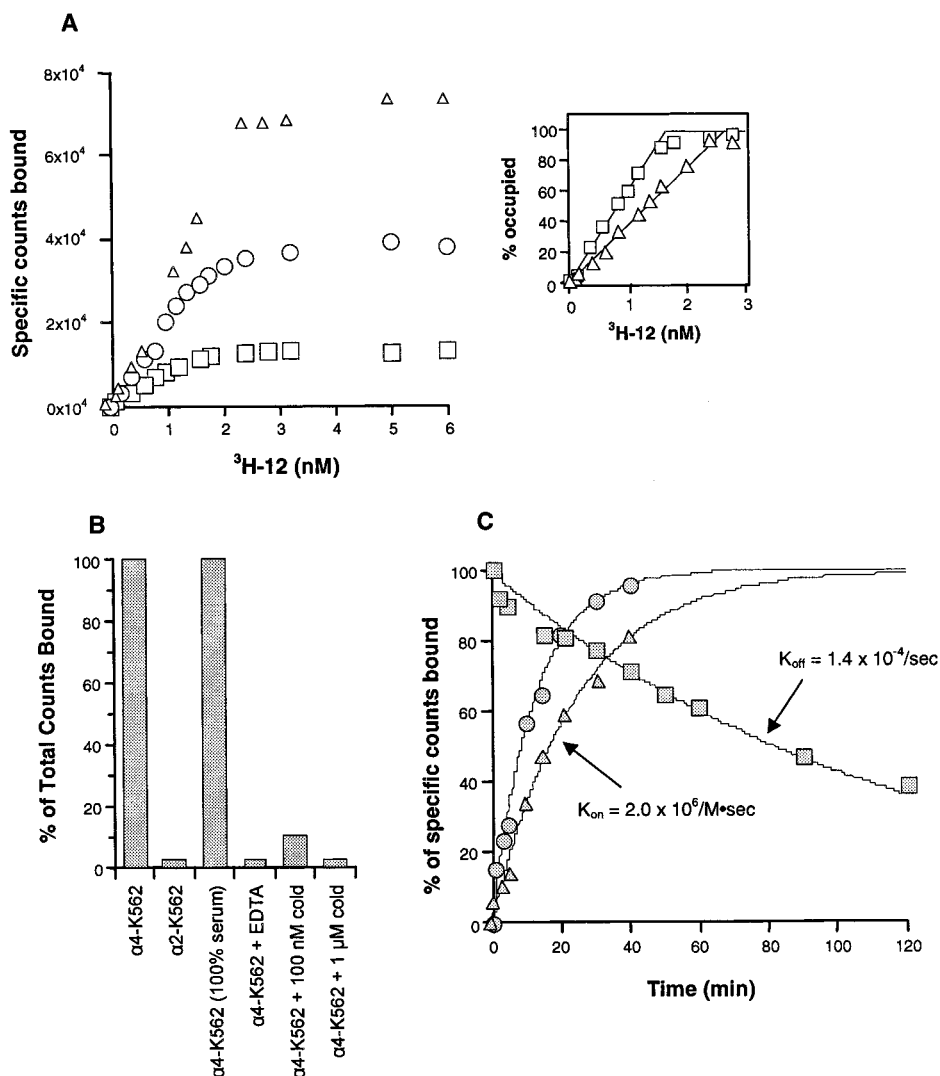


Figure 1. Receptor binding studies with compound **12**. Cells were treated with [^3H]**12** in TBS containing 2 mM Mn^{2+} ; pelleted and bound ligand was quantified by scintillation counting. (A) Saturable binding of increasing concentrations of [^3H]**12** at different, fixed levels of Jurkat cells ($0.75 \times 10^6/\text{mL}$, 0.1 nM $\alpha 4\beta 1$, \square ; $2.25 \times 10^6/\text{mL}$, 0.3 nM $\alpha 4\beta 1$, \circ ; $4.5 \times 10^6/\text{mL}$, 0.6 nM $\alpha 4\beta 1$, \triangle) as a function of total radioligand concentration. The inset shows the same data for 0.1 and 0.6 nM $\alpha 4\beta 1$ samples plotted as a percent of total sites occupied. (B) Specificity of [^3H]**12** binding to $\alpha 4$ -K562 cells or to $\alpha 2$ -K562 cells. Cells were treated with 10 nM [^3H]**12** in buffer containing 2 mM MnCl_2 . Similar incubations included human serum (containing plasma fibronectin) in place of buffer, 5 mM EDTA (integrin-deactivating conditions), and 100 nM or 1 μM unlabeled **12** as competitor. Data are presented as the percent of total counts bound for $\alpha 4$ -K562 cells treated with [^3H]**12**. (C) Association and dissociation kinetics of [^3H]**12** and $\alpha 4\beta 1$. For k_{on} determination, Jurkat cells were treated with either 0.3 nM (\blacktriangle) or 0.9 nM (\bullet) [^3H]**12** for increasing time periods. For k_{off} measurements (\blacksquare), cells were treated with 5 nM [^3H]**12** for 40 min, diluted with excess unlabeled **12**, and further incubated for the indicated times. Data are presented as a percent of maximum specific counts bound as a function of time and are fit to an exponential curve by nonlinear regression; k_{off} is the exponential rate constant, and k_{on} is the observed binding rate constant multiplied by the [^3H]**12** concentration. All experiments were performed at least twice. The data presented are the results from one experiment.

lymphocytes, similar kinetic results were obtained (data not shown). Likewise, when binding experiments were performed with the receptor-activating antibody TS2/16 in $\text{Ca}^{2+}/\text{Mg}^{2+}$ buffer (where receptor is not activated), rather than in Mn^{2+} -containing buffer, concordant results were obtained ruling out the possibility that the slow off-rate was a consequence of the method of receptor activation (data not shown).

Preliminary evidence using affinity-purified $\alpha 4\beta 1$ in an ELISA-type format further confirms the tight-binding properties of **12** (Chen, L., Lew, A., Lobb, R., & Pepinsky, B. unpublished observations). When $\alpha 4\beta 1$ was immobilized directly or captured onto microtiter wells with a nonblocking mAb and, in either case, activated with Mn^{2+} , a dissociation rate constant of 1.0×10^{-4}

s^{-1} was observed, in excellent agreement with the value from cell binding experiments. These cell-free binding results confirm that the observed tight-binding kinetics represent an intrinsic biochemical property of the ligand-receptor interaction, independent of cellular components.

Compound **12** also bound to unactivated $\alpha 4\beta 1$ on Jurkat cells in which case classical equilibrium binding characteristics were observed with $\text{IC}_{50} = 20\text{--}40$ nM (data not shown). Attempts to determine the k_{off} under nonactivating conditions were unsuccessful as ligand dissociation proved too rapid for measurement by the method employed in this study.

Taken together, these kinetic experiments allow us to conclude that compound **12** is a tight-binding inhibi-

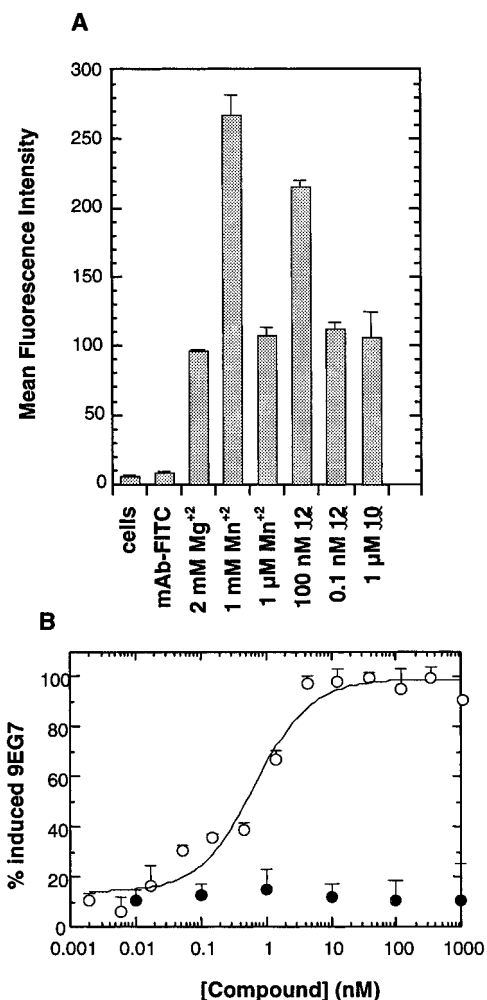


Figure 2. Effect of divalent cations and $\alpha 4\beta 1$ inhibitors on expression of ligand-induced binding sites. LIBS induction on Jurkat cells was determined by FACS analysis using FITC-labeled second antibody to the LIBS-detecting mAb 9EG7. (A) LIBS induction by compound **12**. Control experiments included cells and cells with second antibody, both in the absence of 9EG7. Test conditions included cells incubated with Mg²⁺-TBS (minimal integrin activation), Mg²⁺-TBS containing MnCl₂ (integrin activation), compound **12**, and the inactive, structurally related compound **10**. The means of triplicate determinations \pm SEM are presented. (B) Concentration dependence of LIBS induction by **12** (○) and **10** (●). Data (means of quadruplicate determinations \pm SEM) are presented as the percent of 9EG7 binding induced by compound: 0% = Mg²⁺-TBS control, 100% = binding for 1 μ M **12**; ED₅₀ = 0.68 nM.

tor of $\alpha 4\beta 1$ with a potency $\sim 10^6$ -fold greater than that of the initial peptide lead achieved by simply appending an optimal organic group to the N-terminus of the peptide. Moreover, this inhibitor exhibits >200-fold selectivity for the activated form of the receptor.

Certain mAbs specific for integrin β -subunits bind at increased levels in the presence of ligand thus detecting what are referred to as *ligand induced binding sites* (LIBS). First discovered for $\beta 3$ integrins,²⁸ LIBS antibodies have also been identified for the $\beta 1$ family.^{29–31} These LIBS antibodies bind three different epitopes that span the length of the β -subunit and are generally believed to reflect discrete conformational states related to receptor activation and function. It was important to ascertain if the $\alpha 4\beta 1$ inhibitors of this study are LIBS inducers, a property expected of compounds that are

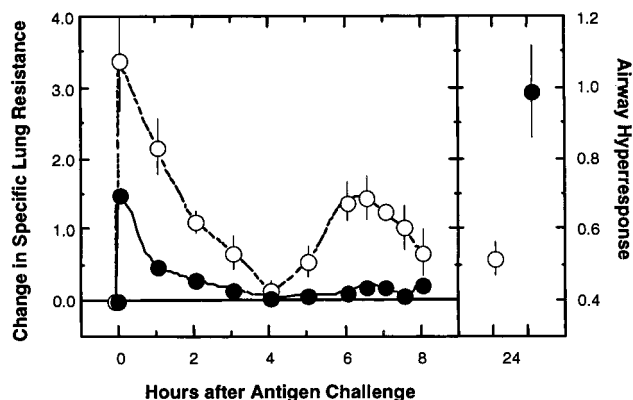


Figure 3. Effect of compound **12** on sheep airway responses to antigen. Naturally allergic Florida Native sheep that were previously shown to exhibit both early and late bronchoconstriction responses to nebulized *A. suum* antigens were used in the study. A 3-mg dose (0.1 mg/kg) of **12** formulated as the Tris salt in saline was administered by an air-jet nebulizer 2 h prior to challenge with nebulized antigen. Specific lung resistance (SR_L) was determined periodically for drug-treated animals (●) over 8 h and is expressed as the change from prechallenge SR_L. Historical control resistance data following antigen challenge (○) is presented for the same animals that received drug in this study. Airway responsiveness was determined 24 h after antigen challenge by determining the cumulative dose of carbachol required to induce a 4-fold increase in SR_L. Results are expressed as the ratio of the carbachol dose in the presence or absence of drug treatment divided by the carbachol dose to induce the same increase before antigen challenge (right panel). The data are plotted as the mean \pm SEM for 4 sheep.

believed to function by occupying ligand-related binding sites.³² When compound **12** was incubated with Jurkat cells in the presence of 2 mM Mg²⁺, LIBS induction ensued as detected by 9EG7²⁹ (Figure 2) and HUTS21³⁰ (data not shown), antibodies that detect distinct LIBS epitopes. The concentration-dependent response for LIBS induction by **12** (EC₅₀ = 0.68 nM) corresponded well with the level of receptor occupancy detected in the direct binding experiments. Moreover, the maximum level of LIBS induction correlated with levels observed with “activating” concentrations of Mn²⁺. These data were also consistent with the LIBS induction observed when K562 cells, expressing integrin $\alpha 5\beta 1$, were incubated with the peptide ligand Gly-Arg-Gly-Asp-Ser-Pro²⁹. Thus, as anticipated, the tight-binding $\alpha 4\beta 1$ inhibitors of this study were potent LIBS inducers.

The pivotal role for the recruitment and activation of $\alpha 4\beta 1$ -expressing leukocytes to the airways of asthmatic patients, where they mediate inflammation characteristic of the disease process, has been extensively documented.^{33,34} Furthermore, mAbs to $\alpha 4$ integrins are efficacious in airway allergen challenge models in multiple species.³⁵ We have previously shown that $\alpha 4$ blockade inhibits antigen-induced bronchoconstriction and airway hyperresponsiveness (AHR) in sheep naturally allergic to parasite antigens.²⁵ Thus, we were hopeful that the unique properties exhibited by **12** would translate to the sheep model preparatory to clinical development and trials in humans. As shown in Figure 3, inhibitor **12** exerted profound effects on airway responses following antigen challenge. A single, nebulized 3-mg dose (0.1 mg/kg) administered 2 h before challenge significantly inhibited the early response (ER) and completely blocked the late-phase response (LR) to

antigen. Importantly, this single administration of **12** also prevented the development of nonspecific AHR to carbachol aerosol. The effect was dose-dependent; decreasing the dose to 1 mg continued to inhibit LR but was inadequate to block the development of AHR. A dose of 0.3 mg was ineffective against all parameters. Increasing the dose to 10 or 30 mg further enhanced inhibition of ER (data not shown). The experiment was also conducted using the inactive, structurally related compound **10** which had no effect on antigen-induced bronchoconstriction or AHR. A recent report details similar results in the sheep model,³⁶ but in this case 30-mg dosing twice daily for 3 days was required to achieve an efficacy profile similar to that of the single dose of compound **12**, consistent with the high affinity, and presumably the tight-binding kinetic properties, of compound **12**.

This report of high-affinity, tight-binding, and selective inhibitors of $\alpha 4\beta 1$ validates this integrin as a viable small-molecule target, inhibitors of which exert important pharmacological effects *in vivo*. Optimal tight-binding inhibitors reported here exhibit a 10^6 enhancement in affinity over the starting peptide. Remarkably, the inhibitors are obtained by simply appending an optimal capping group to the N-terminus of the native ligand LDV. Moreover, this cap delivered greater than 10 000-fold selectivity for all integrins tested except the homologous $\alpha 4\beta 7$ which recognizes the same ligands as $\alpha 4\beta 1$; however, **12** still maintained ~1000-fold selectivity with regard to this integrin. These results, generated against a non-RGD integrin, taken together with the extensive results for RGD-related compounds that inhibit $\beta 3$ integrins,^{37–39} support the hypothesis that integrin receptors may be general small-molecule targets. Moreover, results in the sheep model suggest that compounds like **12** can exert broad pleiotropic activities by acting at $\alpha 4\beta 1$ across a range of leukocyte cell types, to achieve profound effects reminiscent of corticosteroids. Further animal studies and clinical investigations in humans should bear out this mechanistic hypothesis.

Experimental Section

All materials and reagents were obtained from commercial sources unless otherwise noted. K562 and Ramos cells were obtained from the ATCC. CHO cells transfected with the ICAM-1 gene were prepared at Biogen. Integrin $\alpha 4\beta 1$ -expressing Jurkat cells and JY cells were a gift of Dr. S. Burakoff (Dana-Farber Cancer Institute); K562 cells transfected with the $\alpha 2$ and $\alpha 4$ genes and the $\alpha 6$ gene were gifts from Dr. M. Hemler (Dana-Farber Cancer Institute) and Dr. A. Sonnenberg (Netherlands Cancer Institute), respectively. The mAb HUTS 21 was a gift of Dr. F. Sanchez-Madrid (University of Madrid); mAb 9EG7 was from Pharmingen (San Diego). [³H]**12** was synthesized by solid-phase peptide chemistry incorporating [4,5-³H]Leu (50 Ci/mmol) in the peptide and was purified by HPLC. The bovine serum albumin (BSA) conjugate with CS-1 was prepared by reacting CS-1 peptide, terminated with Cys-Tyr at the N-terminus, with maleimide-BSA (Pierce Chemical Co.) at a CS-1-to-BSA ratio of 1:2 (w/w), providing a density of 10 molecules of CS-1/molecule of BSA.

Inhibitor Studies with $\alpha 4\beta 1$. Binding²³ and adhesion assays²⁴ have been detailed elsewhere. Briefly, Jurkat cells (4.0×10^6 /mL) were incubated for 1 h with varying concentrations of inhibitor, or vehicle, in the presence of 1 mM MnCl₂ and 0.1 μ g/mL VCAM-Ig conjugated with alkaline phosphatase (VCAM-Ig-AP). Cells were filtered, washed, and resuspended, and bound VCAM-Ig-AP was quantified with the chromogenic

substrate *p*-nitrophenyl phosphate. For adhesion assays, Jurkat cells (4.0×10^6 /mL) were labeled for 20 min at 37 °C with 2 mM 2',7'-bis(carboxyethyl)-5-(and 6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR), washed, and incubated for 30 min with varying concentrations of inhibitor, or vehicle, in the presence of 1 mM MnCl₂ in microtiter wells previously coated overnight at 4 °C with 1 μ g/mL CS-1 conjugated to BSA. Following washing, bound cells were quantified by fluorescence at 530 nm. IC₅₀ values were determined by fitting the concentration dependence of inhibition to a sigmoid curve (mean of triplicate measurements) by nonlinear regression. Standard error of the mean (SEM) was typically <10% for each experiment.

The level of $\alpha 4\beta 1$ on Jurkat cells was quantified by fluorescence-activated cell sorter (FACS) analysis using the $\alpha 4$ -specific monoclonal antibody HP1/2²⁵ as a reporter for $\alpha 4\beta 1$ expression.

Inhibitor Studies with Alternative Integrins. The selectivity of compounds **8**, **11**, and **12** compared to other integrins was studied in adhesion assays using cells expressing specific integrins with their respective immobilized ligands. Ligand was immobilized in microtiter wells at 4 °C overnight at the indicated concentrations. Cells (4.0×10^6 /mL), labeled with BCECF-AM, were incubated in ligand-coated wells for 30 min at room temperature, unless otherwise noted, with inhibitor, or vehicle, in the presence of 1 mM MnCl₂. After washing, bound cells were quantified by fluorescence at 530 nm. For each integrin assay an appropriate inhibitor was included as positive control. $\alpha 4\beta 1$: CS-1-BSA (1 μ g/mL), K562 cells transfected with $\alpha 4$ integrin gene ($\alpha 4$ -K562); $\alpha 4\beta 7$: CS-1-BSA (1 μ g/mL), JY cells; $\alpha 1\beta 1$: human placental type IV collagen (0.5 μ g/mL), $\alpha 1$ -K562 cells, assay run at 37 °C; $\alpha 5\beta 1$: human 120 KD fibronectin fragment (5 μ g/mL), K562 cells; $\alpha 6\beta 1$: mouse laminin (20 μ g/mL), $\alpha 6$ -K562 cells. For $\alpha L\beta 2$, ICAM-1-transfected CHO cell monolayers (2.5×10^5 /well) were used as ligand with JY cells as receptor source. For $\alpha IIb\beta 3$, human citrated platelet-rich plasma was diluted to 2.0×10^8 platelets/mL with platelet-poor plasma and aggregated with 20 μ M ADP with monitoring in an aggregometer.

Direct Binding Studies of [³H]12** with $\alpha 4\beta 1$.** Binding studies were performed in siliconized 1.5-mL Eppendorf tubes with a standard 1-mL sample volume. For equilibrium binding measurements, Jurkat cells were incubated at room temperature for 40 min with increasing concentrations of [³H]**12** (50 Ci/mmol) in Tris-buffered saline (TBS) containing 2 mM MnCl₂ and pelleted, and bound ligand was quantitated by scintillation counting. Background, determined by incubating identical samples in TBS where integrin is unactivated, was subtracted from total counts bound to determine specific binding. Representative backgrounds for 1 nM [³H]**12** at the cell concentrations of 0.75, 2.25, and 4.5×10^6 cells/mL were 233, 230, and 464 cpm, respectively. For *k*_{on} measurements, separate samples of Mn²⁺-treated Jurkat cells (0.75×10^6 cells/mL) were treated with 0.3 or 0.9 nM [³H]**12**, and after increasing periods of time, individual samples were diluted with 500-fold excess unlabeled **12**. Samples were immediately centrifuged, and cell-associated [³H]**12** was determined by scintillation counting. Backgrounds of 50 and 150 cpm and maximum counts bound of 5 670 and 10 120 cpm were determined for the 0.3 and 0.9 nM samples, respectively. For *k*_{off} measurements, separate samples of Jurkat cells (0.75×10^6 cells/mL) were treated with 5 nM [³H]**12** for 40 min, diluted with a 500-fold excess of unlabeled **12**, and further incubated for increasing times up to 120 min. Cells were centrifuged and counted (background, 1500 cpm; total bound, 19 920 cpm). Maximum specific counts bound as a function of time were fit to an exponential curve by nonlinear regression; *k*_{off} was determined from the exponential rate constant, and *k*_{on} was determined by multiplying the observed binding rate constant by the [³H]**12** concentration. Binding site specificity of [³H]**12** was tested on $\alpha 4$ -K562 cells (2.0×10^6 cells/mL; 250 000 copies of $\alpha 4\beta 1$ /cell) or $\alpha 2$ -K562 cells (2.0×10^6 cells/mL; no detectable $\alpha 4$). Cells were treated with 10 nM [³H]**12** in TBS containing 2 mM MnCl₂ for 1 h at room temperature, pelleted, and counted. Similar incubations were

performed that included 5 mM EDTA, human serum in place of buffer, and unlabeled **12** at concentrations of 100 nM and 1 μ M.

Expression of Ligand-Induced Binding Sites by Inhibitors. Ligand-induced binding site (LIBS) induction was determined by incubating Jurkat cells (8.0×10^6 cells/mL) in TBS containing 2 mM $MgCl_2$ (Mg^{2+} -TBS) for 20 min at 37 °C followed by addition of 9EG7 (10 μ g/mL) with further incubation at 4 °C for 20 min. After two washes, 9EG7 binding was detected by incubating cells for 30 min at 4 °C with goat anti-rat IgG labeled with fluorescein isothiocyanate (FITC) (Jackson Immuno Research, Bar Harbor, ME) followed by FACS analysis. Test conditions for induction included cells incubated in Mg^{2+} -TBS containing $MnCl_2$, different concentrations of **12**, and the inactive, structurally related compound **10**. In two control experiments without 9EG7, FACS analysis was performed on cells in Mg^{2+} -TBS with and without FITC-labeled second antibody. All experiments were performed in triplicate. The concentration dependence of LIBS induction was determined for **12** and for the inactive compound **10** and were fit to a sigmoidal curve by nonlinear regression.

α 4 β 1 Inhibitor Effects on Sheep Airway Responses to Antigen. Methods for assessing drug effects on antigen-induced increases in specific lung resistance (SR_L) are described elsewhere.²⁵ A 3-mg dose of **12** formulated in 5 mL of saline containing 2 mol equiv of Tris (pH 7.0–7.4) was administered by an air-jet nebulizer (Raindrop, Puritan Bennett, Lenexa, KS) 2 h prior to challenge with nebulized *Ascaris suum* antigen. SR_L was determined periodically for drug-treated animals over 8 h. The change in SR_L was calculated for each sheep as the difference from prechallenge baseline SR_L , and values reported are the mean \pm SEM for four sheep. Airway responsiveness was determined 24 h after antigen challenge and is expressed as the cumulative dose of carbachol at 24 h required to induce a 4-fold increase in SR_L in the presence or absence of drug treatment divided by the carbachol dose to induce the same increase before antigen challenge. The protocol used in the sheep study was approved by the Mount Sinai Medical Center Animal Research Committee which is responsible for ensuring the humane care and use of experimental animals.

Chemistry. All starting materials and synthesis reagents were obtained commercially. Abbreviations are as follows: Boc, *tert*-butyloxycarbonyl; DMF, dimethylformamide; EDC, 1-ethyl-3-[3-(*N,N*-dimethylamino)propyl]carbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole hydrate; MPUPA, *o*-methyl phenylureaphenylacetyl; OSu, *N*-hydroxysuccinimide; PA, phenylacetyl; PAA, phenylacetic acid; PUPA, phenylureaphenylacetyl; TEA, triethylamine; TFA, trifluoroacetic acid; Z, benzylloxycarbonyl.

¹H and ¹³C NMR spectra were obtained in DMSO-*d*₆ or CDCl₃ on Bruker AC 300, Bruker DPX 400, Varian Unity-500, or Varian Unity Inova 600 instruments and were referenced to the solvent peaks using chemical shift values of 2.49 and 7.24 ppm, respectively. Mass spectra were obtained on a Fisons VG Platform Quattro II mass spectrometer system and processed with MassLynx software. High-resolution mass spectra were run by M-Scan Inc. (West Chester, PA) on a VG Analytical ZAB 2SE high-field mass spectrometer. IR spectra were recorded on a Perkin-Elmer 1600 Series FTIR spectrometer. Elemental analyses and Karl Fischer analysis for residual water were performed by QTI (Whitehouse, NJ). Residual water reflected in elemental analyses was independently confirmed by KF analysis. Uncorrected melting points were obtained in open capillaries on an Electrothermal IA9000 digital melting point apparatus.

Analytical HPLC was performed on a Perkin-Elmer 200 HPLC system with UV detection at 214 and/or 254 nm with data analysis using either a PE Nelson 1020 or a Hewlett-Packard 3396 Series II integrator. A Vydac C-18 reverse-phase column (5- μ m pore size, 4.5 mm \times 25 cm) eluted at 1.0 mL/min was utilized in all studies using the following solvent systems. System 1: solvent A (H₂O containing 0.1% TFA), solvent B (CH₃CN containing 0.1% TFA), gradients between

10% and 70% solvent B. System 2: solvent C (25 mM sodium phosphate, pH 4.2), solvent D (1:1 CH₃CN:50 mM sodium phosphate, pH 4.2), gradients between 5% and 60% solvent D. Preparative HPLC was performed with a Rainin HPXL solvent delivery system with a Rainin Dynamax UV-1 detector (set at 225 nm) using a Rainin Microsorb C-18 reverse-phase column (5- μ m pore size, 21.4 mm \times 25 cm) eluted at 10 mL/min using solvent system 1. Fractions were collected and analyzed by analytical HPLC, and pure fractions were combined and evaporated in vacuo to provide purified material.

General Procedure for Peptide Synthesis. Preparation of L-(α -*o*-Benzylaspartyl)-L-*o*-benzylvaline Hydrochloride (DV(OBn)₂·HCl). Standard active ester coupling methods were employed using *N*-hydroxysuccinimide esters of Boc-amino acids and amino acid benzyl esters. All reactions were conducted at room temperature unless otherwise noted. To a suspension of Boc-Asp(β -benzyl)-OSu (21.65 g, 51.5 mmol) and valine benzyl ester hydrochloride (13.20 g, 54.1 mmol) in CH₂Cl₂ (50 mL) was added Et₃N (25 mL). The reaction mixture was allowed to stir for 1 h, and it was then diluted with EtOAc (600 mL), washed with 10% citric acid (3 \times), H₂O, saturated NaHCO₃ (3 \times), and brine, dried (Na₂SO₄), and concentrated in vacuo to provide 25.85 g of Boc-DV(OBn)₂ as a viscous oil which was used without further purification.

To Boc-DV(OBn)₂ (25.32 g, 49.43 mmol) was added 4 M HCl in dioxane (60 mL). The reaction mixture was stirred for 2 h, and it was then concentrated in vacuo and crystallized (EtOAc/hexanes) to provide the desired product DV(OBn)₂·HCl (16.26 g, 73%) as a white solid: mp 118.0–118.7 °C; IR (KBr, cm⁻¹) 3390, 3302, 2964, 1734, 1704, 1654; ¹H NMR (300 MHz, CDCl₃) δ 8.64 (br s, 2H), 7.88 (d, *J* = 8.2 Hz, 1H), 7.25 (m, 10H), 5.08 (m, 3H), 5.04 (d, *J* = 12 Hz, 1H), 4.97 (m, 1H), 4.42 (dd, *J* = 7.9, 3.2 Hz, 1H), 3.26 (dd, *J* = 18.0, 3.2 Hz, 1H), 3.11 (dd, *J* = 18.0, 7.5 Hz, 1H), 2.15 (m, 1H), 0.87 (d, *J* = 6.9 Hz, 3H), 0.84 (d, *J* = 6.9 Hz, 3H); MS (EI) 413 (M + H)⁺; HRMS *m/z* (M + H)⁺ calcd 413.2076, obsd 413.2078. Anal. Calcd for C₂₃H₂₈N₂O₅·HCl: C, 61.53; H, 6.51; N 6.24. Found: C, 61.39, H, 6.45; N, 6.12.

***N*-*tert*-Butoxycarbonyl-L-leucyl-L-(α -*o*-benzylaspartyl)-L-valine Benzyl Ester (Boc-LDV(OBn)₂).** Following the general coupling procedure, the reaction of DV(OBn)₂·HCl (10.92 g, 24.32 mmol) and Boc-Leu-OSu (7.59 g, 23.11 mmol) afforded the protected tripeptide (12.37 g, 86%) as a white solid after recrystallization (EtOAc/hexane): mp 106.2–106.6 °C; IR (KBr, cm⁻¹) 3330, 2961, 1748, 1734, 1723, 1684, 1651; ¹H NMR (300 MHz, CDCl₃) δ 7.33 (m, 5H), 7.32 (m, 5H), 7.07 (d, *J* = 8.6 Hz, 1H), 5.12 (q, *J* = 27.5, 12 Hz, 2H), 5.11 (d, *J* = 1.8 Hz, 2H), 4.8 (dt, *J* = 7.6, 4.1 Hz, 1H), 4.76 (m, 1H), 4.47 (dd, *J* = 8.6, 4.7 Hz, 1H), 4.08 (m, 1H), 3.02 (dd, *J* = 4.0, 17.3 Hz, 1H), 2.65 (dd, *J* = 17.3, 6.9 Hz, 1H), 2.16 (m, *J* = 6.9 Hz, 1H), 1.63 (m, 2H), 1.58 (m, 2H), 1.41 (s, 9H), 0.92 (d, *J* = 6.1 Hz, 3H), 0.91 (d, *J* = 6.1 Hz, 3H), 0.88 (d, *J* = 6.9 Hz, 3H), 0.83 (d, *J* = 6.9 Hz, 3H); MS (EI) 625 (M⁺); HRMS *m/z* (M + H)⁺ calcd 626.3441, obsd 626.3438. Anal. Calcd for C₃₄H₄₇N₃O₆: C, 65.26; H, 7.57; N 6.72. Found: C, 65.38; H, 7.53; N, 6.67.

L-Leucyl-L-(α -*o*-benzylaspartyl)-L-valine Benzyl Ester Hydrochloride (LDV(OBn)₂·HCl). Following the general deprotection procedure above, 9.80 g (92%) of the desired product was obtained as a white solid following recrystallization (CH₂Cl₂/Et₂O): mp 170.5–170.9 °C; IR (KBr, cm⁻¹) 3350, 3034, 2962, 1736, 1683, 1656, 1588; ¹H NMR (300 MHz, CDCl₃) δ 8.88 (d, *J* = 7.5 Hz, 1H), 8.35 (d, *J* = 8.2 Hz, 1H), 8.24 (br s, 2H), 7.33 (m, 10H), 5.09 (dd, *J* = 18.5, 12.4 Hz, 2H), 5.08 (q, *J* = 15.6, 12.5 Hz, 2H), 4.78 (m, 1H), 4.19 (d, *J* = 8.12, 6.1 Hz, 1H), 3.75 (m, 1H), 2.77 (dd, *J* = 16.5, 5.5 Hz, 1H), 2.63 (dd, *J* = 16.6, 8.5 Hz, 1H), 2.06 (m, 1H), 1.66 (m, 1H), 1.55 (m, 2H), 0.91 (d, *J* = 5.3 Hz, 3H), 0.87 (d, *J* = 5.3 Hz, 3H), 0.83 (d, *J* = 6.8 Hz, 3H), 0.80 (d, *J* = 6.8 Hz, 3H); MS (EI) 527 (M - Cl)⁺; HRMS *m/z* (M - HCl)⁺ calcd 526.2917, obsd 526.2930. Anal. Calcd for C₂₉H₃₉N₃O₆·HCl·0.93H₂O: C, 60.17; H, 7.29; N, 7.26. Found: C, 60.37; H, 7.27; N, 7.34.

General Procedure for Hydrogenolysis of Benzyl Protecting Groups. Preparation of *N*-*tert*-Butoxycarbonyl-L-leucyl-L-(α -*o*-aspartyl)-L-valine (Boc-LDV). To a solution

of Boc-LDV(OBn)₂ (12.90 g, 20.6 mmol) in THF/H₂O (9:1, 30 mL) was added 10% Pd/C (1.0 g), and the reaction mixture was stirred under H₂ (55 psi) for 22 h. The reaction mixture was filtered through Solka-Floc and concentrated in vacuo to provide 9.8 g (95%) of Boc-LDV as a white solid: mp 92–93 °C; IR (KBr, cm⁻¹) 3319, 2955, 1725, 1696, 1654, 1625; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.35 (d, *J* = 7.8 Hz, 1H), 7.79 (d, *J* = 8.6 Hz, 1H), 7.09 (d, *J* = 8.4 Hz, 1H), 4.79 (m, 1H), 4.31 (dd, *J* = 8.6, 5.3 Hz, 1H), 4.18 (q, *J* = 5.8 Hz, 1H), 2.88 (dd, *J* = 16.6, 5.9 Hz, 1H), 2.69 (m, 1H), 2.22 (m, 1H), 1.78 (m, 1H), 1.61 (m, 2H), 1.56 (s, 9H), 1.03 (m, 12 H); HRMS *m/z* (M + H)⁺ calcd 446.2502, obsd 446.2509. Anal. Calcd for C₂₀H₃₅N₃O₈: C, 53.92; H, 7.91; N 9.43. Found: C, 54.25, H, 8.22; N, 8.83.

L-Leucyl-L- α -aspartyl-L-valine Hydrochloride (LDV·HCl). Acid hydrolysis of Boc-LDV (3.22 g, 7.23 mmol) as above provided product (2.5 g, 93%) as a white solid: mp 216.6–216.9 °C; IR (KBr, cm⁻¹) 3260, 2900, 1721, 1657, 1606; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.90 (d, *J* = 7.4 Hz, 1H), 8.30 (br s, 2H), 7.93 (d, *J* = 8.2 Hz, 1H), 4.67 (m, 1H), 4.10 (dd, *J* = 8.4, 5.7 Hz, 1H), 3.74 (br s, 1H), 2.72 (dd, *J* = 16.9, 5.5 Hz, 1H), 2.54 (dd, *J* = 16.9, 8.2 Hz, 1H), 2.04 (m, 1H), 1.68 (m, 1H), 1.52 (m, 2H), 0.86 (m, 12H); HRMS *m/z* (M - Cl)⁺ calcd 346.1978, obsd 346.1985. Anal. Calcd for C₁₅H₂₇N₃O₆·HCl·0.21H₂O: C, 46.73; H, 7.43; N, 10.99. Found: C, 47.25; H, 7.52; N, 10.68.

N-tert-Butyloxycarbonyl-L-leucyl-L-(α -*o*-benzylaspartate) Benzyl Ester (Boc-LD(OBn)₂). Following the general coupling procedure above, reaction of *p*-Tos·Asp(OBn)₂ (100.6 g, 207.2 mmol) and Boc-Leu-OSu (71.21 g, 217 mmol) provided, after recrystallization (EtOAc/hexanes), 98.2 g (90%) of the product as a white solid: mp 78–79.5 °C; IR (KBr, cm⁻¹) 3433, 3023, 2962, 1733, 1650, 1500; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.36 (d, *J* = 7.9 Hz, 1H), 7.34 (m, 10H), 6.86 (d, *J* = 8.4 Hz, 1H), 5.06 (s, 4H), 4.74 (dd, *J* = 14, 6.7 Hz, 1H), 3.97 (dd, *J* = 9.2, 5.5 Hz, 1H), 2.91 (dd, *J* = 16.6, 6.2 Hz, 1H), 2.78 (dd, *J* = 16.5, 6.9 Hz, 1H), 1.56 (m, *J* = 6.4 Hz, 1H), 1.35 (s, 9H), 1.27 (m, 2H), 0.80 (dd, *J* = 6.6, 2.2 Hz, 6H); MS (EI) 427 (M - Boc + H)⁺; HRMS *m/z* (M⁺) calcd 549.2577, obsd 549.2563. Anal. Calcd for C₂₉H₃₈N₂O₇: C, 66.26; H, 7.22; N, 5.27. Found: C, 66.14, H, 7.27; N, 5.32.

L-Leucyl-L-(α -*o*-benzylaspartate) Benzyl Ester Hydrochloride (LD(OBn)₂·HCl). Removal of the Boc group from Boc-LD(OBn)₂ (95.75 g, 182 mmol) was performed as above to obtain, after crystallization (CH₂Cl₂/Et₂O), 82.72 g (96%) of the desired product as a white solid: mp 152–153 °C; IR (KBr, cm⁻¹) 3448, 2961, 1736, 1709, 1686; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.22 (d, *J* = 7.9 Hz, 1H), 8.41 (br s, 2H), 7.35 (m, 10H), 5.09 (s, 2H), 5.08 (s, 2H), 4.83 (m, 1H), 3.77 (m, 1H), 2.90 (d, *J* = 6.4 Hz, 2H), 1.64 (m, *J* = 6.8 Hz, 1H), 1.50 (t, *J* = 7.1 Hz, 2H), 0.82 (dd, *J* = 6.4, 3.5 Hz, 6H); MS (EI) 427 (M + H)⁺; HRMS *m/z* (M⁺) calcd 427.2233, obsd 427.2227. Anal. Calcd for C₂₄H₃₀N₂O₅·HCl: C, 62.26; H, 6.75; N, 6.05. Found: C, 62.00, H, 6.71; N, 5.96.

L-Isoleucyl-L-leucyl-L-aspartyl-L-valine Hydrochloride (ILDV·HCl) (1). To a solution of Boc-Ile (1.42 g, 2.52 mmol) in DMF (4 mL) were added EDC (584 mg, 3.05 mmol) and HOBT (555 mg, 4.11 mmol). The reaction mixture was allowed to stir for 15 min, and LDV(OBn)₂·HCl (1.42 g, 2.52 mmol) was added followed by Et₃N (1.2 mL, 8.6 mmol). After 14 h, the reaction mixture was poured into 10% citric acid (100 mL). The precipitate was washed with citric acid (3 × 50 mL), H₂O, saturated NaHCO₃ (3 × 50 mL), and H₂O, and dried in vacuo to provide Boc-ILDV(OBn)₂ (1.86 g, 92%) as a white solid: mp 175–176 °C; IR (KBr, cm⁻¹) 3282, 2958, 1740, 1690, 1645, 1544; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.29 (d, *J* = 7.2 Hz, 1H), 8.01 (d, *J* = 8.1 Hz, 1H), 7.83 (d, *J* = 8.1 Hz, 1H), 7.31 (s, 10H), 6.82 (d, *J* = 8.6 Hz, 1H), 5.06 (m, 4H), 4.68 (m, 1H), 4.45 (m, 1H), 4.18 (m, 1H), 3.77 (m, 1H), 3.32 (m, 2H), 2.74 (m, 1H), 2.57 (m, 1H), 2.04 (m, 1H), 1.63 (m, 2H), 1.61 (br s, 1H), 1.39 (s, 9H), 1.05 (m, 1H), 0.84 (m, 18H); MS (EI) 739 (M + H)⁺; HRMS *m/z* (M + H)⁺ calcd 739.4282, obsd 739.4288. Anal. Calcd for C₄₀H₅₈N₄O₉: C, 65.02; H, 7.91; N 9.78. Found: C, 65.08, H, 7.90; N, 7.54.

Hydrogenolysis of Boc-ILDV(OBn)₂ (1.28 g, 1.73 mmol), using the general procedure, provided Boc-ILDV (860 mg, 89%) as a white solid: 139–145 °C dec; IR (KBr, cm⁻¹) 3316, 1718, 1678, 1645, 1527; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.4 (br s, 2H), 8.3 (d, *J* = 7.2 Hz, 1H), 7.82 (d, *J* = 8.2 Hz, 1H), 7.58 (d, *J* = 8.3 Hz, 1H), 6.81 (d, *J* = 8.9 Hz, 1H), 4.58 (m, 1H), 4.43 (m, 1H), 4.22 (m, 1H), 4.08 (m, 1H), 2.67 (m, 1H), 2.45 (m, 1H), 2.02 (m, 1H), 1.62 (m, 2H), 1.44 (m, 1H), 1.41 (m, 2H), 1.36 (s, 9H), 1.25 (m, 1H), 0.83–0.67 (m, 18H); MS (EI) 558 (M⁺); HRMS *m/z* (M + H)⁺ calcd 559.3343, obsd 559.3344. Anal. Calcd for C₂₆H₄₆N₄O₉·0.456H₂O: C, 55.09; H, 8.34; N 9.88. Found: C, 55.27, H, 8.40; N, 9.54.

Following the general procedure, hydrolysis of Boc-ILDV (470.5 mg, 0.84 mmol) provided ILDV·HCl (350 mg, 84%) as a white solid: mp 179–182 °C dec; IR (KBr, cm⁻¹) 3327, 2958, 1723, 1667, 1527; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.51 (m, 1H), 8.49 (m, 1H), 8.47 (m, 1H), 7.65 (d, *J* = 8.4 Hz, 1H), 4.59 (m, 1H), 4.57 (m, 1H), 4.11 (m, 1H), 3.62 (br s, 3H), 2.67 (m, 1H), 2.47 (m, 1H), 2.02 (m, 1H), 1.78 (m, 1H), 1.64 (m, 1H), 1.45 (m, 3H), 1.10 (m, 1H), 0.87–0.83 (m, 18H); ¹³C NMR (100 MHz, DMSO-*d*₆) 172.6 (C=O), 171.6 (C=O), 171.5 (C=O), 170.5 (C=O), 167.5 (C=O), 57.05 (CH), 56.27 (CH), 51.0 (CH), 49.4 (CH), 41.3 (CH), 36.2 (CH), 35.6 (CH), 30.1 (CH₂), 24.1 (CH₂), 23.9 (CH₂), 23.1 (CH₃), 21.8 (CH₃), 19.0 (CH₃), 17.7 (CH₃), 14.5 (CH₃), 11.2 (CH₃); MS (EI) 459 (M + H)⁺; HRMS *m/z* (M + H)⁺ calcd 459.2819, obsd 459.2812. Anal. Calcd for C₂₁H₃₈N₄O₇·HCl·0.46H₂O: C, 50.12; H, 7.99; N, 11.13. Found: C, 50.60; H, 8.18; N, 10.84.

L-Tyrosyl-L-leucyl-L- α -aspartyl-L-valine Hydrochloride (YLDV·HCl) (2). The general coupling procedure above utilizing LDV·HCl (362 mg, 0.95 mmol) and Boc-Tyr-OSu (339 mg, 0.90 mmol) provided Boc-YLDV (496 mg, 86%) as a white solid: mp 134–136 °C dec; IR (KBr, cm⁻¹) 3336, 2958, 1718, 1696, 1678, 1650, 1516; ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.49 (br s, 1H), 8.35 (d, *J* = 8.3 Hz, 1H), 7.89 (d, *J* = 8.1 Hz, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.01 (d, *J* = 7.9 Hz, 2H), 6.84 (d, *J* = 8.6 Hz, 1H), 6.62 (d, *J* = 7.8 Hz, 2H), 4.59 (m, 1H), 4.36 (m, 1H), 4.12 (m, 1H), 4.07 (m, 1H), 2.81 (m, 1H), 2.68 (m, 1H), 2.65 (m, 1H), 2.20 (m, 1H), 1.61 (br s, 1H), 1.43 (br s, 2H), 1.29 (s, 9H), 1.25 (s, 1H), 0.83 (m, 12H); MS (EI) 607 (M - H)⁺; HRMS *m/z* (M + Na)⁺ calcd 631.2955, obsd 631.2952.

Removal of the Boc protecting group from Boc-YLDV (307 mg, 0.50 mmol) as above afforded YLDV·HCl (244 mg, 90%) as a white solid: mp 169–171 °C dec; IR (KBr, cm⁻¹) 3371, 3204, 2967, 1730, 1682, 1653, 1518; ¹H NMR (300 MHz DMSO-*d*₆) δ 9.36 (s, 1H), 8.73 (d, *J* = 8.3 Hz, 1H), 8.53 (d, *J* = 7.5 Hz, 1H), 8.09 (br s, 2H), 7.67 (d, *J* = 8.6 Hz, 1H), 7.04 (d, *J* = 8.4 Hz, 2H), 6.70 (d, *J* = 8.3 Hz, 2H), 4.64 (q, *J* = 6.2 Hz, 1H), 4.40 (q, *J* = 7.1 Hz, 1H), 4.13 (dd, *J* = 8.5, 5.3 Hz, 1H), 3.95 (s, 1H), 3.35 (br s, 2H), 3.03 (m, 1H), 2.80 (t, *J* = 8.2 Hz, 1H), 2.71 (dd, *J* = 17.2, 6.3 Hz, 1H), 2.49 (m, 1H), 2.03 (m, 1H), 1.63 (septet, *J* = 6.7 Hz, 1H), 1.46 (t, *J* = 6.9 Hz, 2H), 0.85 (overlapping doublets, *J* = 6.2, 4.9 Hz, 12H); MS (EI) 509 (M + H)⁺; HRMS *m/z* (M + Na)⁺ calcd for C₂₄H₃₆N₄O₈Na 531.2431, obsd 531.2423.

L-Leucyl-L-(α -*o*-benzylaspartyl)-L-valyl-L-proline Benzyl Ester Hydrochloride (LDVP(OBn)₂·HCl). Reiterative coupling and removal of the Boc group, following the general procedures described above, provided LDVP(OBn)₂·HCl as a white solid: mp 175–177.5 °C; IR (KBr, cm⁻¹) 3249, 3182, 3037, 2958, 1741, 1680, 1657, 1618, 1546; ¹H NMR (600 MHz, DMSO-*d*₆) δ 9.17 (d, *J* = 7.8 Hz, 1H), 8.49 (br s, 2H), 7.97 (d, *J* = 8.4 Hz, 1H), 7.32 (m, 10H), 5.09 (m, 4H), 4.73 (dd, *J* = 14.4, 7.2 Hz, 1H), 4.33 (m, 2H), 3.79 (t, *J* = 6.9 Hz, 1H), 3.71 (dd, *J* = 16.2, 6.6 Hz, 1H), 3.55 (m, 1H), 2.85 (dd, *J* = 16.5, 6.3 Hz, 1H), 2.73 (dd, *J* = 16.5, 7.3 Hz, 1H), 2.14 (m, 1H), 1.97 (m, 1H), 1.87 (m, 2H), 1.80 (m, 1H), 1.72 (m, 1H), 1.59 (m, 2H), 0.88 (d, *J* = 6.6 Hz, 3H), 0.87 (d, *J* = 6.6 Hz, 3H), 0.85 (d, *J* = 6.6 Hz, 3H), 0.81 (d, *J* = 6.6 Hz, 3H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 171.6 (C=O), 169.8 (C=O), 169.6 (C=O), 169.5 (C=O), 169.1 (C=O), 136.0 (C), 135.9 (C), 128.4 (4CH), 128.01 (CH), 127.97 (CH), 127.90 (2CH), 127.8 (2CH), 65.8 (CH₂), 65.7 (CH₂), 58.6 (CH), 55.6 (CH), 50.7 (CH), 49.5 (CH), 46.8 (CH₂), 40.2 (CH₂), 35.8 (CH₂), 30.1 (CH), 28.7 (CH₂), 24.6 (CH₂), 23.6

(CH₂), 22.5 (CH₃), 22.2 (CH₃), 18.9 (CH₃), 17.9 (CH₃); MS (EI) 623 (M⁺). Anal. Calcd for C₃₄H₄₆N₄O₇·HCl: C, 61.95; H, 7.19; N, 8.50. Found: C, 62.06; H, 7.04; N, 8.51.

L-Leucyl-L-α-aspartyl-L-valyl-L-prolyl-L-serinyl-L-threonine Hydrochloride (LDVPST·HCl). A series of reiterative steps, starting with L-Thr-OBn and following the general procedures for coupling, deprotection, and hydrogenolysis, provided the desired product. The synthesis proceeded through the following characterized intermediates:

ST(OBn)₃·HCl: mp 145–146 °C; IR (KBr, cm⁻¹) 3181, 3036, 1740, 1667, 1544; MS (EI) 477 (M + H)⁺, 300, 240; HRMS *m/z* (M + H)⁺ calcd for C₂₈H₃₃N₂O₅ 477.2389, obsd 477.2383.

PST(OBn)₃·HCl: mp 141–142 °C; IR (KBr, cm⁻¹) 3290, 3036, 2856, 1740, 1673, 1639, 1544; MS (EI) 574 (M + H)⁺, 477, 300; HRMS *m/z* (M + H)⁺ calcd for C₃₃H₄₀N₃O₆ 574.2917, obsd 574.2926.

Boc-VPST(OBn)₃: mp 61–63 °C; IR (KBr, cm⁻¹) 3304, 2968, 1746, 1706, 1661, 1628, 1516; MS (EI) 773 (M + H)⁺, 772, 673, 573, 477, 374; HRMS *m/z* (M + Na)⁺ calcd for C₄₃H₅₆N₄O₉Na 795.3945, obsd 795.3929.

VPST(OBn)₃·HCl: mp 66–68 °C; IR (KBr, cm⁻¹) 3315, 2957, 1745, 1656, 1510; MS (EI) 673 (M + H)⁺, 574, 477, 374; HRMS *m/z* (M + H)⁺ calcd for C₃₈H₄₉N₄O₇ 673.3601, obsd 673.3580.

Boc-DVPST(OBn)₄: mp 60–63 °C; IR (KBr, cm⁻¹) 3307, 2966, 1735, 1667, 1622, 1510; MS (EI) 978 (M + H)⁺, 878, 574, 256; HRMS *m/z* (M + Na)⁺ calcd for C₅₄H₆₇N₅O₁₂Na 1000.4684, obsd 1000.4671.

DVPST(OBn)₄·HCl: mp 80–82 °C; IR (KBr, cm⁻¹) 3319, 2966, 1737, 1678, 1631, 1498; MS (EI) 878 (M + H)⁺, 574, 477; HRMS *m/z* (M + Na)⁺ calcd for C₄₉H₅₉N₅O₁₀Na 900.4160, obsd 900.4189.

Boc-LDVPST(OBn)₄: mp 84–85 °C; IR (KBr, cm⁻¹) 3304, 2961, 1742, 1662, 1628, 1516; MS (EI) 1091 (M + H)⁺, 991, 574; HRMS *m/z* (M + Na)⁺ calcd for C₆₀H₇₈N₆O₁₃Na 1113.5525, obsd 1113.5482.

LDVPST·HCl: mp 185–187 °C dec; IR (KBr, cm⁻¹) 3319, 2966, 1684, 1672, 1654, 1631, 1613; ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.75 (d, *J* = 8 Hz, 1H), 8.1 (br s, 2H), 8.05 (d, *J* = 7.6 Hz, 1H), 7.90 (d, *J* = 8.4 Hz, 1H), 7.50 (d, *J* = 8.8 Hz, 1H), 4.65 (dd, *J* = 7.6, 5.6 Hz, 1H), 4.39 (dd, *J* = 8.0, 4.0 Hz, 1H), 4.33–4.26 (m, 2H), 4.20 (dd, *J* = 8.8, 2.8 Hz, 1H), 4.11 (dd, *J* = 6.0, 2.8 Hz, 1H), 3.74 (m, 2H), 3.59 (m, 2H), 3.55 (m, 1H), 2.67 (dd, *J* = 16.8, 5.6 Hz, 1H), 2.50 (dd, *J* = 16.8, 5.6 Hz, 1H), 2.05–1.78 (m, 5H), 1.66 (septet, *J* = 6.4 Hz, 1H), 1.58–1.45 (m, 2H), 1.02 (d, *J* = 6.4 Hz, 3H), 0.90–0.82 (m, 12H); MS (EI) 631 (M + H)⁺; HRMS (*m/z*) (M + H)⁺ calcd for C₂₇H₄₆N₆O₁₁ 631.3303, obsd 631.3333.

Ethyl N-4-[(Benzyloxy)carbonyl]amino]phenylacetate (ZNH-PA-OEt). To a solution of ethyl 4-aminophenylacetate (4.95 g, 27.6 mmol) in CH₂Cl₂ (10 mL) were added benzyloxy-carbonyl *N*-hydroxysuccinimide ester (6.54 g, 26.2 mmol) and Et₃N (6.15 g, 60.7 mmol). The reaction mixture was allowed to stir for 5 h, and it was then concentrated in vacuo to a viscous liquid which was dissolved in EtOAc (150 mL), washed with 10% citric acid (3 × 75 mL), H₂O, saturated NaHCO₃ (2 × 75 mL), and brine, dried (Na₂SO₄), and concentrated. The residue was crystallized (EtOAc/hexanes) to provide 6.56 g (76%) of the product as a white solid: mp 76.1–77.0 °C; IR (KBr, cm⁻¹) 3310, 2970, 1725, 1602, 1531; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.73 (s, 1H), 7.36 (d, *J* = 8.3 Hz, 2H), 7.33 (m, 5H), 7.15 (d, *J* = 8.4 Hz, 2H), 5.13 (s, 2H), 4.04 (q, *J* = 7.1 Hz, 2H), 3.56 (s, 2H), 1.15 (t, *J* = 7.1 Hz, 3H); MS (EI) 336 (M + Na)⁺; HRMS *m/z* (M⁺) calcd 313.1314, obsd 313.1312. Anal. Calcd for C₁₈H₁₉NO₄: C, 68.99; H, 6.11; N, 4.47. Found: C, 69.08, H, 6.10; N, 4.34.

N-4-[(Benzyloxy)carbonyl]amino]phenylacetic Acid (ZNH-PAA). To a solution of ZNH-PA-OEt (3.20 g, 10.2 mmol) in EtOH (50 mL) was added 1 N NaOH (50 mL), and the reaction mixture was allowed to stir for 14 h whereupon it was concentrated in vacuo, diluted with H₂O (50 mL), and acidified to pH 2 (1 N HCl). The resulting precipitate was filtered and washed with 10% citric acid to provide the product (1.87 g, 64%) as a white solid: mp 150.6–151.4 °C; IR (KBr,

cm⁻¹) 3319, 3049, 1707 (br), 1590; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.71 (s, 1H), 7.41 (d, *J* = 8.4 Hz, 2H), 7.35 (m, 5H), 7.14 (d, *J* = 8.4 Hz, 2H), 5.13 (s, 2H), 3.47 (s, 2H); MS (EI) 308 (M + Na)⁺; HRMS *m/z* (M + H)⁺ calcd 286.1079, obsd 286.1092. Anal. Calcd for C₁₆H₁₅NO₄·0.03H₂O: C, 67.22; H, 5.32; N, 4.90. Found: C, 66.56; H, 5.19; N, 4.56.

N-4-[(Benzyloxy)carbonyl]amino]phenylacetate *N*-Hydroxysuccinimidyl Ester (ZNH-PA-OSu). To a solution of ZNH-PAA (500 mg, 1.75 mmol) in DMF (3 mL) were added EDC (404 mg, 2.1 mmol), HOSu (202 mg, 1.25 mmol), and Et₃N (213 mg, 2.1 mmol), and the reaction mixture was allowed to stir for 16 h. The reaction mixture was diluted into EtOAc (60 mL), washed with 10% citric acid (2 × 40 mL) and brine, dried (Na₂SO₄), and concentrated to a yellow oil. Crystallization (EtOAc/hexanes) provided the product (241 mg, 36%) as a white solid: mp 143.4–144.3 °C; IR (KBr, cm⁻¹) 3354, 2998, 1813, 1784, 1731, 1602, 1543; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.78 (s, 1H), 7.44 (d, *J* = 7.6 Hz, 2H), 7.42 (m, 5H), 7.32 (d, *J* = 7.5 Hz, 2H), 5.15 (s, 2H), 4.02 (s, 2H), 2.80 (s, 4H); MS (EI) 405 (M + Na)⁺; HRMS *m/z* (M + H)⁺ calcd 382.1165, obsd 382.1152. Anal. Calcd for C₂₀H₁₈N₂O₆·0.033H₂O: C, 62.72; H, 4.76; N, 7.31. Found: C, 62.01; H, 4.61; N, 6.97.

N-4-(Benzoylamino)phenylacetate *N*-Hydroxysuccinimidyl Ester (PhCONH-PA-OSu). Following the procedure used for the preparation of ZNH-PA-OEt, the reaction of ethyl 4-aminophenylacetate (5.27 g, 29.4 mmol) and benzoyl chloride (3.93 g, 27.9 mmol) provided PhCONH-PA-OEt (3.70 g, 47%) as a white solid: ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.94 (d, *J* = 8.1 Hz, 2H), 7.71 (d, *J* = 8.4 Hz, 2H), 7.58 (m, 3H), 7.23 (d, *J* = 8.4 Hz, 2H), 4.07 (q, *J* = 7.1 Hz, 2H), 3.62 (s, 2H), 1.18 (t, *J* = 7.1 Hz, 3H); MS (EI) 306 (M + Na)⁺, 283 (M⁺); HRMS *m/z* (M + H)⁺ calcd 284.1287, obsd 284.1288. Anal. Calcd for C₁₇H₁₇NO₃: C, 72.06; H, 6.04; N, 4.94. Found: C, 72.06; H, 5.89; N, 4.84.

Following the procedure used for the preparation of ZNH-PAA, alkaline hydrolysis of PhCONH-PA-OEt (3.50 g, 12.3 mmol) afforded PhCONH-PAA (3.2 g) in quantitative yield as a white solid: mp 193.3–194.3 °C dec; ¹H NMR (300 MHz, DMSO-*d*₆) δ 10.24 (s, 1H), 7.96 (d, *J* = 6.9 Hz, 2H), 7.72 (d, *J* = 8.3 Hz, 2H), 7.54 (m, 3H), 7.23 (d, *J* = 8.3 Hz, 2H), 3.54 (s, 2H); MS (EI) 256 (M + H)⁺; HRMS *m/z* (M + H)⁺ calcd 256.0974, obsd 256.0986.

Following the procedure used for the preparation of ZNH-PA-OSu, reaction of PhCONH-PAA (1.40 g, 5.48 mmol) and HOSu (820 mg, 7.13 mmol) in the presence of EDC (1.37 g, 7.13 mmol) in DMF (1 mL) provided PhCONH-PA-OSu (712 mg, 37%) as a white solid: mp 193.3–194.3 °C; IR (KBr, cm⁻¹) 3413, 3001, 1813, 1778, 1731, 1661, 1596, 1525; ¹H NMR (300 MHz, DMSO-*d*₆) δ 10.19 (s, 1H), 7.85 (d, *J* = 6.7 Hz, 2H), 7.66 (d, *J* = 8.5 Hz, 2H), 7.46 (m, 3H), 7.23 (d, *J* = 8.5 Hz, 2H), 3.98 (s, 2H), 2.71 (s, 4H); MS (EI) 353 (M + H)⁺; HRMS *m/z* (M + H)⁺ calcd 353.1137, obsd 353.1156. Anal. Calcd for C₁₉H₁₆N₂O₅: C, 64.77; H, 4.58; N, 7.95. Found: C, 64.76; H, 4.42; N, 7.81.

4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenylacetic Acid (MPUPA-OH) (9). To a suspension of *p*-amino-phenylacetic acid (56.8 g, 376 mmol) in DMF (150 mL) was added *o*-tolyl isocyanate (50.0 g, 376 mmol) dropwise. The reaction mixture was allowed to stir 1 h, and it was poured into EtOAc (1.75 L) with stirring. The precipitate was collected and washed with EtOAc (400 mL) and MeCN (400 mL) to provide MPUPA (80 g, 75%) as a white solid: mp 210–215 °C dec; IR (KBr, cm⁻¹) 3295 (br), 3034 (br), 1707, 1637, 1603, 1551; ¹H NMR (600 MHz, DMSO-*d*₆) δ 12.28 (br s, 1H), 9.00 (s, 1H), 7.91 (s, 1H), 7.88 (d, *J* = 7.8 Hz, 1H), 7.43 (d, *J* = 8.4 Hz, 2H), 7.19 (d, *J* = 8.4 Hz, 2H), 7.16 (m, 2H), 6.94 (dd, *J* = 7.8, 8.4 Hz, 1H), 3.51 (s, 2H), 2.25 (s, 3H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 173.0 (C=O), 152.7 (C=O), 138.5 (C), 137.5 (C), 130.2 (CH), 129.8 (C), 128.3 (2CH), 127.5 (C), 126.2 (CH), 122.7 (CH), 121.0 (CH), 118.1 (2CH), 40.1 (CH₂), 17.9 (CH₃); MS (EI) *m/z* 285 (M + H)⁺; HRMS *m/z* (M + Na)⁺ calcd 285.1239, obsd 285.1237. Anal. Calcd for C₁₅H₁₆N₂O₃: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.60; H, 5.70; N, 10.01.

4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenylacetate *N*-Hydroxysuccinimide Ester (MPUPA-OSu). To a refluxing suspension of MPUPA-OH (150 g, 0.501 mol) in CH₃CN (600 mL) was added SOCl₂ (41 mL, 0.558 mol) over 10 min with vigorous stirring. Large amounts of HCl evolved. The reaction mixture was cooled to room temperature, solid HOSu (75.5 g, 0.636 mol) was added in one portion, and Et₃N (174 mL) was then added dropwise. Stirring was continued for 2 h, and then H₂O (500 mL) was added to precipitate the product which was filtered and washed with H₂O (2 L) and CH₃CN (2 × 200 mL). Recrystallization (CH₃CN) gave 129 g (68%) of MPUPA-OSu as a white powder: mp 211.2–211.8 °C; IR (KBr, cm⁻¹) 3905–3203 (br), 1816, 1783, 1654; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.04 (s, 1 H), 7.92 (s, 1 H), 7.82 (d, 1 H), 7.44 (d, *J* = 8.5 Hz, 2 H), 7.24 (d, *J* = 8.5 Hz, 2 H), 7.15 (m, 2 H), 6.93 (dd, *J* = 7.4, 7.3 Hz, 1 H), 4.02 (s, 2 H), 2.80 (s, 4 H), 2.23 (s, 3 H); MS (EI) *m/z* 382 (M + H)⁺; HRMS *m/z* (M + Na)⁺ calcd 382.1403, obsd 382.1390. Anal. Calcd for C₂₀H₁₉N₃O₅: C, 63.15; H, 5.02; N, 11.02. Found: C, 62.96; H, 4.84; N, 11.04.

4-[(Phenylamino)carbonyl]amino]phenylacetic Acid (PUPA-OH). Following the procedure used for the preparation of MPUPA-OH, the reaction of 4-aminophenylacetic acid (20.0 g, 132 mmol) and phenyl isocyanate (15.7 g, 132 mmol) provided 31.3 g (88%) of product as a white solid: mp 230.8–231.5 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.85 (br s, 1H), 8.62 (s, 1H), 8.61 (s, 1H), 7.43 (dd, *J* = 7.5, 0.8 Hz, 2H), 7.37 (d, *J* = 8.5 Hz, 2H), 7.26 (t, *J* = 8.4 Hz, 2H), 7.15 (d, *J* = 8.5 Hz, 2H), 6.95 (t, *J* = 7.3 Hz, 1H), 3.48 (s, 2H); MS (EI) 271 (M + H)⁺; HRMS *m/z* (M + H)⁺ calcd 271.1083, obsd 271.1082. Anal. Calcd for C₁₅H₁₄N₂O₃: C, 66.66; H, 5.22; N, 10.36. Found: C, 66.70; H, 5.14; N, 10.29.

4-[(Phenylamino)carbonyl]amino]phenylacetate *N*-Hydroxysuccinimide Ester (PUPA-OSu). Following the procedure used for the preparation of MPUPA-OSu, reaction of PUPA-OH (30.0 g, 111 mmol) with SOCl₂ (14.5 g, 122 mmol) and then with *N*-hydroxysuccinimide (16.0 g, 139 mmol) in the presence of Et₃N (28.1 g, 278 mmol) provided 12 g (29%) of desired product as a white solid following recrystallization (CH₃CN): mp 204.2–205 °C; IR (KBr, cm⁻¹) 3338, 1800, 1770, 1741, 1645, 1597; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.69 (s, 1H), 8.66 (s, 1H), 7.44 (d, *J* = 6.0 Hz, 2H), 7.40 (d, *J* = 8.0 Hz, 2H), 7.27 (m, 2H), 7.25 (d, *J* = 8 Hz, 2H), 6.96 (t, *J* = 7.3 Hz, 1H), 4.02 (s, 2H), 2.80 (s, 4H); MS (EI) 368 (M + H)⁺; HRMS *m/z* (MH)⁺ calcd 368.1246, obsd 368.1253. Anal. Calcd for C₁₉H₁₇N₃O₅: C, 62.12; H, 4.66; N, 11.44. Found: C, 61.82; H, 4.56; N, 11.29.

***N*-[[4-[[[(Phenylamino)carbonyl]amino]phenyl]acetyl]-L-leucyl-L-(α -*o*-benzylaspartyl)-L-*o*-benzylvaline (PUPA-LDV(OBn)₂).** To a solution of LDV(OBn)₂·HCl (679 mg, 1.21 mmol) and PUPA-OSu (423 mg, 1.15 mmol) in DMF (0.75 mL) was added TEA (0.34 mL, 2.42 mmol), and the mixture was allowed to stir at room temperature for 3 h. The reaction mixture was poured into vigorously stirring 60% NaHCO₃ (100 mL). The precipitate was filtered, washed with saturated NaHCO₃ (2 × 100 mL), H₂O (100 mL), 10% citric acid (3 × 100 mL), and brine (100 mL), and dried in vacuo to provide the product as a white solid. This material was dissolved in THF/H₂O (25 mL, 1.5 mL). The product was precipitated by slow addition of Et₂O (100 mL), collected by filtration, and dried in vacuo to afford 703 mg (78%) of the product as a white solid: mp 195.5–197.0 °C; IR (KBr, cm⁻¹) 3295, 2959, 1735, 1646, 1540; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.16 (s, 1H), 9.12 (s, 1H), 8.40 (d, *J* = 7.6 Hz, 1H), 8.20 (d, *J* = 8.0 Hz, 1H), 7.93 (d, *J* = 8.1 Hz, 1H), 7.43 (d, *J* = 7.6 Hz, 2H), 7.34 (m, 12H), 7.28 (d, *J* = 6.9 Hz, 1H), 7.23 (d, *J* = 8.2 Hz, 1H), 7.12 (d, *J* = 8.5 Hz, 2H), 6.92 (m, 1H), 5.09 (m, 2H), 5.04 (m, 2H), 4.67 (m, 1H), 4.24 (m, 1H), 4.17 (m, 1H), 3.42 (d, *J* = 14.2 Hz, 1H), 3.32 (d, *J* = 14.2 Hz, 1H), 2.88 (dd, *J* = 16.4, 5.5 Hz, 1H), 2.62 (dd, *J* = 16.4, 7.8 Hz, 1H), 2.02 (m, 1H), 1.56 (m, 1H), 1.41 (m, 2H), 0.83 (d, *J* = 6.6 Hz, 3H), 0.81 (d, *J* = 7.4 Hz, 6H), 0.78 (d, *J* = 6.6 Hz, 3H); MS (EI) 778 (M⁺); HRMS *m/z* (M + H)⁺ calcd

778.3816, obsd 778.3824. Anal. Calcd for C₄₄H₅₁N₅O₈·0.39H₂O: C, 67.32; H, 6.65; N, 8.92. Found: C, 67.17; H, 6.72; N, 8.98.

***N*-[[4-[[[(Phenylamino)carbonyl]amino]phenyl]acetyl]-L-leucyl-L-(α -aspartyl)-L-valine (7).** To a solution of PUPA-LDV(OBn)₂ (621 mg, 0.80 mmol) in THF/H₂O (10:1, 5 mL) was added 10% Pd-C (100 mg), and the reaction mixture was stirred under H₂ (55 psi) for 20 h. The reaction mixture was filtered through SolkaFloc, and the filtrate was concentrated and precipitated in vigorously stirring Et₂O (100 mL). The solid was filtered, washed with Et₂O (3 × 100 mL), and dried in vacuo to provide the desired product (379 mg, 79%) as a white powder, mp 203.4–203.6 °C; IR (KBr, cm⁻¹) 3287, 3066, 2961, 1717, 1640; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.61 (s, 1H), 8.58 (s, 1H), 8.37 (d, *J* = 7.6 Hz, 1H), 8.18 (d, *J* = 8.1 Hz, 1H), 7.55 (d, *J* = 8.5 Hz, 1H), 7.43 (d, *J* = 7.9 Hz, 2H), 7.34 (d, *J* = 8.4 Hz, 2H), 7.26 (t, *J* = 7.8 Hz, 2H), 7.13 (d, *J* = 8.4 Hz, 2H), 6.95 (t, *J* = 7.3 Hz, 1H), 4.60 (dd, *J* = 13.3, 5.9 Hz, 1H), 4.30 (dd, *J* = 15.1, 7.9 Hz, 1H), 4.11 (dd, *J* = 8.6, 5.3 Hz, 1H), 3.43 (d, *J* = 14.0 Hz, 1H), 3.33 (d, *J* = 14.0 Hz, 1H), 2.70 (dd, *J* = 16.6, 5.7 Hz, 1H), 2.49 (m, 1H), 2.01 (m, 1H), 1.57 (m, 1H), 1.43 (m, 2H), 0.84 (d, *J* = 6.8 Hz, 3H), 0.83 (d, *J* = 6.1 Hz, 3H), 0.81 (m, 6H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 172.44 (C=O), 172.36 (C=O), 171.62 (C=O), 170.35 (C=O), 170.28 (C=O), 152.51 (C=O), 139.69 (C), 137.92 (C), 129.69 (C), 129.24 (2CH), 128.67 (2CH), 121.72 (CH), 118.17 (2CH), 118.11 (2CH), 57.07 (CH), 50.97 (CH), 49.39 (CH), 41.37 (CH₂), 41.15 (CH₂), 35.51 (CH₂), 29.99 (CH), 24.14 (CH), 22.90 (CH₃), 21.52 (CH₃), 18.87 (CH₃), 17.66 (CH₃); MS (EI) 597 (M⁺); HRMS *m/z* (M + H)⁺ calcd 598.2877, obsd 598.2867. Anal. Calcd for C₃₀H₃₉N₅O₈·0.05H₂O: C, 60.20; H, 6.58; N, 11.70. Found: C, 60.06; H, 6.60; N, 11.59.

***N*-[[4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenyl]acetyl]-L-leucyl-L-(α -*o*-benzylaspartyl)-L-*o*-benzylvaline (MPUPA-LDV(OBn)₂).** Following the procedure used for the preparation of PUPA-LDV(OBn)₂, the reaction of LDV(OBn)₂·HCl (699 mg, 1.24 mmol) and MPUPA-OSu (443 mg, 1.16 mmol) provided the desired product (773 mg, 84%) as a white solid: mp 218.4–219.0 °C; IR (KBr, cm⁻¹) 3284, 2960, 1737, 1643, 1590, 1537; ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.98 (s, 1H), 8.37 (d, *J* = 7.7 Hz, 1H), 8.18 (d, *J* = 7.8 Hz, 1H), 7.94 (d, *J* = 8.2 Hz, 1H), 7.90 (s, 1H), 7.83 (d, *J* = 7.9 Hz, 1H), 7.34 (m, 12H), 7.13 (m, 4H), 6.92 (t, *J* = 7.3 Hz, 1H), 5.10 (dd, *J* = 18, 12.5 Hz, 2H), 5.05 (t, *J* = 2.8 Hz, 2H), 4.68 (m, 1H), 4.28 (m, 1H), 4.19 (m, 1H), 3.37 (m, 1H), 3.32 (m, 1H), 2.78 (dd, *J* = 16.4, 5.5 Hz, 1H), 2.62 (dd, *J* = 16.4, 8.6 Hz, 1H), 2.20 (s, 3H), 2.10 (m, 1H), 1.57 (m, 1H), 1.42 (t, *J* = 8.2 Hz, 2H), 0.84 (d, *J* = 6.6 Hz, 6H), 0.79 (d, *J* = 6.9 Hz, 6H); HRMS *m/z* (M + H)⁺ calcd 792.3972, obsd 792.3930. Anal. Calcd for C₄₅H₅₃N₅O₈·0.19H₂O: C, 67.95; H, 6.77; N, 8.80. Found: C, 67.67; H, 6.74; N, 8.73.

***N*-[[4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenyl]acetyl]-L-leucyl-L-(α -aspartyl)-L-valine (8).** Following the procedure for preparation of PUPA-LDV, hydrogenolysis of MPUPA-LDV(OBn)₂ (640 mg, 0.81 mmol) provided the desired product (403 mg, 82%) as a white powder: mp 209.5–209.9 °C; IR (KBr, cm⁻¹) 3293, 3067, 2962, 1719, 1649, 1535; ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.00 (s, 1H), 8.36 (d, *J* = 7.8 Hz, 1H), 8.18 (d, *J* = 8.2 Hz, 1H), 7.93 (s, 1H), 7.82 (d, *J* = 8.0 Hz, 1H), 7.54 (d, *J* = 8.6 Hz, 1H), 7.35 (d, *J* = 8.4 Hz, 2H), 7.15 (m, 4H), 6.92 (t, *J* = 7.6 Hz, 1H), 4.58 (m, 1H), 4.30 (m, 1H), 4.10 (dd, *J* = 8.8, 5.3 Hz, 1H), 4.42 (d, *J* = 14 Hz, 1H), 3.33 (d, *J* = 14 Hz, 1H), 2.70 (dd, *J* = 16.5, 5.8 Hz, 1H), 2.50 (dd, *J* = 16.5, 8.4 Hz, 1H), 2.22 (s, 3H), 2.00 (m, 1H), 1.57 (m, 1H), 1.44 (m, 2H), 0.84 (d, *J* = 7.0 Hz, 3H), 0.83 (d, *J* = 6.8 Hz, 3H), 0.82 (d, *J* = 6 Hz, 3H), 0.79 (d, *J* = 6.1 Hz, 3H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 172.5 (C=O), 172.3 (C=O), 171.6 (C=O), 170.2 (2C=O), 153.6 (C=O), 138.1 (C), 137.4 (C), 130.0 (CH), 129.5 (C), 129.2 (2CH), 127.6 (C), 125.97 (CH), 122.5 (CH), 121.1 (CH), 117.9 (2CH), 57.1 (CH), 50.9 (CH), 49.4 (CH), 41.3 (CH₂), 41.1 (CH₂), 35.6 (CH₂), 30.0 (CH), 24.1 (CH), 22.9 (CH₃), 21.5 (CH₃), 18.9 (CH₃), 17.7 (CH₃), 17.6 (CH₃); MS (EI) 612 (M⁺); HRMS *m/z* (M + H)⁺ calcd 612.3033, obsd 612.3032.

Anal. Calcd for $C_{31}H_{41}N_5O_8 \cdot 0.24H_2O$: C, 60.43; H, 6.78; N, 11.36. Found: C, 60.21; H, 6.65; N, 11.14.

N-[[4-(4-Hydroxyphenyl)acetyl]-L-leucyl-L-(α -*o*-benzyl-aspartyl)-L-*o*-valine (4-HO-PA-LDV(OBn)₂). To a solution of 4-hydroxyphenylacetic acid (253 mg, 1.66 mmol) and LDV(OBn)₂·HCl (919 mg, 1.63 mmol) in DMF (2 mL) was added HOBT (335 mg, 2.48 mmol) followed by EDC (383 mg, 2.0 mmol) and TEA (1.06 mL, 3.32 mmol). The reaction mixture was allowed to stir for 14 h, and it was then poured into vigorously stirring 60% saturated NaHCO₃ (150 mL). The resulting precipitate was filtered and washed with saturated NaHCO₃ (2 × 100 mL), H₂O (100 mL), 10% citric acid (2 × 100 mL), H₂O (100 mL), brine (100 mL), and Et₂O (200 mL) to provide a tan solid. Recrystallization (THF/Et₂O) provided the desired product (707 mg, 66%) as a white solid: mp 113.4–117.3 °C; IR (KBr, cm⁻¹) 3276, 2962, 1736, 1639, 1547; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.20 (br s, 1H), 8.35 (d, *J* = 7.8 Hz, 1H), 8.10 (d, *J* = 8.0 Hz, 1H), 7.92 (d, *J* = 8.2 Hz, 1H), 7.34 (m, 10H), 7.01 (d, *J* = 8.4 Hz, 2H), 6.64 (d, *J* = 8.4 Hz, 2H), 5.09 (m, 2H), 5.05 (m, 2H), 4.67 (m, 1H), 4.25 (m, 1H), 4.18 (dd, *J* = 8.0, 6.1 Hz, 1H), 3.35 (d, *J* = 14 Hz, 1H), 3.33 (d, *J* = 14 Hz, 1H), 2.78 (dd, *J* = 16.3, 5.5 Hz, 1H), 2.61 (dd, *J* = 16.3, 8.6 Hz, 1H), 2.01 (m, 1H), 1.55 (m, 1H), 1.40 (t, *J* = 7.0 Hz, 2H), 0.82 (d, *J* = 6.1 Hz, 3H), 0.80 (d, *J* = 6.4 Hz, 3H), 0.79 (d, *J* = 6.1 Hz, 3H), 0.77 (d, *J* = 6.4 Hz, 3H); MS (EI) 660 (M⁺); HRMS *m/z* (M + H)⁺ calcd 660.3285, obsd 660.3278. Anal. Calcd for $C_{37}H_{45}N_3O_8 \cdot 0.30H_2O$: C, 66.81; H, 6.91; N, 6.32. Found: C, 66.43; H, 6.89; N, 6.56.

N-[[4-(4-Hydroxyphenyl)acetyl]-L-leucyl-L- α -aspartyl-L-valine (3). Following the procedure for preparation of PUPA-LDV, hydrogenolysis of HO-PA-LDV(OBn)₂ (560 mg, 0.85 mmol) provided the desired product (352 mg, 86%) as a white powder. Purification by HPLC (system 1, 15% solvent B (1 min), then 15% to 30% B (60 min)) followed by recrystallization (THF/Et₂O) afforded an analytical sample as a white solid: mp 109.6–110.3 °C; IR (KBr, cm⁻¹) 3306, 2965, 1742, 1724, 1712, 1666, 1651, 1639; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.17 (s, 1H), 8.34 (d, *J* = 7.6 Hz, 1H), 8.10 (d, *J* = 8.1 Hz, 1H), 7.52 (d, *J* = 8.0 Hz, 1H), 7.01 (d, *J* = 8.5 Hz, 2H), 6.64 (d, *J* = 8.5 Hz, 2H), 4.55 (m, 1H), 4.27 (m, 1H), 4.10 (m, 1H), 3.37 (m, 2H), 2.69 (dd, *J* = 16.6, 5.7 Hz, 1H), 2.48 (dd, *J* = 16.6, 8.5 Hz, 1H), 2.00 (m, 1H), 1.53 (m, 1H), 1.41 (m, 2H), 0.82 (d, *J* = 6.1 Hz, 3H), 0.80 (d, *J* = 6.4 Hz, 3H), 0.79 (d, *J* = 6.1 Hz, 3H), 0.77 (d, *J* = 6.4 Hz, 3H); MS (EI) 479 (M⁺); HRMS *m/z* (M + H)⁺ calcd 480.2346, obsd 480.2346. Anal. Calcd for $C_{23}H_{33}N_3O_8$: C, 57.61; H, 6.94; N, 8.76. Found: C, 57.36; H, 7.30; N, 8.11.

N-[[4-[[[(Benzyloxy)carbonyl]amino]phenyl]acetyl]-L-leucyl-L- α -aspartyl-L-valine (6). To a solution of LDV·HCl (253 mg, 0.66 mmol) and ZNH-PA-OSu (237 mg, 0.62 mmol) in DMF (1.5 mL) was added TEA (0.19 mL, 1.32 mmol), and the reaction was allowed to stir for 14 h. The reaction mixture was partitioned between ethyl acetate (125 mL) and water (25 mL), and the organic layer was separated, washed with 5% citric acid (3 × 50 mL), water (50 mL), and brine (50 mL), dried (Na₂SO₄), and evaporated in vacuo to provide the desired product (274 mg, 72%) as a white solid. Recrystallization (EtOAc/Et₂O) afforded an analytical sample as a white solid: mp 215.0–215.4 °C; IR (KBr, cm⁻¹) 3302, 2961, 1702, 1637, 1534; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.72 (s, 1H), 8.41 (d, *J* = 7.6 Hz, 1H), 8.22 (d, *J* = 8.6 Hz, 1H), 7.59 (d, *J* = 8.6 Hz, 1H), 7.41 (m, 7H), 7.18 (d, *J* = 8.6 Hz, 2H), 5.17 (s, 2H), 4.61 (m, 1H), 4.33 (dd, *J* = 14.9, 8.0 Hz, 1H), 4.15 (dd, *J* = 9.6, 3.4 Hz, 1H), 3.47 (d, *J* = 14.2 Hz, 1H), 3.41 (d, *J* = 14.2 Hz, 1H), 2.75 (dd, *J* = 16.6, 5.7 Hz, 1H), 2.54 (m, 1H), 2.05 (m, 1H), 1.59 (m, 1H), 1.47 (t, *J* = 6.8 Hz, 2H), 0.85 (m, 12H); MS (EI) 611 (M - H)⁺; HRMS *m/z* (M + H)⁺ calcd 613.2874, obsd 613.2864. Anal. Calcd for $C_{31}H_{40}N_4O_9$: C, 60.77; H, 6.58; N, 9.14. Found: C, 60.40; H, 6.59; N, 8.90.

N-[[4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenyl]acetyl]-L-leucyl-L- α -aspartyl-L-valyl-L-prolyl-L-serinyl-L-threonine (MPUPA-LDVPST) (13). Following the procedure used for the preparation of ZNH-PA-LDV, reaction of LDVPST·HCl (299 mg, 0.45 mmol) and MPUPA-OSu (154 mg, 0.40 mmol) provided 216 mg (60%) of the desired product

as a white solid: mp 184–186 °C dec; IR (KBr, cm⁻¹) 3307, 3072, 2966, 1731, 1649; ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.95 (d, *J* = 4.6 Hz, 1H), 8.36 (d, *J* = 7.5 Hz, 1H), 8.19 (d, *J* = 7.7 Hz, 1H), 8.07 (d, *J* = 7.8 Hz, 1H), 7.89 (s, 1H), 7.83 (d, *J* = 7.5 Hz, 1H), 7.51 (dd, *J* = 14.4, 8.7 Hz, 2H), 7.36 (d, *J* = 8.4 Hz, 2H), 7.15 (m, 4H), 6.93 (t, *J* = 7.4 Hz, 1H), 4.55 (m, 1H), 4.42 (dd, *J* = 7.8, 3.7 Hz, 1H), 4.33 (m, 2H), 4.22 (m, 1H), 4.13 (dd, *J* = 6.4, 3.1 Hz, 1H), 3.60 (m, 6H), 3.44 (d, *J* = 13.6 Hz, 1H), 3.35 (d, *J* = 14.1 Hz, 1H), 2.71 (dd, *J* = 16.2, 6.2 Hz, 1H), 2.49 (m, 1H), 2.24 (s, 3H), 2.03 (m, 1H), 1.93 (m, 2H), 1.82 (m, 1H), 1.57 (m, 1H), 1.43 (m, 2H), 1.01 (d, *J* = 6.5 Hz, 3H), 0.90 (d, *J* = 6.1 Hz, 3H), 0.86 (d, *J* = 7.1 Hz, 3H), 0.82 (d, *J* = 5.5 Hz, 3H), 0.81 (d, *J* = 6.1 Hz, 3H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 172.4 (C=O), 172.3 (C=O), 171.9 (C=O), 171.7 (C=O), 171.5 (C=O), 170.3 (C=O), 170.1 (C=O), 169.4 (C=O), 152.6 (C=O), 138.1 (C), 137.5 (C), 130.2 (CH), 129.6 (C), 129.3 (CH), 127.4 (C), 126.1 (CH), 122.6 (CH), 120.9 (CH), 120.8 (CH), 117.9 (CH), 117.8 (CH), 66.4 (CH), 61.4 (CH₂), 59.2 (CH), 57.3 (CH), 55.4 (CH), 55.0 (CH), 50.8 (CH), 49.3 (CH), 47.1 (CH₂), 41.4 (CH₂), 41.2 (CH₂), 35.4 (CH₂), 30.2 (CH), 29.1 (CH₂), 24.4 (CH₂), 24.2 (CH), 23.0 (CH₃), 21.5 (CH₃), 20.3 (CH₃), 19.1 (CH₃), 17.9 (2CH₃); MS (EI) 897 (M + H)⁺; HRMS *m/z* (M + Na)⁺ calcd 919.4178, obsd 919.4185. Anal. Calcd for $C_{43}H_{60}N_8O_{13} \cdot 1.62H_2O$: C, 55.76; H, 6.88; N, 12.09. Found: C, 55.56; H, 6.85; N, 11.43.

N-[[4-(Benzoylamino)phenyl]acetyl]-L-leucyl-L- α -aspartyl-L-valine (5). To a mixture of LDV·HCl (376 mg, 0.98 mmol) and PhCONH-PA-OSu (327.6 mg, 0.93 mmol) in DMF (2 mL) was added TEA (0.27 mL, 1.98 mmol), and the reaction mixture was stirred for 4 h after which it was poured into vigorously stirring 5% citric acid (125 mL). The resulting precipitate was filtered, washed with 5% citric acid (3 × 50 mL), water (50 mL), and brine (50 mL), and dried in vacuo to provide the desired product (439 mg, 81%) as a white powder. Purification by preparative HPLC (system 1, 20% solvent B (1 min), then 20% to 45% B (50 min)) followed by recrystallization (THF/Et₂O) afforded an analytical sample: mp 207.1–207.5 °C; IR (KBr, cm⁻¹) 3319, 2961, 1711, 1654, 1522; ¹H NMR (300 MHz, DMSO-*d*₆) δ 10.16 (s, 1H), 8.36 (d, *J* = 7.6 Hz, 1H), 8.19 (d, *J* = 8.2 Hz, 1H), 7.91 (dd, *J* = 8.1, 1.4 Hz, 2H), 7.63 (dd, *J* = 8.0, 1.4 Hz, 2H), 7.55 (m, 4H), 7.18 (d, *J* = 8.5 Hz, 2H), 4.55 (dd, *J* = 13.4, 7.7 Hz, 1H), 4.29 (dd, *J* = 14.9, 8.1 Hz, 1H), 4.09 (dd, *J* = 9.6, 4.4 Hz, 1H), 3.46 (d, *J* = 14.1 Hz, 1H), 3.35 (d, *J* = 14.1 Hz, 1H), 2.68 (dd, *J* = 16.6, 5.8 Hz, 1H), 2.47 (dd, *J* = 16.6, 7.9 Hz, 1H), 1.99 (m, 1H), 1.55 (m, 1H), 1.41 (m, 2H), 0.82 (m, 12H); MS (EI) 582 (M⁺); HRMS *m/z* (M + H)⁺ calcd 583.2768, obsd 583.2759. Anal. Calcd for $C_{30}H_{38}N_4O_8 \cdot 0.333H_2O$: C, 61.21; H, 6.62; N, 9.51. Found: C, 61.76; H, 6.85; N, 9.12.

N-[[4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenyl]acetyl]-L-leucine (10). To a solution of L-leucine (504 mg, 3.8 mmol) and TEA (0.75 mL, 4.8 mmol) in DMF (6 mL) was added MPUPA-OSu (1.31 g, 3.45 mmol), and the reaction was allowed to stir for 2 h. The reaction mixture was then added dropwise to vigorously stirring 5% citric acid (125 mL), and the precipitate was filtered, washed with water (500 mL), and dried in vacuo to afford the crude product which was dissolved in THF (10 mL) and precipitated with Et₂O (200 mL). The precipitate was filtered and dried in vacuo to afford the desired product (891 mg, 65%) as a white solid: mp 170–173 °C dec; IR (KBr, cm⁻¹) 3308, 2950, 1729, 1710, 1595; ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.94 (s, 1H), 8.28 (d, *J* = 7.7 Hz, 1H), 7.87 (s, 1H), 7.83 (d, *J* = 7.6 Hz, 1H), 7.36 (d, *J* = 7.9 Hz, 2H), 7.14 (m, 4H), 6.92 (t, *J* = 7.3 Hz, 1H), 4.22 (m, 1H), 3.40 (m, 3H), 2.22 (s, 3H), 1.60 (m, 1H), 1.50 (m, 2H), 0.88 (d, *J* = 5.9 Hz, 3H), 0.81 (d, *J* = 6.3 Hz, 3H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 174.0 (C=O), 170.4 (C=O), 152.7 (C=O), 138.1 (C), 137.4 (C), 130.1 (CH), 129.6 (C), 129.2 (2CH), 127.5 (C), 126.0 (CH), 122.6 (CH), 121.1 (CH), 117.9 (2CH), 50.3 (CH), 41.3 (CH₂), 40.1 (CH₂), 24.3 (CH), 22.7 (CH₃), 21.3 (CH₃), 17.8 (CH₃); MS (EI) 397 (M⁺); HRMS *m/z* (M + H)⁺ calcd 398.2080, obsd 398.2082. Anal. Calcd for $C_{22}H_{27}N_3O_4$: C, 66.48; H, 6.85; N, 10.57. Found: C, 66.26; H, 6.86; N, 10.44.

L-Leucyl-L-(α -*o*-benzylaspartyl)-L-valyl-prolinamide Hydrochloride (LD(OBn)VP-NH₂·HCl). Following the method for coupling and deprotection of LDVP(OBn)₂·HCl but starting with L-prolinamide, the desired product was obtained as a white solid: mp 146–148 °C; IR (KBr, cm⁻¹) 3307, 3201, 3048, 2966, 1737, 1684, 1637; ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.00 (d, *J* = 7.6 Hz, 1H), 8.37 (bm, 2H), 7.93 (d, *J* = 8.5 Hz, 1H), 7.35 (m, 5H), 7.26 (m, 1H), 6.84 (br s, 1H), 5.08 (s, 2H), 4.71 (m, 1H), 4.31 (m, 1H), 4.20 (m, 1H), 3.62 (m, 3H), 2.82 (dd, *J* = 16.5, 6.3 Hz, 1H), 2.67 (dd, *J* = 16.5, 7.5 Hz, 1H), 1.75 (m, 8H), 0.84 (m, 12H); MS (EI) 533 (M - Cl)⁺; HRMS *m/z* (M⁺) calcd for C₂₇H₄₁N₅O₆·HCl 532.3135, obsd 532.3130.

N-[[4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenyl]acetyl]-L-leucyl-L- α -aspartyl-L-valyl-L-prolinamide (14). To a solution of LD(OBn)VP-NH₂·HCl (535 mg, 0.94 mmol) and TEA (0.4 mL, 2.82 mmol) in DMF (5 mL) was added MPUPA-OSu (288 mg, 0.75 mmol), and the suspension was stirred for 2 h. The reaction mixture was then added dropwise to vigorously stirring 5% citric acid (125 mL), and the precipitate was filtered, washed with water (100 mL), 5% NaHCO₃ (100 mL), and brine (100 mL), and dried in vacuo to afford the crude product (540 mg, 90%) as a light tan solid, mp 181–183 °C. The crude material was dissolved in DMF (2 mL) and diluted with EtOAc (10 mL), and 10% Pd-C (100 mg) was added after which the mixture was stirred under H₂ (50 psi) for 4 h. The reaction mixture was filtered through Celite, and the filtrate was concentrated to ~3 mL volume and purified by preparative HPLC (system 1, 20% to 25% solvent B (6 min), 25% to 50% B (20 min), 50% to 80% B (24 min)) to afford the desired product (218 mg, 46%) as a white solid: mp 183–185 °C dec; IR (KBr, cm⁻¹) 3300, 2961, 1700, 1639, 1543; ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.99 (bm, 1H), 8.35 (d, *J* = 7.6 Hz, 1H), 8.18 (d, *J* = 8.4 Hz, 1H), 7.91 (s, 1H), 7.84 (m, 1H), 7.50 (d, *J* = 8.6 Hz, 1H), 7.36 (d, *J* = 8.7 Hz, 2H), 7.24 (s, 1H), 7.13 (m, 4H), 6.93 (t, *J* = 7.2 Hz, 1H), 6.84 (s, 1H), 4.54 (m, 1H), 4.32 (m, 2H), 4.22 (m, 1H), 3.43 (d, *J* = 14 Hz, 1H), 3.36 (d, *J* = 14 Hz, 1H), 3.33 (m, 3H), 2.70 (dd, *J* = 17.0, 6.4 Hz, 1H), 2.48 (dd, *J* = 17.0, 7.4 Hz, 1H), 2.23 (s, 3H), 2.02 (m, 2H), 1.97 (m, 1H), 1.90 (m, 2H), 1.82 (m, 1H), 1.76 (m, 2H), 0.88 (d, *J* = 6.6 Hz, 3H), 0.85 (d, *J* = 6.4 Hz, 3H), 0.80 (d, *J* = 6.7 Hz, 6H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 173.2 (C=O), 172.2 (C=O), 171.5 (C=O), 170.1 (C=O), 169.9 (C=O), 169.2 (C=O), 152.6 (C=O), 138.1 (C), 137.3 (C), 130.0 (CH), 129.5 (C), 129.2 (2CH), 127.3 (C), 126.0 (CH), 122.5 (CH), 121.0 (CH), 117.8 (CH), 117.7 (CH), 59.2 (CH), 55.2 (CH), 50.8 (CH), 49.2 (CH), 46.8 (CH₂), 41.3 (CH₂), 41.0 (CH₂), 35.4 (CH₂), 30.0 (CH), 29.0 (CH₂), 24.4 (CH₂), 24.1 (CH), 22.9 (CH₃), 21.4 (CH₃), 19.1 (CH₃), 17.7 (CH₃), 17.5 (CH₃); MS (EI) 707 (M⁺); HRMS *m/z* (M + H)⁺ calcd 708.3721, obsd 708.3727. Anal. Calcd for C₃₆H₄₉N₇O₈·0.57H₂O: C, 60.21; H, 7.04; N, 13.65. Found: C, 60.57; H, 6.94; N, 13.41.

N-(Phenylacetyl)-L-leucyl-L- α -(*o*-benzylaspartyl)-L-*o*-benzylvaline (PA-LDV(OBn)₂). To a solution of LDV(OBn)₂·HCl (480 mg, 0.86 mmol) and TEA (0.25 mmol, 1.72 mmol) in DMF (3 mL) was added phenylacetic acid (179 mg, 0.77 mmol) followed by HOBt (155 mg, 1.15 mmol) and EDC (185 mg, 0.96 mmol), and the suspension was stirred for 2 h. The reaction mixture was diluted with DMF (2 mL) and purified by preparative HPLC (system 1, 20% to 25% solvent B (6 min), 25% to 50% B (20 min), 50% to 80% B (24 min)) to afford the desired product (254 mg, 51%) as a white solid: mp 155–157 °C; IR (KBr, cm⁻¹) 3625, 3138, 2890, 1711, 1681, 1590; ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.36 (d, *J* = 7.6 Hz, 1H), 8.23 (d, *J* = 8.0 Hz, 1H), 7.92 (d, *J* = 8.1 Hz, 1H), 7.26 (m, 15H), 5.12 (d, *J* = 12.4 Hz, 1H), 5.07 (d, *J* = 12.4 Hz, 1H), 5.06 (d, *J* = 12.5 Hz, 1H), 5.02 (d, *J* = 12.6 Hz, 1H), 4.67 (m, 1H), 4.27 (m, 1H), 4.17 (m, 1H), 3.48 (d, *J* = 13.9 Hz, 1H), 3.39 (d, *J* = 13.9 Hz, 1H), 2.77 (dd, *J* = 16.1, 5.4 Hz, 1H), 2.61 (dd, *J* = 16.1, 8.5 Hz, 1H), 2.01 (m, 1H), 1.55 (m, 1H), 1.41 (m, 2H), 0.80 (m, 12H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 172.1 (C=O), 170.7 (C=O), 170.2 (C=O), 170.0 (C=O), 169.7 (C=O), 136.2 (C), 135.8 (C), 135.7 (C), 128.8 (2CH), 128.2 (2CH), 127.9 (3CH), 127.8 (2CH), 127.7 (2CH), 127.6 (2CH), 126.1 (2CH), 65.8 (CH₂), 65.5 (CH₂), 57.3 (CH), 51.0 (CH), 49.2 (CH), 41.9 (CH₂),

40.9 (CH₂), 35.6 (CH₂), 29.8 (CH), 24.0 (CH), 22.8 (CH₃), 21.5 (CH₃), 18.6 (CH₃), 17.8 (CH₃); MS (EI) 644 (M + H)⁺; HRMS *m/z* (M + H)⁺ calcd 644.3336, obsd 644.3336. Anal. Calcd for C₃₇H₄₅N₃O₇: C, 68.97; H, 7.04; N, 6.52. Found: C, 68.96; H, 6.87; N, 6.46.

N-(Phenylacetyl)-L-leucyl-L- α -aspartyl-L-valine (4). To a solution of PA-LDV(OBn)₂ (104 mg, 0.16 mmol) in EtOAc (5 mL) was added Pd-C (100 mg), and the suspension was stirred under H₂ (50 psi) for 12 h. The reaction mixture was filtered through Celite, the solvent was removed in vacuo, and the product was dissolved in CH₃CN (2 mL) and purified by preparative HPLC (system 1, 20% to 25% solvent B (6 min), 25% to 50% B (20 min), 50% to 80% (24 min)) to afford the desired product (36 mg, 48%) as a white solid: mp 143–144 °C; IR (KBr, cm⁻¹) 3291, 2968, 1717, 1644, 1539; ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.37 (d, *J* = 7.7 Hz, 1H), 8.24 (d, *J* = 8.4 Hz, 1H), 7.55 (d, *J* = 8.4 Hz, 1H), 7.29–7.19 (m, 5H), 4.59 (m, 1H), 4.32 (m, 1H), 4.12 (m, 1H), 3.51 (d, *J* = 13.9 Hz, 1H), 3.42 (d, *J* = 13.9 Hz, 1H), 2.71 (dd, *J* = 16.5, 5.9 Hz, 1H), 2.50 (dd, *J* = 16.5, 7.7 Hz, 1H), 2.02 (m, 1H), 1.55 (m, 1H), 1.45 (m, 2H), 0.85–0.79 (m, 12H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 172.6 (C=O), 172.4 (C=O), 171.7 (C=O), 170.5 (C=O), 170.0 (C=O), 136.4 (C), 129.0 (2CH), 128.1 (2CH), 126.2 (CH), 57.1 (CH), 50.9 (CH), 49.4 (CH), 42.0 (CH₂), 41.3 (CH₂), 35.5 (CH₂), 30.0 (CH), 24.2 (CH), 23.1 (CH₃), 21.5 (CH₃), 19.0 (CH₃), 17.7 (CH₃); MS (EI) 463 (M⁺); HRMS *m/z* (M + H)⁺ calcd for C₂₃H₃₃N₃O₇ 464.2397, obsd 464.2383. Anal. Calcd for C₂₃H₃₃N₃O₇: C, 59.60; H, 7.18; N, 9.05. Found: C, 59.29; H, 7.24; N, 8.89.

N-[[4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenyl]acetyl]-L-leucyl-L- α -(*o*-benzylaspartyl)-L-valyl-*o*-benzylproline (MPUPA-LDVP(OBn)₂). To a solution of EDC (69.8 g, 0.364 mol) in DMF (600 mL) were added HOBt (55.9 g, 0.364 mol) and TEA (127 mL). The resulting mixture was stirred at room temperature for 45 min, and MPUPA-OH (86.3 g, 0.304 mol) was added followed by LDVP(OBn)₂·HCl (200 g, 0.30 mol). After 20 h the reaction mixture was poured into vigorously stirring water (3 L), and the precipitate which formed was stirred for an additional 45 min after which it was filtered and washed with water (2 × 0.5 L). The filter cake was stirred with 1:1 CH₃CN/H₂O (3 L, containing 0.1% TFA) for 4 h followed by filtration and further trituration with methanol (6.0 L) for 3 h. The product was finally filtered, rinsed with methanol (0.5 L), and air-dried on a funnel for 12 h. The white solid was crushed and dried for an additional 12 h to afford the desired product (228.9 g, 85%) as white fine powder: mp 199.6–200.8 °C; IR (KBr, cm⁻¹) 3285, 3053, 2961, 1740, 1642, 1590; ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.95 (s, 1H), 8.43 (d, *J* = 7.8 Hz, 1H), 8.21 (d, *J* = 7.8 Hz, 1H), 7.89 (s, 1H), 7.86 (d, *J* = 8.4 Hz, 1H), 7.67 (d, *J* = 8.4 Hz, 1H), 7.39 (d, *J* = 6 Hz, 2H), 7.34 (m, 10H), 7.15 (m, 4H), 6.94 (t, *J* = 7.5 Hz, 1H), 5.12 (m, 2H), 5.05 (m, 2H), 4.65 (dd, *J* = 14.1, 7.5 Hz, 1H), 4.36 (m, 3H), 3.72 (dd, *J* = 16.5, 6.9 Hz, 1H), 3.57 (dd, *J* = 15.9, 6.9 Hz, 1H), 3.45 (d, *J* = 14.4 Hz, 1H), 3.37 (d, *J* = 14.4 Hz, 1H), 2.85 (dd, *J* = 16.5, 6.2 Hz, 1H), 2.66 (dd, *J* = 16.1, 7.7 Hz, 1H), 2.24 (s, 3H), 2.16 (m, 1H), 1.95 (m, 1H), 1.88 (m, 2H), 1.82 (m, 1H), 1.60 (m, 1H), 1.46 (m, 2H), 0.85 (m, 12H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 172.4 (C=O), 171.6 (C=O), 170.3 (C=O), 170.0 (C=O), 169.9 (C=O), 169.6 (C=O), 152.6 (C=O), 138.2 (C), 137.5 (C), 136.0 (C), 135.9 (C), 130.2 (CH), 129.6 (C), 129.4 (2CH), 128.4 (2CH), 128.3 (CH), 128.0 (2CH), 127.9 (2CH), 127.8 (2CH), 127.7 (2CH), 127.3 (C), 126.1 (CH), 122.5 (CH), 120.9 (CH), 117.9 (CH), 65.9 (CH₂), 65.7 (CH₂), 58.6 (CH), 55.3 (CH), 50.9 (CH), 49.3 (CH), 46.8 (CH₂), 41.4 (CH₂), 41.1 (CH₂), 35.6 (CH₂), 30.2 (CH), 28.7 (CH₂), 24.7 (CH), 24.2 (CH₂), 23.0 (CH₃), 21.6 (CH₃), 18.9 (CH₃), 17.9 (CH₃), 17.7 (CH₃); MS (EI) 889 (M-H)⁺. Anal. Calcd for C₅₀H₆₀N₆O₉·0.30H₂O: C, 67.14; H, 6.83; N, 9.39. Found: C, 67.40; H, 6.78; N, 9.46.

N-[[4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenyl]acetyl]-L-leucyl-L- α -aspartyl-L-valyl-L-proline (12). To a solution of MPUPA-LDVP(OBn)₂ (22 g, 24.7 mmol) in THF (150 mL) and purified water (30 mL) was added 10% Pd-C (1 g). The reaction mixture was stirred under H₂ (45

psi) for 3 h. The catalyst was filtered on a filter cloth and washed with a 4:1 THF/H₂O (50 mL). The combined filtrate was concentrated in vacuo at 45 °C to 45 mL. After cooling to 20 °C, the solution was poured into vigorously stirring toluene (50 mL), the slurry was allowed to stir for 30 min, and the precipitate was filtered, washed with toluene (250 mL), and dried in a vacuum oven at 15 mbar at 55 °C. The solid was further dried under vacuum at 1.5 mbar to constant weight to afford the desired product (16 g, 91%) as a fine white powder: mp 175–178 °C dec; IR (KBr, cm⁻¹) 3302, 2962, 1723, 1642, 1542; ¹H NMR (600 MHz, DMSO-*d*₆) δ 12.4 (bs, 1H), 8.97 (s, 1H), 8.38 (d, *J* = 7.8 Hz, 1H), 8.20 (d, *J* = 8.4 Hz, 1H), 7.91 (s, 1H), 7.84 (d, *J* = 8.1 Hz, 1H), 7.55 (d, *J* = 8.4 Hz, 1H), 7.38 (d, *J* = 8.4 Hz, 2H), 7.17 (d, *J* = 8.4 Hz, 1H), 7.14 (m, 3H), 6.93 (t, *J* = 7.3 Hz, 1H), 4.56 (dd, *J* = 14.1, 6.9 Hz, 1H), 4.37 (t, *J* = 8.1, Hz, 1H), 4.33 (dd, *J* = 14.3, 8.8, Hz, 1H), 4.24 (dd, *J* = 7.8, 5.4 Hz 1H), 3.69 (dd, *J* = 16.1, 6.9 Hz, 1H), 3.55 (dd, *J* = 15.9, 6.9 Hz, 1H), 3.45 (d, *J* = 13.8 Hz 1H), 3.36 (d, *J* = 13.8 Hz, 1H), 2.71 (dd, *J* = 16.5, 6.3 Hz, 1H), 2.51 (dd, *J* = 16.2, 7.2 Hz 1H), 2.24 (s, 3H), 2.14 (m, 1H), 1.95 (m, 1H), 1.90 (m, 2H), 1.82 (m, 1H), 1.45 (m, 2H), 0.91 (d, *J* = 6.6 Hz, 3H), 0.87 (d, *J* = 6.6 Hz, 3H), 0.83 (d, *J* = 6.6 Hz 3H), 0.81 (d, *J* = 6.6 Hz, 3H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 173.3 (C=O), 172.4 (C=O), 171.8 (C=O), 170.4 (C=O), 170.2 (C=O), 169.4 (C=O), 152.7 (C=O), 138.2 (C), 137.5 (C), 130.2 (CH), 129.7 (C), 129.4 (2CH), 127.4 (C), 126.2 (CH), 122.6 (CH), 121.0 (CH), 118.0 (2CH), 58.6 (CH), 55.2 (CH), 50.9 (CH), 49.4 (CH), 46.8 (CH₂), 41.4 (CH₂), 41.2 (CH₂), 35.6 (CH₂), 30.3 (CH), 28.7 (CH₂), 24.6 (CH₂), 24.2 (CH), 23.1 (CH₃), 21.5 (CH₃), 19.0 (CH₃), 17.9 (CH₃), 17.7 (CH₃); HRMS *m/z* (M + H)⁺ calcd 709.3561, obsd 709.3555. Anal. Calcd for C₃₆H₄₈N₆O₉·0.24H₂O: C, 60.63; H, 6.85; N, 11.78. Found: C, 60.32; H, 6.91; N, 11.66.

Dibenzyl *N*-[[[4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenyl]acetyl]-L-leucyl-L-α-aspartate (MPUPA-LD(OBn)₂). To a solution of LD(OBn)₂·HCl (92.5 g, 0.20 mol) and TEA (56 mL, 0.40 mol) in DMF (500 mL) was added MPUPA-OSu (73.9 g, 0.19 mol), and the reaction was stirred for 3 h. The reaction mixture was then added to vigorously stirring 60% NaHCO₃ (3 L), and the precipitate was filtered and washed with 5% NaHCO₃ (2 × 1 L), water (4 × 1 L), 5% citric acid (3 × 1 L), water (2 × 1 L), brine (2 × 1 L), CH₃CN (2 × 1 L), and THF (2 × 1 L). The material was dried in vacuo to afford the desired product (129.4 g, 96%) as a white solid: mp 155 °C dec; IR (KBr, cm⁻¹) 3277, 2956, 1741, 1722, 1637, 1592; ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.97 (s, 1H), 8.56 (d, *J* = 7.6 Hz, 1H), 8.18 (d, *J* = 8.6 Hz, 1H), 7.90 (s, 1H), 7.85 (d, *J* = 7.7 Hz, 1H), 7.38 (d, *J* = 8.1 Hz, 2H), 7.33 (m, 10H), 7.15 (m, 4H), 6.93 (t, *J* = 7.2 Hz, 1H), 5.07 (s, 4H), 4.77 (m, 1H), 4.36 (m, 1H), 3.43 (d, *J* = 14 Hz, 1H), 3.36 (d, *J* = 14 Hz, 1H), 2.92 (dd, *J* = 16.7, 6.2 Hz, 1H), 2.80 (dd, *J* = 16.7, 7.1 Hz, 1H), 2.24 (s, 3H), 1.56 (m, 1H), 1.41 (m, 2H), 0.82 (d, *J* = 6.5 Hz, 3H), 0.77 (d, *J* = 6.5 Hz, 3H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 172.1 (C=O), 170.2 (C=O), 170.1 (C=O), 169.6 (C=O), 152.6 (C=O), 138.1 (C), 137.4 (C), 136.1 (C), 135.5 (C), 130.0 (CH), 129.5 (C), 129.2 (2CH), 128.3 (2CH), 128.2 (2CH), 127.9 (2CH), 127.8 (2CH), 127.6 (2CH), 127.4 (C), 126.0 (CH), 122.5 (CH), 121.0 (CH), 117.9 (2CH), 66.3 (CH₂), 65.9 (CH₂), 50.7 (CH), 48.5 (CH), 41.4 (CH₂), 41.0 (CH₂), 35.6 (CH₂), 24.1 (CH), 22.8 (CH₃), 21.5 (CH₃), 17.7 (CH₃); MS (EI) 693 (M + H)⁺. Anal. Calcd for C₄₀H₄₄N₄O₇·0.19H₂O: C, 68.95; H, 6.46; N, 8.04. Found: C, 68.56; H, 6.31; N, 7.87.

***N*-[[[4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenyl]acetyl]-L-leucyl-L-α-aspartic Acid (11).** To a solution of MPUPA-LD(OBn)₂ (128 g, 0.185 mol) in 10:1 THF/0.1N HCl (1.3 L) was added 10% Pd-C (3.6 g), and the mixture was stirred under H₂ (50 psi) overnight. Upon completion the reaction mixture was filtered through Celite, and the filtrate was transferred to a separatory funnel and diluted with Et₂O (3 L). The aqueous layer was separated and added slowly to stirring Et₂O (12 L) to precipitate the product. The precipitate thus formed was filtered, washed with Et₂O (2 L), and dried in a vacuum oven (1 mmHg, 45 °C) to afford the desired product (81.8 g, 86%) as a white solid: mp 177–179 °C dec; IR (KBr, cm⁻¹) 3298, 1728, 1699, 1643, 1615; ¹H NMR (600

MHz, DMSO-*d*₆) δ 8.96 (s, 1H), 8.25 (d, *J* = 7.6 Hz, 1H), 8.15 (d, *J* = 8.4 Hz, 1H), 7.89 (s, 1H), 7.83 (d, *J* = 7.6 Hz, 1H), 7.37 (d, *J* = 8.0 Hz, 2H), 7.14 (m, 4H), 6.93 (t, *J* = 7.3 Hz, 1H), 4.52 (m, 1H), 4.34 (m, 1H), 3.34 (d, *J* = 14.0 Hz, 1H), 3.37 (d, *J* = 14.0 Hz, 1H), 2.69 (dd, *J* = 16.5, 5.8 Hz, 1H), 2.58 (dd, *J* = 16.5, 7.3 Hz, 1H), 2.23 (s, 3H), 1.58 (m, 1H), 1.45 (m, 2H), 0.86 (d, *J* = 6.2 Hz, 3H), 0.80 (d, *J* = 6.5 Hz, 3H); ¹³C NMR (150 MHz, DMSO-*d*₆) δ 172.1 (C=O), 171.9 (C=O), 171.5 (C=O), 170.1 (C=O), 152.7 (C=O), 138.1 (C), 137.4 (C), 130.1 (CH), 129.6 (C), 129.3 (2CH), 127.5 (C), 126.0 (CH), 122.6 (CH), 121.1 (CH), 118.0 (2 CH), 50.8 (CH), 48.6 (CH), 41.4 (CH₂), 41.0 (CH₂), 35.9 (CH₂), 24.1 (CH), 23.0 (CH₃), 21.6 (CH₃), 17.8 (CH₃); MS (EI) 513 (M⁺); HRMS *m/z* (M + H)⁺ calcd 513.2349, obsd 513.2360. Anal. Calcd for C₂₆H₃₂N₄O₇·0.25H₂O: C, 60.36; H, 6.51; N, 10.83. Found: C, 60.73; H, 6.42; N, 10.74.

6-[*N*-(Benzyloxycarbonyl)amino]hexanoic Acid. To a solution of 6-aminohexanoic acid (8 g, 61 mmol) in water (18 mL) stirring in an ice bath were added sodium carbonate (3.2 g, 61 mmol) and benzyl chloroformate (9.6 mL, 67.1 mL). The reaction was stirred at 0 °C for 15 min while 2 N sodium hydroxide (34 mL, 67.1 mmol) was added in portions. The reaction was stirred for 1 h with warming to room temperature. The reaction mixture was extracted with Et₂O (2 × 20 mL), and the aqueous layer was acidified to pH = 1–2 with cooling on ice. The resulting precipitate was filtered, washed with cold water (3 × 20 mL), and dried in vacuo to afford the desired product (5.1 g, 32%) as a white solid: mp 54.6–55.2 °C; ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.33 (m, 5H), 4.99 (s, 2H), 3.4 (br s, 1H), 2.96 (dd, *J* = 12.8, 6.6 Hz, 2H), 2.17 (t, *J* = 7.2 Hz, 2H), 1.35 (m, 6H); HRMS *m/z* (M⁺) calcd 226.1395, obsd 226.1392. This material was used in the following reaction without purification.

***N*_ε-[6-[*N*-(Benzyloxycarbonyl)amino]hexanoyl]-*N*_α-(*tert*-butyloxycarbonyl)-L-lysine Methyl Ester (Boc-Lys(Z-Nhex)-OCH₃).** To a solution of 6-[*N*-(benzyloxycarbonyl)amino]hexanoic acid (5 g, 18.9 mmol), HOBt (3.6 g, 26.4 mmol), and *N*_α-Boc-L-lysine methyl ester hydrochloride (5.6 g, 18.9 mmol) in DMF (25 mL) was added TEA (6.5 mL, 47.1 mmol) followed by EDC (4.3 g, 22.6 mmol), and the reaction was stirred for 2 h. The reaction mixture was diluted with EtOAc (100 mL) and washed with 60% NaHCO₃ (3 × 40 mL), water (40 mL), 5% citric acid (3 × 40 mL), and brine (3 × 40 mL). The organic layer was dried (Na₂SO₄) and concentrated in vacuo to afford the desired product as a pale-yellow oil (5.6 g, 58%): ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.34 (m, 5H), 5.8 (br s, 1H), 5.1 (d, *J* = 8.0 Hz, 1H), 5.0 (s, 2H), 4.95 (br s, 1H), 4.2 (m, 1H), 3.65 (s, 3H), 3.15 (m, 4H), 2.17 (t, *J* = 7.2 Hz, 2H), 1.75 (m, 2H), 1.45 (m, 19H), which was used without further purification.

***N*_ε-[6-[*N*-(Benzyloxycarbonyl)amino]hexanoyl]-*N*_α-(*tert*-butyloxycarbonyl)-L-lysine (Boc-Lys(Z-Nhex)-OH).** To a solution of Boc-Lys(Z-Nhex)-OCH₃ (5.40 g, 10.6 mmol) in methanol (40 mL) was added 2 N lithium hydroxide (26.6 mL, 53.2 mmol), and the reaction mixture was stirred at room temperature for 16 h. The methanol was removed in vacuo. The residue was dissolved in water (100 mL), washed with Et₂O (2 × 40 mL), poured into 5% citric acid (150 mL), and extracted with EtOAc (5 × 50 mL). The EtOAc layers were combined and washed with water (50 mL), dried (Na₂SO₄), and evaporated in vacuo to afford the desired product (4.8 g, 92%) as an oil: ¹H NMR (300 MHz, DMSO-*d*₆) δ 12.30 (s, 1H), 7.70 (t, *J* = 4 Hz, 1H), 7.33 (m, 5H), 7.20 (m, 1H), 7.00 (d, *J* = 8.0 Hz, 1H), 5.00 (s, 2H), 3.70 (m, 1H), 2.95 (m, 4H), 1.99 (m, 2H), 1.69–1.31 (m, 6H), 1.3 (s, 9H), 1.29–1.11 (m, 6H); HRMS *m/z* (M⁺) calcd 494.2866, obsd 494.2860.

***N*_ε-[6-[*N*-(Benzyloxycarbonyl)amino]hexanoyl]-*N*_α-(*tert*-butyloxycarbonyl)-L-lysiny-L-(α-*o*-benzylaspartyl)-L-valyl-L-proline Benzyl Ester (Boc-Lys(Z-Nhex)-DVP(OBn)₂).** To a solution of Boc-Lys(Z-Nhex)-OH (2.29 g, 4.66 mmol) and DVP(OBn)₂·HCl (2.8 g, 5.13 mmol) in DMF (20 mL) containing HOBt (0.95 g, 7.0 mmol) and TEA (2.9 mL, 21 mmol) cooled to 0 °C was added EDC (1.1 g, 5.6 mmol). The reaction was stirred at 0 °C for 15 min and then for 2 h at room temperature. The reaction mixture was diluted with EtOAc (100 mL), washed with 60% NaHCO₃ (3 × 40 mL), water (40 mL), 5%

citric acid (3 × 40 mL), and brine (3 × 40 mL), and then dried (Na_2SO_4) and evaporated in vacuo to afford the desired product (4.7 g, 100%) as a white solid: mp 60–63 °C; IR (KBr, cm^{-1}) 3307, 2954, 1743, 1707, 1642; ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 8.20 (m, 1H), 7.70 (m, 2H), 7.33 (m, 15H), 7.15 (m, 1H), 6.75 (d, $J = 8.0$ Hz, 1H), 5.10 (s, 2H), 5.05 (s, 2H), 4.95 (s, 2H), 4.75 (m, 1H), 4.35 (m, 2H), 3.85 (m, 1H), 3.70 (m, 1H), 3.55 (m, 1H), 3.0 (m, 4H), 2.85 (m, 1H), 2.70 (m, 1H), 2.2 (m, 1H), 1.95 (m, 6H), 1.40 (m, 21H), 0.80 (d, $J = 7.0$ Hz, 3H), 0.79 (d, $J = 7.0$ Hz, 3H); HRMS m/z (M^+) calcd 985.5286, obsd 985.5292.

N_α -[[6-*N*-(Benzyloxycarbonyl)amino]hexanoyl]-L-lysinyll- α -(*o*-benzylaspartyl)-L-valyl-L-proline Benzyl Ester (Lys-(Z-Nhex)-DVP(OBn)₂). To a solution of Boc-Lys(Z-NHex)-DVP(OBn)₂ (4.70 g, 4.77 mmol) in methylene chloride (20 mL) was added 4 N HCl in dioxane (8.3 mL), and the reaction was allowed to stir overnight. The dioxane was removed in vacuo, and the residue was dissolved in EtOAc (100 mL), washed with saturated NaHCO_3 (3 × 20 mL), dried (Na_2SO_4), and evaporated in vacuo. The resulting material was dissolved in EtOAc (~3 mL) and purified on a silica gel column, (10% MeOH/EtOAc) to afford the desired product (2.8 g, 66%) as a white solid: mp 186.5–187.5 °C; IR (KBr, cm^{-1}) 3311, 3050, 2944, 1742, 1682, 1647, 1540; ^1H NMR (300 MHz $\text{DMSO}-d_6$) δ 8.3 (br s, 1H), 7.85 (d, $J = 8.4$ Hz, 1H), 7.70 (m, 1H), 7.30 (m, 15H), 7.22 (m, 1H), 5.09 (s, 2H), 5.05 (s, 2H), 4.99 (s, 2H), 4.66 (d, $J = 6.2$ Hz, 1H), 4.33 (m, 2H), 3.73 (m, 1H), 3.5 (m, 1H), 3.36 (br s, 2H), 3.12 (m, 1H), 2.98 (m, 4H), 2.71 (m, 2H), 2.14 (m, 1H), 1.9 (m, 6H), 1.3 (m, 12H), 0.83 (d, $J = 6.6$ Hz, 3H), 0.79 (d, $J = 6.5$ Hz, 3H); MS (EI) 885 (M^+); HRMS (m/z) (M^+) calcd for $\text{C}_{48}\text{H}_{64}\text{N}_6\text{O}_{10}$ 885.4762, obsd 885.4734.

N_α -[[4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenyl]acetyl]- N_ϵ -[6-*N*-(benzyloxycarbonyl)amino]hexanoyl]-L-lysinyll- α -(*o*-benzylaspartyl)-L-valyl-L-proline Benzyl Ester (MPUPA-Lys(Z-Nhex)-DVP(OBn)₂). To a solution of Lys(Z-Nhex)-DVP(OBn)₂ (3.8 g, 4.3 mmol) and MPUPA-OSu (1.6 g, 4.3 mmol) in DMF (9 mL) was added TEA (1.2 mL, 8.6 mmol), and the reaction was stirred for 2.5 h. The reaction mixture was added dropwise to 60% NaHCO_3 (215 mL) with stirring, and the resulting precipitate was filtered and washed with water (4 × 20 mL), 5% citric acid (5 × 20 mL), and water (5 × 20 mL). The product was dissolved in hot ethanol (200 mL), and the precipitate that formed upon cooling was filtered and dried in vacuo to afford the desired product (4.0 g, 71%) as a white solid: mp 192–193 °C; IR (KBr, cm^{-1}) 3294, 2936, 1740, 1637, 1589; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 9.00 (s, 1H), 8.38 (d, $J = 7.1$ Hz, 1H), 8.17 (d, $J = 8.06$ Hz, 1H), 7.91 (s, 1H), 7.84 (d, $J = 8.4$ Hz, 1H), 7.71 (m, 2H), 7.37 (m, 2H), 7.32 (m, 15H), 7.20 (m, 1H), 7.14 (m, 4H), 6.91 (m, 1H), 5.10 (br s, 2H), 5.05 (br s, 2H), 4.99 (br s, 2H), 4.64 (m, 1H), 4.34 (m, 2H), 4.23 (m, 1H), 3.70 (m, 1H), 3.55 (m, 1H), 3.40 (m, 2H), 2.96 (m, 4H), 2.81 (m, 1H), 2.65 (m, 1H), 2.23 (s, 3H), 2.12 (m, 1H), 2.01 (m, 2H), 1.85 (m, 2H), 1.48 (m, 4H), 1.29 (m, 10H), 0.84 (d, $J = 6.6$ Hz, 3H), 0.78 (d, $J = 6.1$ Hz, 3H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ 171.8 (C=O), 171.7 (C=O), 171.4 (C=O), 170.3 (C=O), 169.9 (C=O), 169.7 (C=O), 169.5 (C=O), 156.0 (C=O), 152.6 (C=O), 138.1(C), 137.4 (C), 137.2 (C), 135.9 (C), 135.8 (C), 130.0 (2CH), 129.5 (C), 129.2 (3CH), 128.2 (4CH), 127.9 (CH), 127.8 (CH), 127.7 (4 CH), 127.6 (2CH), 125.9 (2CH), 122.5 (CH), 121.0 (CH), 120.9 (C), 117.9 (2CH), 65.8 (CH₂), 65.6 (CH₂), 65.0 (CH₂), 58.5 (CH), 55.3 (CH), 52.4 (CH), 49.3 (CH), 46.7 (CH), 41.4 (CH₂), 40.2 (CH₂), 38.2 (CH₂), 35.7 (CH₂), 35.4 (CH₂), 31.9 (CH₂), 30.1 (CH₂), 29.2 (CH₂), 28.9 (CH₂), 28.6 (CH₂), 26.0 (CH₂), 25.0 (CH₂), 24.6 (CH₂), 22.7 (CH₂), 18.6 (CH₃), 17.9 (CH₃), 17.8 (CH₃); MS (FAB) 1173 ($\text{M} + \text{Na}^+$), 1151 ($\text{M} + \text{H}^+$); HRMS m/z ($\text{M} + \text{Na}^+$) calcd 1173.5637, obsd 1173.5649. Anal. Calcd for $\text{C}_{64}\text{H}_{78}\text{N}_8\text{O}_{12} \cdot 0.37\text{H}_2\text{O}$: C, 66.36; H, 6.78; N, 9.67. Found: C, 66.70; H, 6.72; N, 9.58.

N_α -[[4-[[[(2-Methylphenyl)amino]carbonyl]amino]phenyl]acetyl]- N_ϵ -(6-aminohexanoyl)-L-lysinyll- α -aspartyl-L-valyl-L-proline Hydrochloride (15). To a solution of MPUPA-Lys(Z-Nhex)-DVP(OBn)₂ (2.25 g, 1.95 mmol) in THF (54 mL) and water (1.5 mL) was added 0.5 N HCl (3.8 mL)

followed by 20% $\text{Pd}(\text{OH})_2\text{-C}$ (0.5 g), and the reaction was stirred under H_2 (60 psi) for 2 h. The reaction mixture was filtered through Celite and concentrated in vacuo. The residue was dissolved in 1:1 THF/ H_2O (10 mL) and added dropwise to vigorously stirring Et_2O (200 mL). The supernatant was decanted from the gelatinous product which was dried in vacuo over P_2O_5 to afford the desired product (1.7 g, 99%) as an off-white solid: mp 160 °C dec; IR (KBr, cm^{-1}) 3300, 2950, 1700, 1650, 1560; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 9.42 (s, 1H), 8.31 (br s 1H), 8.19 (s, 1H), 8.14 (br s 1H), 7.82 (br m, 1H), 7.72 (br, 1H), 7.54 (br s, 1H), 7.38 (d, $J = 8.0$ Hz, 2H), 7.14 (d, $J = 7.7$ Hz, 2H), 7.11 (m, 2H), 6.91 (t, $J = 7.3$ Hz, 1H), 4.53 (m, 1H), 4.34 (m, 1H), 4.21 (m, 2H), 3.67 (m, 1H), 3.53 (m, 1H), 3.45 (d, $J = 14$ Hz, 1H), 3.35 (d, $J = 14$ Hz, 1H), 3.31 (br s, 4H), 2.96 (m, 2H), 2.70 (m, 2H), 2.66 (m, 1H), 2.28 (m, 2H), 2.25 (s, 3H), 2.13 (m, 1H), 2.04 (m, 2H), 1.90(m, 4H), 1.61 (m, 1H), 1.49 (m, 4H), 1.33 (m, 2H), 1.25 (m, 4H), 0.89 (d, $J = 6.3$ Hz, 3H), 0.81 (d, $J = 6.5$ Hz, 3H); ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ 173.0 (C=O), 171.8 (C=O), 171.6 (C=O), 171.5 (2C=O), 170.3 (C=O), 169.2 (C=O), 152.5 (C=O), 138.3 (C), 137.2 (C), 130.0 (CH), 129.3 (C), 129.2 (2CH), 125.9 (CH), 122.4 (CH), 120.9 (CH), 120.7 (C), 117.7 (CH), 117.6 (CH), 58.5 (CH), 55.2 (CH), 52.4 (CH), 49.3 (CH), 46.7 (CH₂), 41.3 (CH₂), 38.5 (CH₂), 38.1 (CH₂), 35.6 (CH₂), 35.0 (CH₂), 31.7 (CH₂), 30.2 (CH₂), 30.1 (CH), 28.7 (CH₂), 26.6 (CH₂), 25.4 (CH₂), 24.6 (CH₂), 24.5 (CH₂), 22.6 (CH₂), 18.9 (CH₃), 18.1 (CH₃), 17.7 (CH₃); MS (EI) 837 (M^+); HRMS m/z ($\text{M} + \text{H}^+$) calcd 837.4511, obsd 837.4540. Anal. Calcd for $\text{C}_{42}\text{H}_{60}\text{N}_8\text{O}_{10} \cdot \text{HCl} \cdot 1.58 \text{H}_2\text{O}$: C, 56.01; H, 7.38; N, 12.43; Cl, 3.93. Found: C, 56.04; H, 7.19; N, 11.92; Cl, 3.76.

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