# *N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)nonanoyl]-L-phenylalanine Ethyl Ester as a Stable Ester-Type Inhibitor of Chymotrypsin-like Serine Proteases: Structural Requirements for Potent Inhibition of α-Chymotrypsin

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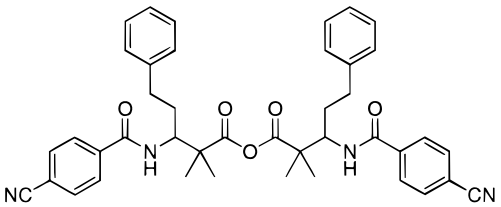
We introduce a new potent inhibitor, *N*-[2,2-dimethyl-3-(*N*-(4-cyanobenzoyl)amino)nonanoyl]-L-phenylalanine ethyl ester (**3**), which preferentially inhibits serine proteases belonging to a chymotrypsin superfamily. This inhibitor, despite consisting of a stable ethyl ester structure, showed strong inhibitory activities toward bovine α-chymotrypsin, human cathepsin G, and porcine elastase by acting as an acylating agent. The calculated inactivation rate constant ($k_{inact}$) and enzyme−inhibitor dissociation constant ($K_i$) against α-chymotrypsin were 0.0028 $s^{-1}$ and 0.0045 $\mu$M, respectively ($k_{inact}/K_i = 630\ 000\ M^{-1}\ s^{-1}$). These kinetic parameters indicate that this inhibitor is one of the most powerful α-chymotrypsin inactivators ever reported. On the basis of structure−activity relationship (SAR) and structure−stability relationship studies of analogues of **3**, which were modified in three parts of the molecule, i.e., the 4-cyanophenyl group, $\beta$-substituent at the $\beta$-amino acid residue, and ester structure, we suggest that the potent inhibitory activity of **3** is due to the following structural features: (1) the ethyl ester which enforces specific acyl-enzyme formation, (2) the *n*-hexyl group at the $\beta$-position and 4-cyanophenyl group which stabilize the acyl-enzyme, and (3) the phenylalanine residue which functions for the specific recognition of S1 site in the enzyme. In particular, the action of **3** as a potent inhibitor, but poor substrate, can be ascribed largely to the very slow deacylation rate depending on the structure factors cited in feature 2. The results of inhibition by **3** and its analogues against different serine proteases such as chymase, cathepsin G, and elastase suggest that these compounds recognize common parts in the active sites among these chymotrypsin-like serine proteases, and **3** is one of the most suitable structures to recognize those common parts. Our results provide an intriguing basis for further developments in the design of a stable ester-based selective serine protease inhibitor.

## Introduction

Serine proteases play important roles in numerous physiological processes, and some of them are involved in pathological processes. The development of effective and selective low-molecular-weight synthetic inhibitors for these proteases has been the focus of several studies of enzyme mechanisms, in vivo biochemical investigations, and development of potential therapeutic drugs.[1,2] Chymotrypsin-like serine proteases are implicated in a wide variety of pathological states including inflammation for cathepsin G[3,4] and cardiomyopathy for mast cell chymase.[5] Bovine α-chymotrypsin (EC 3.4.21.1) constitutes a model for the evaluation of new synthetic inhibitors directed against chymotrypsin-like serine proteases and a comprehensive approach to elucidate the biological roles of such targeted proteases. Many types of inhibitors such as substrate inhibitors, transition-state inhibitors, affinity labels, active-site alkylating agents, and mechanism-based enzyme inactivators have been developed for effective and selective inhibition of serine proteases.[1,2,6−9]

Recently, we reported that *N*-modified trisubstituted $\beta$-amino acid anhydride **1** [2,2-dimethyl-3-(*N*-(4-cyanobenzoyl)amino)-5-phenylpentanoic anhydride] was a potent inhibitor of α-chymotrypsin ($k_{inact}/K_i = 242\ 000\ M^{-1}\ s^{-1}$, $k_{inact} = 0.017\ s^{-1}$, and $K_i = 0.071\ \mu M$).[10] Its inhibitory action appears to involve the acyl transfer of the anhydride to the hydroxyl group of serine-195, generating a stable acyl-enzyme intermediate. The anhydride structure is important for the activation of carbonyl reactivity of **1**, since 2,2-dimethyl-3-(*N*-(4-cyanobenzoyl)amino)-5-phenylpentanoic acid, which is an acid form of **1**, shows no inhibitory activity for α-chymotrypsin. **1** possesses a bulky anhydride structure with dimethyl groups on two symmetric α-carbons. This may contribute to the stability against hydrolysis of the anhydride structure in a buffer solution such as Tris-HCl (pH 8.1). However, anhydride still seems to be too reactive to use in in vivo studies.




**1**

To improve the in vivo stability, i.e., to prevent

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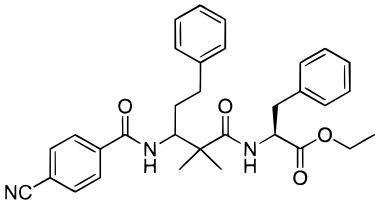
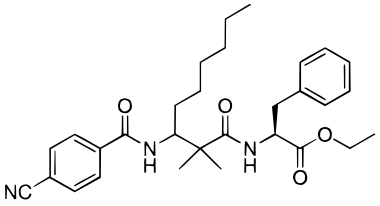
nonspecific acylation toward nontargeted proteins or self-hydrolysis under physiological conditions, it is necessary that the electron density at the carbonyl carbon of the reactive center of the inhibitor is increased. Such increase in electron density, however, will decrease the reactivity of the carbonyl carbon with the active-site serine residue, resulting in a reduced rate of formation of the intermediate complex of the inhibitor and enzyme. One way to overcome this negative effect is to incorporate the acyl and leaving group moiety that interact with the respective binding sites more favorably into the molecule.[11] In particular, such interactions with the leaving group binding sites are known to increase the rate of hydrolysis of the normal substrate.[12] It is, therefore, likely that these interactions will also increase the rate of formation of the acyl-enzyme. In addition, a favorable interaction of the acyl moiety with its binding site in proteases will also compensate for the reduced rate of acyl-enzyme formation.

A trisubstituted *β*-amino acid in anhydride **1** is not a native amino acid, which has a dimethyl group on the α-carbon and another substituent on the *β*-carbon. These trisubstituted *β*-amino acids were originally used for the development of fibrinogen receptor antagonists and found to be effective in restricting the conformational mobility by steric repulsion.[13,14] In anhydride **1**, two trisubstituted *β*-amino acids will also restrict the free rotation of the backbone of the molecule and fix the conformation, which probably enables the molecule to interact with enzymes with a higher affinity. Therefore, we have synthesized an extensive series of compounds containing an α,α-dimethyl-*β*-alkyl-*β*-amino acid derivative and a leaving group with modified reactivity such as esters or amides instead of the anhydride structure.

In such modifications, one of the phenylalanine residue-inserted derivatives, *N*-[2,2-dimethyl-3-(*N*-(4-cyanobenzoyl)amino)-5-phenylpentanoyl]-L-phenylalanine ethyl ester (**2**), exhibited a relatively strong inhibitory activity. We modified this compound and found a new potent inhibitor for chymotrypsin-like serine proteases, *N*-[2,2-dimethyl-3-(*N*-(4-cyanobenzoyl)amino)nonanoyl]-L-phenylalanine ethyl ester (**3**), which exhibits anti-α-chymotrypsin activity comparable to that of anhydride **1**.

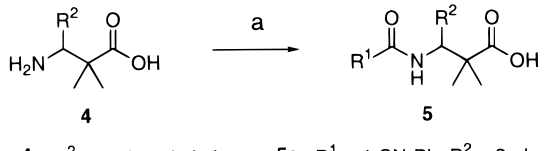


**2**



**3**

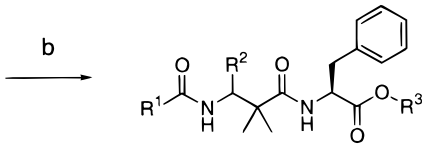
**Scheme 1**[a]



**4** **5**

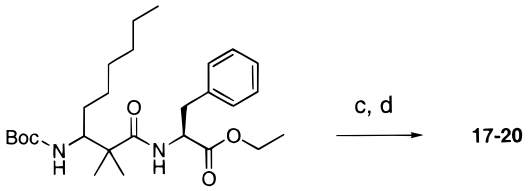
**4a** $R^2$ = 2-phenylethyl
**4b** $R^2$ = *n*-hexyl
**4c** $R^2$ = Et
**4d** $R^2$ = *n*-Pr
**4e** $R^2$ = *iso*-Pr
**4f** $R^2$ = *n*-Bu
**4g** $R^2$ = *iso*-Bu
**4h** $R^2$ = *n*-pentyl
**4i** $R^2$ = *n*-heptyl
**4j** $R^2$ = *n*-octyl
**4k** $R^2$ = *n*-decyl
**4l** $R^2$ = *n*-dodecyl

**5a** $R^1$ = 4-CN-Ph, $R^2$ = 2-phenylethyl
**5b** $R^1$ = 4-CN-Ph, $R^2$ = *n*-hexyl
**5c** $R^1$ = 4-CN-Ph, $R^2$ = Et
**5d** $R^1$ = 4-CN-Ph, $R^2$ = *n*-Pr
**5e** $R^1$ = 4-CN-Ph, $R^2$ = *iso*-Pr
**5f** $R^1$ = 4-CN-Ph, $R^2$ = *n*-Bu
**5g** $R^1$ = 4-CN-Ph, $R^2$ = *iso*-Bu
**5h** $R^1$ = 4-CN-Ph, $R^2$ = *n*-pentyl
**5i** $R^1$ = 4-CN-Ph, $R^2$ = *n*-heptyl
**5j** $R^1$ = 4-CN-Ph, $R^2$ = *n*-octyl
**5k** $R^1$ = 4-CN-Ph, $R^2$ = *n*-decyl
**5l** $R^1$ = 4-CN-Ph, $R^2$ = *n*-dodecyl
**5m** $R^1$ = Ph, $R^2$ = *n*-hexyl
**5n** $R^1$ = *tert*-butoxy, $R^2$ = *n*-hexyl



**2,3, 6-16, 21-26**

$R^3$ : Me, Et, *n*-Pr, *iso*-Pr, Bn, *tert*-Bu



**21** **17-20**

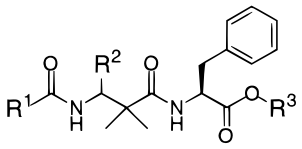
[a] (a) 4-CN-Bz-OSu, Bz-Cl, or $(Boc)_2O$, $Et_3N$, DMF; (b) H-Phe-OEt, -OMe, -O-*n*-Pr, -O-*i*-Pr, -OBzl, or -O-*t*-Bu, WSCD·HCl, HOBt, $CH_2Cl_2$; (c) 4 N HCl−dioxane, 0 °C; (d) aryl-COOH, HOBt, WSCD·HCl, $CH_2Cl_2$.

In this study, we report a stable ester-type potent inhibitor of chymotrypsin-like proteases and a comprehensive investigation of the structural factors necessary for its potent activity and the kinetics of inhibition of α-chymotrypsin by **3**.

## Chemistry

Inhibitor **3** and its analogues were generally synthesized by the method shown in Scheme 1 as a diastereomeric mixture (a 50:50 mixture of *RS* and *SS*). In Scheme 1, *β*-substituted-α,α-dimethyl-*β*-alanine **4**[14] was coupled with the *N*-hydroxysuccinimide ester of 4-cyanobenzoic acid, benzoyl chloride, or $(Boc)_2O$ in the presence of triethylamine to yield the corresponding amides or *N*-Boc-*β*-substituted-α,α-dimethyl-*β*-alanine **5**, which was coupled with phenylalanine ethyl ester or corresponding other esters such as methyl, *n*-propyl, isopropyl, benzyl, and *tert*-butyl using 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (WSCD·HCl)[15] in the presence of 1-hydroxybenzotriazole (HOBt)[16] to yield the target compounds. The 4-cyanophenyl-modified or -substituted analogues **17**−**20** were synthesized from **21** by the deprotection of the *t*-Boc group of **21** by 4 N HCl−dioxane, followed by

**Table 1.** Structure−Activity Relationships of the Analogues of **3**



| compd | $R^1$ | $R^2$ | $R^3$ | $IC_{50}$ ($\mu$M)[a] α-chymotrypsin | $IC_{50}$ ($\mu$M)[a] trypsin | stability[b] (%) |
|---|---|---|---|---|---|---|
| **1** | na[c] | na | na | 0.0021 | 0.30 | nd[d] |
| **2** | 4-CN-Ph[e] | 2-phenylethyl | Et | 0.25 | ni[f] | nd |
| **3** | 4-CN-Ph | *n*-hexyl (C6)[g] | Et | 0.0026 | 0.54 | >99 |
| **6** | 4-CN-Ph | Et (C2) | Et | 3.4 | ni | 91 |
| **7** | 4-CN-Ph | *n*-propyl (C3) | Et | ni | ni | 11 |
| **8** | 4-CN-Ph | isopropyl (C3) | Et | 2.2 | 60 | 90 |
| **9** | 4-CN-Ph | *n*-butyl (C4) | Et | 27 | ni | 89 |
| **10** | 4-CN-Ph | isobutyl (C4) | Et | ni | ni | 41 |
| **11** | 4-CN-Ph | *n*-pentyl[h] (C5) | Et | ni | ni | 3 |
| **12** | 4-CN-Ph | *n*-heptyl (C7) | Et | ni | ni | <1 |
| **13** | 4-CN-Ph | *n*-octyl (C8) | Et | 8.6 | ni | 61 |
| **14** | 4-CN-Ph | *n*-decyl (C10) | Et | ni | ni | 16 |
| **15** | 4-CN-Ph | *n*-dodecyl (C12) | Et | 4.2 | 220 | nd |
| **16** | Ph | *n*-hexyl (C6) | Et | 17 | 560 | <1 |
| **17** | 3,4-diCl-Ph[i] | *n*-hexyl (C6) | Et | 7.0 | ni | nd |
| **18** | 3,4-diF-Ph[j] | *n*-hexyl (C6) | Et | 1.5 | ni | nd |
| **19** | 2-F-4-CN-Ph[k] | *n*-hexyl (C6) | Et | ni | ni | 6 |
| **20** | 2-furyl | *n*-hexyl (C6) | Et | 12 | ni | nd |
| **21** | *tert*-butoxy | *n*-hexyl (C6) | Et | 1.0 | 6.3 | nd |
| **22** | 4-CN-Ph | *n*-hexyl (C6) | Me | 1.1 | ni | 98 |
| **23** | 4-CN-Ph | *n*-hexyl (C6) | *n*-propyl | 0.23 | 17 | 97 |
| **24** | 4-CN-Ph | *n*-hexyl (C6) | isopropyl | 0.086 | 6.7 | 98 |
| **25** | 4-CN-Ph | *n*-hexyl (C6) | benzyl | 230 | ni | nd |
| **26** | 4-CN-Ph | *n*-hexyl (C6) | *tert*-butyl | 73 | ni | nd |

[a] Inhibition of bovine α-chymotrypsin and bovine trypsin. The $IC_{50}$ values of chymostatin for α-chymotrypsin and leupeptin for trypsin are 0.02 and 0.44 $\mu$M, respectively. All experiments were performed in triplicate. Standard deviations are less than 15% of the mean values with the exception of compounds **1**, **6**, **16**, **21**, and **22** (29%, 19%, 17%, 18%, and 31% in α-chymotrypsin assay, respectively). [b] Percentages of intact compounds after α-chymotryptic cleavage at 37 °C for 120 min. [c] na, not applicable. [d] nd, not determined. [e] 4-CN-Ph, 4-cyanophenyl. [f] ni, no inhibition at 1 mM. [g] Number of carbon atoms at $R^2$ position. [h] *normal*-Pentyl. [i] 3,4-diCl-Ph, 3,4-dichlorophenyl. [j] 3,4-diF-Ph, 3,4-difluorophenyl. [k] 2-F-4-CN-Ph, 2-fluoro-4-cyanophenyl.

coupling with 3,4-dichlorobenzoic acid, 3,4-difluorobenzoic acid, 2-fluoro-4-cyanobenzoic acid, and 2-furoic acid for **17**−**20**, respectively, using the above WSCD−HOBt procedure. Purification was carried out using silica gel column chromatography.

## Results

**Structure−Activity Relationship Studies of Analogues of 3.** As shown in Table 1, **3** inhibited both α-chymotrypsin and trypsin with an $IC_{50}$ of 2.6 and 540 nM, respectively, indicating that this ester is about 200 times more selective against α-chymotrypsin than against trypsin, and these activities were almost comparable to those of anhydride inhibitor **1** ($IC_{50}$ = 2.1 and 300 nM, respectively). Since **3** is a mixture of two diastereomers due to the existence of an asymmetric carbon atom at the $\beta$-position, in addition to the L-phenylalanine residue, both diastereomers (*SS* and *RS*) were separated by normal phase HPLC, and their inhibitory activities against chymotrypsin-like serine proteases were measured. As shown in Table 3, both diastereomers exhibited almost the same inhibitory activities against those proteases, indicating that the potent inhibition of **3** is nonenantioselective at the $\beta$-position.

To elucidate the structural factors responsible for the potent activity of **3**, we focused on three parts of the molecule, the 4-cyanophenyl group, the $\beta$-substituent at the $\beta$-amino acid residue, and the ester structure, and synthesized a series of analogues of **3**. In the modification of the $R^1$ position (4-cyanophenyl group), deletion of the cyano group (**16**) resulted in a 6500 time decrease in the inhibitory activity against α-chymotrypsin compared with that of **3**. Introduction of halogen atoms (**17** and **18**) or substitutions of the 4-cyanophenyl group with a 2-furyl or *tert*-butoxy group (**20** and **21**) also decreased the activity. Moreover, introduction of a fluorine atom at the 2-position of the 4-cyanophenyl group (**19**) resulted in total loss of the activity. Although we extensively modified the same part in **2** with substituents such as 4-halophenyl, 4-nitrophenyl, 4-(methoxycarbonyl)phenyl, and 4-(aminosulfonyl)phenyl, these analogues were more than 2000 times less active than **2** (data not shown). These results indicate that the 4-cyanobenzoyl group is one of the best substituents in the $R^1$ position for a potent anti-α-chymotryptic activity.

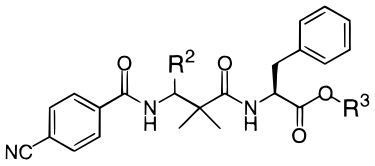
The $R^2$ position ($\beta$-substituent) was also modified with

**Table 2.** Kinetic Parameters for Inactivation of Bovine α-Chymotrypsin by Inhibitor **3** and Its Ester Analogues

| compd | ester form | $K_i$[a] ($\mu$M) | $k_{inact}$[a] ($s^{-1}$) | $k_{inact}/K_i$ ($M^{-1}\,s^{-1}$) | $IC_{50}$[b] ($\mu$M) |
|---|---|---|---|---|---|
| **3** | ethyl | 0.0045 | 0.0028 | 630000 | 0.0026 |
| **22** | methyl | 12 | 0.020 | 1600 | 1.1 |
| **23** | *n*-propyl | 0.34 | 0.0057 | 17000 | 0.23 |
| **24** | isopropyl | 0.070 | 0.0035 | 51000 | 0.086 |

[a] The progress curves of the substrate hydrolysis were analyzed by the zero-time method described by Hart and O'Brien.[18] The concentration of inhibitors used here were 0.1 $\mu$M for compound **3**, 10 $\mu$M for compound **22**, 1 $\mu$M for compound **23**, and 0.33 $\mu$M for compound **24**. [b] See Table 1.

**Table 3.** Inhibitory Activities of **3** and Its Analogues against Serine Proteases



| | | | $IC_{50}$ ($\mu$M)[a] | | | |
|---|---|---|---|---|---|---|
| compd | $R^2$ | $R^3$ | α-chymotrypsin | chymase | cathepsin G | elastase |
| **3** | *n*-hexyl | ethyl | 0.0026 | 0.44 | 0.0087 | 0.0070 |
| **3-F**[b] | *n*-hexyl | ethyl | 0.0016 | 0.22 | 0.0084 | 0.0086 |
| **3-R**[c] | *n*-hexyl | ethyl | 0.0051 | 0.36 | 0.010 | 0.014 |
| **22** | *n*-hexyl | methyl | 1.1 | 19 | 1.5 | 4.2 |
| **23** | *n*-hexyl | *n*-propyl | 0.23 | 3.2 | 0.20 | 0.27 |
| **24** | *n*-hexyl | isopropyl | 0.086 | 0.15 | 0.077 | 0.20 |
| **9** | *n*-butyl | ethyl | 27 | 510 | 83 | 710 |
| **11** | *n*-pentyl | ethyl | ni[d] | ni | ni | ni |
| **12** | *n*-heptyl | ethyl | ni | ni | 440 | ni |
| **13** | *n*-octyl | ethyl | 8.6 | 62 | 6.6 | 2.7 |

[a] Inhibition of bovine α-chymotrypsin, human chymase, human cathepsin G, and porcine elastase. The $IC_{50}$ values of chymostatin for α-chymotrypsin, chymase, and cathepsin G and elastatinal for elastase are 0.02, 0.44, 0.025, and 0.22 $\mu$M, respectively. All experiments were performed in triplicate. Standard deviations (SEM) are less than 20% of the mean values with the exception of compound **22** (α-chymotrypsin), **13** (chymase), and **13** (elastase), in which those SEM values are 31%, 21%, and 21% of the mean values, respectively. [b] **3-F**, a diastereomer of **3**, which was eluted as a front peak by normal phase HPLC. [c] **3-R**, a diastereomer of **3**, which was eluted as a rear peak by normal phase HPLC. [d] ni, no inhibition at 1 mM.

alkyl groups of different lengths. The inhibitory activity was observed in analogues bearing the linear alkyl chains, whose carbon number was even, such as C2, C4, C6, and C8 (**6**, **9**, **3**, and **13**). Maximum inhibition was noted after the introduction of a *n*-hexyl group (**3**), and compounds with shorter or larger alkyl chains were less potent. However, analogues with linear alkyl chains, whose carbon number was odd, such as C3, C5, and C7 (**7**, **11**, and **12**), showed no inhibitory activity. We also evaluated the inhibitory activity of the analogues with the branched alkyl groups such as isopropyl **8** and isobutyl **10**. The isopropyl analogue **8** containing odd-numbered (three) carbon atoms showed an inhibitory activity with an $IC_{50}$ value of 2.2 $\mu$M, which was comparable to that of the ethyl analogue **6** (3.4 $\mu$M), although the *n*-propyl analogue **5** showed no inhibitory activity. Isobutyl analogue **10** containing even-numbered (four) carbon atoms showed no activity similar to the *n*-propyl analogue **7** (no activity), although the *n*-butyl analogue **9** showed considerable anti-chymotryptic activity. These results imply that the inhibitory activity of analogues with the branched alkyl groups is dependent on the length of their main chains, not the total number of carbon atoms.

In modification of the $R^3$ position (ester), compounds with alkyl groups such as methyl, *n*-propyl, and isopropyl groups (**22**−**24**) were 30−400 times less active than **3**, and compounds with the benzyl and *tert*-butyl groups (**25** and **26**) showed very weak activity, indicating that the ethyl ester in this position is one of the best functional groups associated with the potent anti-α-chymotryptic activity.

**Chymotryptic Cleavage of Analogues of 3.** The mechanism underlying the marked change in the inhibitory activity observed with only slight modifications of the structure, including the 4-cyanophenyl group, the side chain at the $\beta$-position, or the ester, is not clear. One possible explanation is that some of the analogues may be cleaved by the enzyme as a substrate, resulting in loss of activity. To investigate such a possibility, we examined the stability of each compound against α-chymotrypsin by analyzing the formation of degraded products, especially the corresponding free acid. As shown in Table 1, analogues of **3** bearing linear alkyl chains, whose carbon numbers were odd, such as C3, C5, and C7 (**7**, **11** and **12**), were effectively hydrolyzed by the protease, yielding only the corresponding acids. However, those analogues bearing the linear alkyl chains, whose carbon numbers were even, such as C2, C4, and C6 (**6**, **9**, and **3**), strongly resisted hydrolysis to the corresponding acids by the protease, except C8 (**13**) and C10 (**14**) in which 40% and 85% of the acid formation was observed during 2 h of incubation, respectively. In particular, compound **3** with a linear alkyl chain of C6 was rarely hydrolyzed during this period (less than 1%) and even less so in the 3-h incubation (not shown). Although these analogues contain one asymmetric carbon atom at the $\beta$-position in addition to the L-phenylalanine residue, i.e., mixture of two diastereomers, both diastereomers, whose corresponding acids can be detected separately by HPLC, showed almost the same behavior against chymotryptic cleavage. (The percent values of stability shown in Table 1 are the average of both diastereomers, and each deviation was less than 1% with the exception of compounds **6** and **7** with a deviation of 5% and 12%, respectively.) These results indicate that the recognition of compounds as substrates by α-chymotrypsin is non-enantioselective at the $\beta$-position.

The relationship between anti-α-chymotryptic activity and stability against chymotryptic cleavage is plotted in Figure 1. As this figure shows, anti-α-chymotryptic activities clearly correlated with the stability against chymotrypsin, suggesting that the reduced inhibitory activities observed when the length of the alkyl chains was varied were largely dependent on the instability of the acyl-enzyme. This is also true with compounds with the branched alkyl chains such as isopropyl and isobutyl and compounds with relatively long linear alkyl chains such as C8 (**13**) and C10 (**14**) at the $\beta$-position.

As shown in Table 1, compounds with the cyano group deleted and the fluorine atom added (**16** and **19**) were

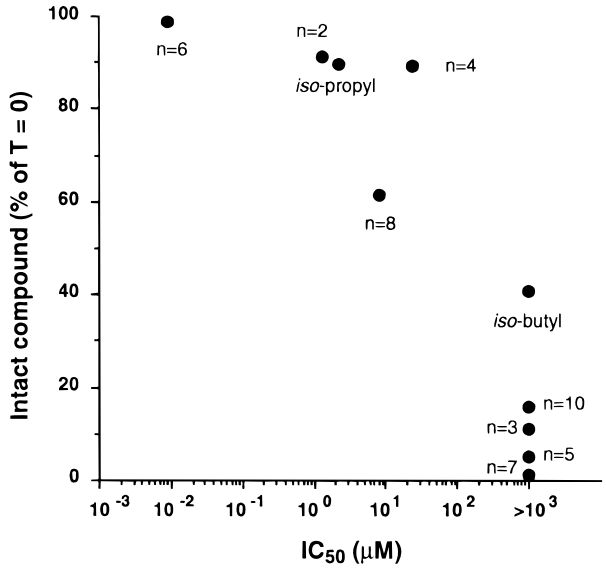



**Figure 1.** Relationship between $IC_{50}$ values and stability. The $IC_{50}$ value of each compound was plotted against its stability against chymotryptic cleavage. The number of carbon atoms is indicated near the symbol.

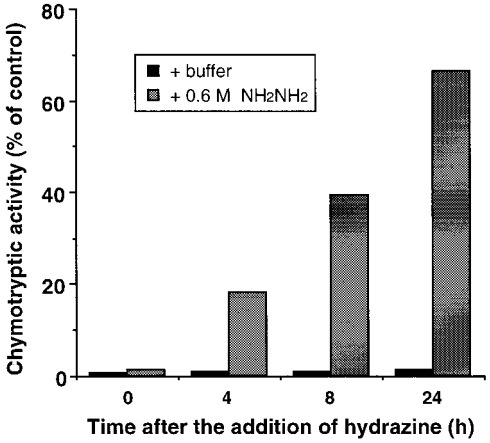



**Figure 2.** Reactivation of chymotrypsin by the addition of hydrazine. Chymotrypsin (1.25 $\mu$g/mL) was inactivated by treatment with 5 $\mu$M compound **3** for 20 min at room temperature and was dialyzed overnight against HEPES buffer (pH 7.4) containing 100 mM NaCl at 4 °C. Hydrazine solution was added to the dialysate (final concentration = 0.6 M) followed by incubation at room temperature. The activity of reactivated chymotrypsin was determined by adding a chromogenic substrate (final concentration = 750 $\mu$M). α-Chymotrypsin without incubation with compound **3** was used as a control, and the chymotryptic activity of each sample is expressed as percentage of the control.

effectively hydrolyzed to the corresponding acids, indicating that the 4-cyanophenyl group was also an important unit for resisting chymotryptic cleavage. Since these modifications resulted in a reduction of anti-α-chymotryptic activities, the reduced activities will also be largely dependent on the instability of the acyl-enzyme.

We also examined the effects of ester structures on chymotryptic cleavage. Formation of the corresponding acids was 2.4% (methyl), 3.1% (*n*-propyl), and 2.7% (isopropyl) after incubation with chymotrypsin for 2 h, respectively, which were comparable to that of **3** (ethyl, <1%).

**Competitive Inhibition Studies of 3.** In the next step, we investigated the kinetics of inhibition of α-chymotrypsin by **3** using a chromogenic substrate, *N*-succinyl-Ala-Ala-Pro-Phe-pNA.[17] In competitive binding assays, inhibitor **3** appeared to be an active-site-directed reagent. **3** behaved as a competitive inhibitor of α-chymotrypsin. The initial $K_i$ value for **3**, determined from the double-reciprocal plot (see Figure 3, Supporting Information), was 13.6 $\mu$M.

**Inhibition Kinetics of 3 and Its Ester Analogues.** As shown in Figure 4 (Supporting Information), inhibition of α-chymotrypsin by inhibitor **3** was time-dependent and the hydrolysis rate of the substrate did not reach a steady state at the end of the experimental period (300 s). Therefore, $K_i$ (final) and $k_{inact}$ values were calculated by the progress curve method.[18] The method used to calculate these values is described in the Experimental Section. Using a semilogarithmic plot of velocity−time, our analysis showed that the rate of substrate hydrolysis was linear (data not shown). Using this relationship, the estimated $K_i$ and $k_{inact}$ values were 0.0045 $\mu$M and 0.0028 $s^{-1}$, respectively, resulting in a $k_{inact}/K_i$ of 630 000 $M^{-1}$ $s^{-1}$. Table 2 shows the kinetic constants including $K_i$, $k_{inact}$, and $k_{inact}/K_i$ values, calculated by the same method, for inhibition of α-chymotrypsin by inhibitor **3** and its ester analogues. The $K_i$ values, not $k_{inact}$, were largely affected by the ester forms, and the inhibitory activity of each compound was dependent on $K_i$ values.

**Recovery of Enzyme Activity by Hydrazine.** The kinetic studies showed that compound **3** inhibited α-chymotrypsin in a competitive manner, suggesting that **3** may interact with the active site of the enzyme and form a stable acyl-enzyme. To examine this possibility, the reactivation kinetics of the inactivated enzyme was studied. α-Chymotrypsin, fully inactivated by treatment with **3**, was dialyzed at 4 °C overnight against HEPES buffer (pH 7.4), and the chymotryptic activity was determined by the addition of the chromogenic substrate. As shown in Figure 2, the hydrolytic activity was not recovered even after dialysis, and additional incubation at room temperature for 24 h also failed to reactivate the enzyme, indicating that the deacylation rate constant of **3** is too small to be determined. These results indicated that the inhibition was irreversible. However, reactivation of the inactivated enzyme occurred when the enzyme was treated with buffered hydrazine. Although the reactivation rate was not very fast, about 70% of chymotryptic activity was recovered after a 24-h incubation. These results suggest that compound **3** inhibits α-chymotrypsin by forming an irreversible acyl-enzyme, probably by reacting with the hydroxyl group of the active site Ser residue (195) or with the imidazole ring of the His residue (57).

**Inhibition of Other Serine Proteases.** The $IC_{50}$ values of inhibitor **3** and its analogues against several serine proteases are shown in Table 3. **3** inhibited cathepsin G and elastase at nanomolar concentrations, and the inhibitory activity against human chymase was less potent. We also examined the effects of modification of both the $\beta$-position and the ester structure and found that the profiles of inhibitory potency against cathepsin G, elastase, and chymase were almost similar to those against α-chymotrypsin.

## Discussion

In our previous study,[10] we developed a series of inhibitors for chymotrypsin-like enzymes, which possess an anhydride structure. We demonstrated that one of the most potent inhibitors, anhydride **1** [2,2-dimethyl-3-(*N*-(4-cyanobenzoyl)amino)-5-phenylpentanoic anhydride], inhibited chymotrypsin-like enzymes including chymotrypsin, chymase, and cathepsin G by competing with the substrates and irreversibly acylating the active site of the enzymes. Because of the anhydride structure, anhydride **1** is very reactive, making nonspecific acylating reactions inevitable in vivo.

In the present study, we report a stable ester-type inhibitor, *N*-[2,2-dimethyl-3-(*N*-(4-cyanobenzoyl)amino)-nonanoyl]-L-phenylalanine ethyl ester (**3**), which is a trisubstituted *β*-amino acid derivative, and its analogues. As shown in Figure 3 (Supporting Information), inhibitor **3** is a competitive inhibitor of α-chymotrypsin. Dialysis of the inactivated enzyme showed that the action of inhibitor **3** is irreversible and reactivation by treatment with hydrazine suggests the formation of the acyl-enzyme. Although the exact acylation site is not known, the Ser residue at the active site of the enzyme is probably acylated. Inhibition of α-chymotrypsin by inhibitor **3** is time-dependent, suggesting that it is a relatively slow binding inhibitor. Analysis of the progress curve (Figure 4, Supporting Information) resulted in kinetic constants of $k_{inact} = 0.0028\ s^{-1}$ and $K_i = 0.0045\ \mu M$, yielding a $k_{inact}/K_i$ value of 630 000 $M^{-1}\ s^{-1}$. These kinetic constants are almost comparable to those of anhydride inhibitor **1** ($k_{inact}/K_i = 242\ 000\ M^{-1}\ s^{-1}$, $k_{inact} = 0.017\ s^{-1}$, and $K_i = 0.071\ \mu M$). The ethyl ester structure in **3** will be more stable under physiological conditions compared with the anhydride structure, due to the reduced electrophilicity at the carbonyl carbon, which is a reactive center and a target of the active-site Ser-195 in α-chymotrypsin. Because a reduced reactivity of the reactive center will generally result in a reduced inhibitory activity by slowing the rate of acyl-enzyme formation, the potent activity of inhibitor **3** suggests that other structural factors may compensate the reduced reactivity.

To investigate the structural factors involved in the interaction between inhibitors and the enzyme, we conducted a structure−activity relationship (SAR) study and a structure−stability relationship study among inhibitor **3** and its analogues. We focused on three parts of the molecule: the 4-cyanophenyl group, the *β*-substituent at the trisubstituted *β*-amino acid residue, and the ester structure. Since the phenylalanine residue in inhibitor **3** is the most typical amino acid residue in peptide substrates recognized by chymotrypsin-like enzymes, this residue would interact with the S1 site in acyl binding sites of α-chymotrypsin. In the present study, this moiety was not modified.

Modification of the *β*-substituent at the trisubstituted *β*-amino acid residue largely affected the inhibitory activity as shown in Table 1. The inhibitory activity against α-chymotrypsin is dependent on the number of carbon atoms of the alkyl chains introduced into the *β*-position. Introduction of the linear alkyl chain with an odd carbon number resulted in complete loss of activity. On the other hand, the structure−stability relationship study showed that these inactive compounds were hydrolyzed to the free acid, suggesting that the acyl-enzyme is formed but the deacylation rate is very high. In contrast, in compounds with an alkyl chain and even carbon numbers, the acyl-enzyme was stable and the deacylation rate was very slow. The mechanism that determines the stability of the acyl-enzyme is not known. Baek et al.[19] discussed the factors that affect the deacylation rate of the acyl-enzyme in their study on alternate substrate inhibitors with an enol lactone structure. These factors are (a) a reactivity of ester in the acyl-enzyme,[20−22] (b) a steric blockade or ionic repulsion of the attacking water molecules,[23−26] (c) a conformational demand that pulls the acyl-enzyme carbonyl oxygen out of the oxyanion hole, a site where the reactivity with nucleophiles is thought to be enhanced,[27,28] and (d) a conformational alternation of other portions of the catalytic machinery of the enzyme such as His-57 moving from the active "in" position to the "out" position.[29] In the case of the compounds presented here, it is probable that the catalytic inertness derived from mechanisms such as factors b−d may be dependent on the alternate positioning of the compound, caused by different lengths of the alkyl chains at the *β*-position, in the narrow pocket of the active site of the enzyme. We are about to begin a study of crystallization of **3** and α-chymotrypsin complex to elucidate the precise binding mechanism. This approach may provide the possible answer to explain the effects of the length of the alkyl chains at the *β*-position on the inhibitory activity.

The ethyl ester structure is also an interesting feature of inhibitor **3**, since the inhibitory activity was markedly reduced following the conversion of the structure of this ester to other similar alkyl esters such as methyl, *n*-propyl, isopropyl, and *tert*-butyl esters, although these analogues were resistant to enzymatic cleavage. Determination of the kinetic constants in the inhibition of α-chymotrypsin by compound **3** and its ester form derivatives showed that $K_i$ values, not $k_{inact}$ values, were largely affected by the deviation of the ester form (Table 2). These results suggest that the rate of transition from the intermediate inhibitor−enzyme complex to the stable acyl-enzyme may not be dependent to a large extent on the ester form. The leaving group of the ester moiety may play at least two opposite roles in these steps. First, the leaving group may be involved in the interaction between the compound and the binding sites on the enzyme, through which the compound binds more tightly to the enzyme. Second, the leaving group may donate electrons onto the carbonyl carbon, which reduces the reactivity of that carbon with the active site of the enzyme. Based on our finding of a potent activity of inhibitor **3** which has an ethyl ester, it is likely that the ethyl ester interacts strongly with the leaving group binding sites on the enzyme and overcomes the negative effects of its electron donation.

The 4-cyanophenyl group was also very important moiety for the potent inhibitory activity. **16**, a compound without the cyano group, and **19**, a compound with an additional fluorine atom at the 2-position of the 4-cyanophenyl ring, exhibited a markedly reduced inhibitory activity as well as stability against enzymatic cleavage. These results suggest that this cyanophenyl group may be responsible for the stabilization of the acyl-enzyme. Since the phenyl group would be located at the P3

position of peptide substrates, this group may be implicated in the interaction with the S3 site of the enzyme. Therefore, it is likely that the 4-cyano group stabilizes the acyl-enzyme by interacting with a site in or near the S3 pocket or by modifying the distribution of the $\pi$-electrons on the phenyl ring. Such interaction may lead to the above-mentioned catalytic inertness resulting in a reduced deacylation rate of the acyl-enzyme.

Small synthetic molecules, such as Bz-Phe-OEt, are known to be better substrates for α-chymotrypsin with a relatively higher deacylation rate (5500 $min^{-1}$).[30] However, the deacylation rate of inhibitor **3**, which contains the same Phe-OEt structure, seems to be very slow, although the exact rate was not determined. These results also suggest the significance of the *n*-hexyl group at the $\beta$-position and the 4-cyanophenyl group in the slow deacylation.

The importance of trisubstituted $\beta$-amino acids as a conformation-restricting linear template was first shown in our studies on fibrinogen receptor (GPIIb/IIIa) antagonists. In these studies, we confirmed that trisubstituted $\beta$-amino acids were very useful in restricting the free rotation of the backbone structure of the molecules and fixing the molecular conformation. Trisubstituted $\beta$-amino acids have also shown to be useful for the introduction of a functional group into the molecule, by substituting the group at the $\beta$-position. On the basis of these findings, we expected that trisubstituted-$\beta$-amino acids will be generally applicable to the amide bond-based small molecules for the purpose of fixing the molecular conformation and the addition of the interaction site. Protease inhibitors are one of the possible targets, because to develop potent and selective protease inhibitors, the molecule should have multiple binding sites recognized by the enzyme and should be tightly fixed to the appropriate conformation so as to position these multiple functional groups in a proper way. Therefore, in the present study we introduced a trisubstituted $\beta$-amino acid to the inhibitors for fixing the molecules and introducing the P2 moiety, which is known as the interaction site recognized by the S2 site of the enzyme. The present study showed that only a minor modification, such as the addition or deletion of a C1 unit at the $\beta$-substituent and the introduction of a substituted group to the phenyl group, resulted in a major reduction in the inhibitory activity. This hypersensitivity may be attributed to the conformational rigidity of our compounds, a property thought to be derived from that of the trisubstituted $\beta$-amino acid introduced into the molecules. Further molecular modeling studies are necessary to demonstrate the significance of the trisubstituted $\beta$-amino acids as a conformation-restricting unit. These studies are currently being conducted in our laboratory.

The inhibition profiles of inhibitor **3** and its analogues against several serine proteases including chymotrypsin, chymase, cathepsin G, and elastase suggested a similar structure of the active sites, recognized by this series of compounds, in these proteases and the presence of a common inhibitory mechanism.

In conclusion, we synthesized a novel potent inhibitor, *N*-[2,2-dimethyl-3-(*N*-(4-cyanobenzoyl)amino)nonanyl]-L-phenylalanine ethyl ester (**3**), for chymotrypsin-like serine proteases and defined the structural factors necessary for its potent activity. The structural factors responsible for the potent inhibitory activity of **3** elucidated in the present study can be summarized as follows: (1) the ethyl ester that functions as a group enforcing specific acyl-enzyme formation, (2) the *n*-hexyl group at the $\beta$-position and the 4-cyanophenyl group, which function as stabilizers for the acyl-enzyme, and (3) the phenylalanine residue which functions as a moiety involved in the specific recognition by the enzyme upon acyl-enzyme formation. **3** is one of the ideal potent substrate inhibitors with a high binding affinity and intensely slow deacylation rate. These results provide an intriguing basis for further developments in the design of specific serine protease inhibitors.

## Experimental Section

**Enzyme Assays.** The inhibitory effects of each compound on the enzymatic activities of serine proteases were evaluated using purified enzymes and chromogenic substrates. Enzymes and their substrates used in the present study were as follows: *N*-succinyl-Ala-Ala-Pro-Phe-pNA[17] (1.5 mM) for bovine pancreatic α-chymotrypsin (47 ng/mL, type I-S; Sigma Chemical Co., St. Louis, MO); *N*-succinyl-Ala-Ala-Pro-Phe-pNA (3 mM) for human leukocyte cathepsin G (2.5 U/mL; Elastin Products Co., Inc.); *N*-succinyl-Ala-Ala-Ala-pNA (1 mM) for porcine pancreatic elastase (0.8 $\mu$g/mL, Sigma type III); Bz-L-Arg-pNA (1 mM) for porcine pancreatic trypsin (1 U/mL; Wako Pure Chemicals); *N*-succinyl-Ala-Ala-Pro-Phe-pNA (1.5 mM) for human recombinant chymase. All experiments were carried out in 50 mM HEPES buffer (pH 7.4) containing 0.1 mM NaCl, except for the buffer used for chymase, which contained 50 mM Tris-HCl (pH 8.0) and 1 M KCl. The enzyme solutions were mixed with substrate solutions in the presence of various concentrations of each compound and incubated at 37 °C for 120 min, and absorbance at 405 nm was measured to determine enzymatic activities. The concentrations of enzymes and substrates mentioned above gave an increment of the absorbance unit at 405 nm to 1.0 without inhibitors under our assay conditions. $IC_{50}$ values were calculated from the dose−inhibition curves. Chymostatin was used as a control compound for the inhibition of α-chymotrypsin, cathepsin G, and chymase. Elastatinal and leupeptin were also used for elastase and trypsin, respectively. We confirmed that deviations of $IC_{50}$ values were less than 10% from repetitive determinations of $IC_{50}$ values of these control compounds.

**Competitive Inhibition Assay of α-Chymotrypsin.** The inhibitory effect of the compounds against chymotrypsin was estimated from the effects on the initial velocity of hydrolysis of the substrate; 2 $\mu$L of DMSO solution of the inhibitor was added to 400 $\mu$L of 60 nM bovine α-chymotrypsin solubilized in HEPES buffer (pH 7.4) containing 100 mM NaCl, and immediately after mixing, 400 $\mu$L of the substrate (*N*-succinyl-Ala-Ala-Pro-Phe-pNA) solution was added at various concentrations. An increase in absorbance at 405 nm was followed for 30 s at room temperature to determine the initial velocity of the substrate hydrolysis.

**Kinetic Studies by Progress Curve Method**. Because inhibition of α-chymotrypsin by the present compounds was time-dependent, the kinetics of the inhibition was analyzed by the progress curve methods described by Hart and O'Brien.[18] Briefly, 400 $\mu$L of 60 nM bovine α-chymotrypsin was mixed with 400 $\mu$L of chromogenic substrate solution in the presence of an appropriate concentration of inhibitors, and increased absorbance at 405 nm was followed for 300 s at 25 °C. Using a semilogarithmic plot, the velocity of hydrolysis of the substrate ($v$) at each time point was plotted as a function of time, which gave a linear regression line. $K_i$ is calculated by using the following equation:

$$K_i = K_m[I]/((K_m + [S])(v_c/v_0 - 1))$$

where $K_m$ is the Michaelis constant (41 $\mu$M; determined by the standard method), [ I ] is the inhibitor concentration, [S] is the substrate concentration (200 $\mu$M), $v_c$ is the velocity of a control reaction carried out in the absence of inhibitor, and $v_0$ is the velocity of the reaction at time zero, which was determined from the *y*-intercept of the time−ln *v* plot. $k_{inact}$ is also obtained from the equation:

$$k_{inact} = (\Delta \ln v/\Delta t)(K_i/([I](1 - \alpha)) + 1)$$

where $(\Delta \ln v/\Delta t)$ is the slope of the regression line in the semilogarithmic plot mentioned above, $\alpha = [S]/(K_m + [S])$, and $K_i$ is the calculated dissociation constant.

**Stability of Inhibitor 3 Analogues against Chymotryptic Cleavage.** Ten microliters of 5 mM inhibitor solution was mixed with 990 $\mu$L of 625 ng/mL bovine α-chymotrypsin solution. After incubation of the mixture at 37 °C for 120 min, an aliquot of the sample was applied to HPLC, and the intact compound and cleaved free acid were determined. HPLC was carried out using a Jasco system (800 series) equipped with a UV/vis detector and an integrator. The solvent system used for analytical HPLC consisted of a binary system, water containing 0.1% TFA, and acetonitrile containing the same TFA as the organic modifier. The dimensions of the column used for analytical chromatography were 4.6 × 250 mm (Wakosil-II 5C18 HG). The analytical conditions were a linear gradient with 50% to 75% acetonitrile in 0.1% TFA over 25 min at a flow rate of 1.0 mL/min. Stability was expressed as percent of the intact compound remaining in the sample relative to the initial amount.

**Recovery of Enzyme Activity by Hydrazine.** α-Chymotrypsin (1.25 $\mu$g/mL) was incubated with 5 $\mu$M **3** for 20 min at room temperature and dialyzed against HEPES buffer (containing 0.1 M NaCl, pH 7.4) overnight. Hydrazine solution (HEPES buffer solution, pH 7.4, final concentration was 0.6 M) was added to the dialysates, and enzyme solutions were incubated at room temperature. An equal volume of the enzyme solution and the substrate solution (final concentration was 750 $\mu$M) were mixed, and the increase in absorbance at 405 nm was monitored. α-Chymotrypsin solution without incubation with **3** was used as a control, and chymotryptic activity of each group was expressed as percent of control.

**Chemistry.** $^1$H NMR and $^{13}$C NMR spectra were recorded on a JEOL GSX270J spectrometer. The spectra were recorded with tetramethylsilane ($\delta = 0.0$ for $^1$H), $CD_3OD$ ($\delta = 49.8$ for $^{13}$C), or $CDCl_3$ ($\delta = 77.0$ for $^{13}$C) as internal reference. Mass spectra (electrospray ionization, methanol as the mobile phase) were analyzed with a Finnigan SSQ 7000 spectrometer. High-resolution fast atom bombardment mass spectra were analyzed with a JEOL JMS-DX303 spectrometer. A silica gel column chromatography was performed using Merck 70-230 mesh silica gel 60. Normal phase HPLC on a semipreparative scale for the separation of diastereomers of **3** was performed with a column (Merck, LiChrospher Si 60, 10 × 250 mm, 10 $\mu$m) employing the solvent system with AcOEt−hexane (2:3) at a flow rate of 4 mL/min on a Waters system (600E series).

**General Procedure A for Preparation of *β*-Substituted-α,α-dimethyl-*β*-alanines. 3-Amino-2,2-dimethylnanoic Acid (4b) (Hydrochloride).** To 14 mL (66 mmol) of 1,1,1,3,3,3-hexamethyldisilazane in 20 mL of dry tetrahydrofuran was added 40 mL (66 mmol) of 1.65 M *n*-butyllithium in hexane at 0 °C over a 10-min period. The mixture was stirred at 0 °C for 20 min, and the solvent was removed in vacuo until a white precipitate appeared. To the resulting slurry was added dropwise a solution of 7.7 mL (55 mmol) of *n*-heptanal in 15 mL of tetrahydrofuran at −20 °C over a 5-min period. The resulting solution of trimethylsilylimine was used directly in the following reaction. To 9.1 mL (65 mmol) of diisopropylamine in 30 mL of tetrahydrofuran was added 36.4 mL (60 mmol) of 1.65 M *n*-butyllithium in hexane at −70 °C. The mixture was stirred at −70 °C for 20 min, followed by addition of 6.7 mL (50 mmol) of ethyl isobutyrate in 10 mL of tetrahydrofuran over a 10-min period. The solution was stirred at −70 °C for 60 min, followed by addition of the solution of trimethylsilylimine via a cannula at a rate such that the temperature did not exceed −50 °C. The mixture was stirred at −70 °C for 60 min, allowed to warm to room temperature, and stirred for an additional 18 h. In the next step, 100 mL of saturated aqueous $NH_4Cl$ was added to the reaction mixture, and the mixture was extracted three times with 50 mL of diethyl ether. The combined organic layers were washed with saturated NaCl, dried over $Na_2SO_4$, and concentrated in vacuo. The residual pale-yellow oil was applied to a silica gel column (5 × 50 cm) and eluted with EtOAc−hexane (1:4) to give 4.78 g (52%) of corresponding azetidinone as a pale-yellow oil: $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.90 (t, $J$ = 8.4 Hz, 3H), 1.17 (s, 3H), 1.31 (s, 3H), 1.21−1.40 (m, 8H), 1.42−1.64 (m, 2H), 3.29 (dd, $J$ = 5.9, 8.3 Hz, 1H), 5.91 (br s, 1H, NH); MS (ESI) *m*/*z* 184 $(M + H)^+$, 206 $(M + Na)^+$. A mixture of this azetidinone (1.0 g, 5.5 mmol) and 6 N HCl (100 mL) was stirred at room temperature for 48 h. The solution was condensed under reduced pressure, and then toluene (30 mL) was added to the residue and evaporated twice to give 1.28 g (98%) of a hydrochloride salt of 4-*n*-hexyl-3,3-dimethyl-2-azetidinone (**4b**) as a white solid: mp 150−152 °C; MS (ESI) *m*/*z* 202 $(M + H)^+$. Anal. ($C_{11}H_{23}NO_2$·0.9HCl) C, H, N.

Compounds **4a**, **c**−**l** were prepared according to procedure A described for preparation of **4b**. See ref 14 for the syntheses and physicochemical data for compounds **4a**−**h**.

**3-Amino-2,2-dimethyldecanoic acid (4i) (hydrochloride):** 69% yield from ethyl isobutyrate; white solid; mp 159−161 °C; MS (ESI) *m*/*z* 216 $(M + H)^+$. Anal. ($C_{12}H_{25}NO_2$·HCl) C, H, N.

**3-Amino-2,2-dimethylundecanoic acid (4j) (hydrochloride):** 66% yield from ethyl isobutyrate; white solid; mp 158−160 °C; MS (ESI) *m*/*z* 230 $(M + H)^+$. Anal. ($C_{13}H_{27}NO_2$·HCl·0.25$H_2O$) C, H, N.

**3-Amino-2,2-dimethyltridecanoic acid (4k) (hydrochloride):** 11% yield from ethyl isobutyrate; white solid; mp 141−143 °C; MS (ESI) *m*/*z* 258 $(M + H)^+$. Anal. ($C_{15}H_{31}NO_2$·HCl) C, H, N.

**3-Amino-2,2-dimethylpentadecanoic acid (4l) (hydrochloride):** 11% yield from ethyl isobutyrate; white solid; mp 137−139 °C; MS (ESI) *m*/*z* 286 $(M + H)^+$.

**General Procedure B for Preparation of 5. 3-(*N*-(4-Cyanobenzoyl)amino)-2,2-dimethylnanoic Acid (5b).** To a solution of 4-*n*-hexyl-3,3-dimethyl-2-azetidinone hydrochloride (**4b**) (1.0 g, 4.2 mmol) in DMF (40 mL) were added triethylamine (1.3 mL, 9.3 mmol) and *N*-succinimidyl 4-cyanobenzoate (1.2 g, 5.0 mmol) at 0 °C, and the resulting mixture was stirred at room temperature for 18 h. The solvent was removed by evaporation, and the residue was dissolved in EtOAc (50 mL). The organic phase was washed with 5% citric acid and saturated NaCl, dried over $Na_2SO_4$ and concentrated in vacuo. The residual crude powder (1.45 g) was applied to a silica gel column (2 × 17 cm) and eluted with EtOAc−hexane (1:3) to give 0.92 g (66%) of **5b** as a white solid: mp 142−144 °C; MS (ESI) *m*/*z* 331 $(M + H)^+$, 353 $(M + Na)^+$. Anal. ($C_{19}H_{26}N_2O_3$·0.125$H_2O$) C, H, N.

Compounds **5a,c**−**n** were prepared according to procedure B described for the preparation of **5b** starting from the appropriate intermediates **4**. In the preparation of **5m**,**n**, benzoyl chloride and $(Boc)_2O$ were used instead of *N*-succinimidyl 4-cyanobenzoate, respectively. See ref 14 for the syntheses and physicochemical data for compounds **5a**,**c**−**h**.

**3-(*N*-(4-Cyanobenzoyl)amino)-2,2-dimethyldecanoic acid (5i):** 75% yield from **4i**; white solid; mp 119−120 °C; MS (ESI) *m*/*z* 343 $(M - H)^+$. Anal. ($C_{20}H_{28}N_2O_3$) C, H, N.

**3-(*N*-(4-Cyanobenzoyl)amino)-2,2-dimethylundecanoic acid (5j):** 70% yield from **4j**; white solid; mp 96−97 °C; MS (ESI) *m*/*z* 357 $(M - H)^+$. Anal. ($C_{21}H_{30}N_2O_3$·0.125$H_2O$) C, H, N.

**3-(*N*-(4-Cyanobenzoyl)amino)-2,2-dimethyltridecanoic acid (5k):** 81% yield from **4k**; white solid; mp 132−133 °C; MS (ESI) *m*/*z* 409 $(M + Na)^+$. Anal. ($C_{23}H_{34}N_2O_3$) C, H, N.

**3-(*N*-(4-Cyanobenzoyl)amino)-2,2-dimethylpentadecanoic acid (5l):** 66% yield from **4l**; colorless oil; MS (ESI) *m*/*z* 413 $(M - H)^-$. Anal. ($C_{25}H_{38}N_2O_3$·0.25$H_2O$) C, H, N.

**3-(*N*-Benzoylamino)-2,2-dimethylnanoic acid (5m):** 54% yield from **4b**; white solid; mp 140−142 °C; MS (ESI) *m*/*z* 306 $(M + H)^+$. Anal. ($C_{18}H_{27}NO_3$) C, H, N.

**3-(*N*-(Butyloxycarbonyl)amino)-2,2-dimethylnanoic acid (5n):** 82% yield from **4b**; white solid; mp 120−122 °C; MS (ESI) *m*/*z* 302 $(M + H)^+$. Anal. ($C_{16}H_{31}NO_4 \cdot 0.75H_2O$) C, H, N.

**General Procedure C for Preparation of Target Compounds. *N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)nonanoyl]-L-phenylalanine Ethyl Ester (3).** To a solution of **5b** (0.99 g, 3.0 mmol) and L-phenylalanine ethyl ester hydrochloride (0.92 g, 4.0 mmol) in $CH_2Cl_2$ (30 mL) were added 1-hydroxybenzotriazole (HOBt; 0.55 g, 3.6 mmol) and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (WSCD·HCl; 0.75 g, 3.9 mmol) at 0 °C, and the mixture was stirred at room temperature for 18 h. After the solvent was removed in vacuo, the residue was dissolved in EtOAc (50 mL) and washed with 5% citric acid, 5% sodium bicarbonate, and saturated NaCl, dried over $Na_2SO_4$, and concentrated in vacuo. The residual oil was applied to a silica gel column (2.2 × 20 cm) and eluted with EtOAc−hexane (1:3 to 1:2) to give 1.47 g (75%) of **3** as a colorless oil: 75% yield from **5b**; white foam; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.83 (t, $J$ = 6.8 Hz, 1.5H), 0.85 (t, $J$ = 6.8 Hz, 1.5H), 1.19 (s, 3H), 1.22 (s, 3H), 1.30 (t, $J$ = 7.3 Hz, 1.5H), 1.31 (t, $J$ = 7.3 Hz, 1.5H), 1.05−1.42 (m, 9H), 1.49−1.68 (m, 1H), 3.07−3.26 (m, 2H), 3.92−4.04 (m, 1H), 4.23 (q, $J$ = 7.3 Hz, 2H), 4.77−4.91 (m, 1H), 6.11 (d, $J$ = 8.3 Hz, 0.5H, NH), 6.20 (d, $J$ = 7.8 Hz, 0.5H, NH), 7.07−7.14 (m, 2H), 7.20−7.32 (m, 3H), 7.70 (d, $J$ = 8.3 Hz, 1H), 7.74 (d, $J$ = 8.3 Hz, 1H), 7.80 (d, $J$ = 9.8 Hz, 0.5H, NH), 7.88 (d, $J$ = 8.3 Hz, 1H), 7.94 (d, $J$ = 8.3 Hz, 1H), 7.86−7.96 (m, 0.5H, NH); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 14.0, 14.1, 22.5, 22.6, 22.96, 22.99, 25.1, 25.8, 26.4, 26.5, 29.1, 30.8, 31.0, 31.6, 37.4, 37.6, 45.1, 45.4, 52.66, 52.72, 57.56, 57.64, 61.8, 61.9, 114.67, 114.73, 118.2, 127.3, 127.4, 127.7, 128.6, 128.7, 129.0, 129.2, 132.3, 132.4, 135.4, 138.4, 138.6, 165.1, 165.2, 171.2, 171.8, 176.6, 177.1; MS (ESI) *m*/*z* 506 $(M + H)^+$, 528 $(M + Na)^+$; HRMS (FAB) calcd for $C_{30}H_{40}N_3O_4$ $(M + H)^+$ 506.3019, found 506.2998. Anal. ($C_{30}H_{39}N_3O_4$) C, H, N.

Compounds **2, 6**−**16**, and **20**−**25** were prepared according to general procedure C described for the preparation of **3** starting from the appropriate intermediates **5** by coupling with L-phenylalanine methyl, ethyl, *n*-propyl, isopropyl, benzyl, and *tert*-butyl esters.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)-5-phenylpentanoyl]-L-phenylalanine ethyl ester (2):** 58% yield from **5a**; colorless oil; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 1.20 (s, 3H), 1.22 (s, 3H), 1.24 (t, $J$ = 7.3 Hz, 1.5H), 1.30 (t, $J$ = 7.3 Hz, 1.5H), 1.38−1.65 (m, 1H), 1.77−1.98 (m, 1H), 2.54−2.72 (m, 2H), 3.04−3.24 (m, 2H), 4.01−4.13 (m, 1H), 4.14−4.28 (m, 2H), 4.75−4.88 (m, 1H), 6.06 (d, $J$ = 7.8 Hz, 0.5H, NH), 6.18 (d, $J$ = 7.8 Hz, 0.5H, NH), 7.03−7.31 (m, 10H), 7.71 (d, $J$ = 8.8 Hz, 1H), 7.75 (d, $J$ = 8.8 Hz, 1H), 7.87 (d, $J$ = 8.8 Hz, 1H), 7.94 (d, $J$ = 8.8 Hz, 1H), 7.84−7.95 (m, 0.5H, NH), 8.04 (d, $J$ = 9.3 Hz, 0.5H, NH); MS (ESI) *m*/*z* 526 $(M + H)^+$, 548 $(M + Na)^+$. Anal. ($C_{32}H_{35}N_3O_4 \cdot 0.75H_2O$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)pentanoyl]-L-phenylalanine ethyl ester (6):** 55% yield from **5c**; white foam; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.86 (t, $J$ = 7.3 Hz, 1.5H), 0.91 (t, $J$ = 7.3 Hz, 1.5H), 1.19 (s, 3H), 1.22 (s, 3H), 1.15−1.34 (m, 1H), 1.296 (t, $J$ = 7.3 Hz, 1.5H), 1.303 (t, $J$ = 7.3 Hz, 1.5H), 1.54−1.78 (m, 1H), 3.07−3.25 (m, 2H), 3.86−3.98 (m, 1H), 4.227 (q, $J$ = 7.3 Hz, 1H), 4.230 (q, $J$ = 7.3 Hz, 1H), 4.76−4.91 (m, 1H), 6.10 (d, $J$ = 8.3 Hz, 0.5H, NH), 6.19 (d, $J$ = 8.3 Hz, 0.5H, NH), 7.06−7.13 (m, 2H), 7.20−7.31 (m, 3H), 7.71 (d, $J$ = 8.3 Hz, 1H), 7.74 (d, $J$ = 8.3 Hz, 1H), 7.81 (d, $J$ = 9.8 Hz, 0.5H, NH), 7.89 (d, $J$ = 8.3 Hz, 1H), 7.95 (d, $J$ = 8.3 Hz, 1H), 7.86−7.98 (m, 0.5H, NH); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 11.0, 11.1, 14.1, 22.9, 23.0, 23.8, 24.0, 25.2, 25.9, 37.5, 37.7, 45.0, 45.3, 52.6, 52.7, 59.0, 59.1, 61.8, 61.9, 114.7, 114.8, 118.2, 127.36, 127.39, 127.7, 128.66, 128.72, 129.1, 129.2, 132.3, 132.4, 135.4, 138.4, 138.6, 165.3, 165.5, 171.3, 171.8, 176.6, 177.1; MS (ESI) *m*/*z* 450 $(M + H)^+$. Anal. ($C_{26}H_{31}N_3O_4 \cdot H_2O$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)hexanoyl]-L-phenylalanine ethyl ester (7):** 73% yield from **5d**; white foam; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.877 (t, $J$ = 7.3 Hz, 1.5H), 0.882 (t, $J$ = 7.3 Hz, 1.5H), 1.19 (s, 3H), 1.22 (s, 3H), 1.04−1.42 (m, 3H), 1.30 (t, $J$ = 6.8 Hz, 1.5H),), 1.31 (t, $J$ = 6.8 Hz, 1.5H), 1.44−1.63 (m, 1H), 3.08−3.26 (m, 2H), 3.94−4.06 (m, 1H), 4.23 (q, $J$ = 6.8 Hz, 2H), 4.78−4.92 (m, 1H), 6.11 (d, $J$ = 7.8 Hz, 0.5H, NH), 6.20 (d, $J$ = 7.3 Hz, 0.5H, NH), 7.07−7.15 (m, 2H), 7.18−7.34 (m, 3H), 7.70 (d, $J$ = 8.3 Hz, 1H), 7.74 (d, $J$ = 8.3 Hz, 1H), 7.82 (d, $J$ = 9.8 Hz, 0.5H, NH), 7.88 (d, $J$ = 8.3 Hz, 1H), 7.94 (d, $J$ = 8.3 Hz, 1H), 7.88−7.97 (m, 0.5H, NH); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 13.91, 13.94, 14.1, 14.2, 19.7, 19.8, 22.9, 23.0, 25.1, 25.8, 33.0, 33.2, 37.5, 37.6, 45.1, 45.3, 52.66, 52.72, 57.3, 57.4, 61.8, 61.9, 114.68, 114.74, 118.2, 127.3, 127.4, 127.7, 128.6, 128.7, 129.0, 129.2, 132.3, 132.4, 135.4, 138.3, 138.5, 165.1, 165.3, 171.2, 171.8, 176.6, 177.1; MS (ESI) *m*/*z* 464 $(M + H)^+$, 486 $(M + Na)^+$. Anal. ($C_{27}H_{33}N_3O_4$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)-4-methylpentanoyl]-L-phenylalanine ethyl ester (8):** 37% yield from **5e**; white foam; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.63 (d, $J$ = 6.8 Hz, 1.5H), 0.75 (d, $J$ = 6.8 Hz, 1.5H), 0.95 (br t, $J$ = 6.8 Hz, 3H), 1.225 (s, 1.5H), 1.233 (s, 1.5H), 1.26 (s, 1.5H), 1.271 (s, 1.5H), 1.273 (d, $J$ = 6.8 Hz, 1.5H), 1.31 (d, $J$ = 6.8 Hz, 1.5H), 1.93−2.15 (m, 1H), 3.07−3.26 (m, 2H), 3.95−4.02 (m, 1H), 4.13−4.28 (m, 2H), 4.78−4.88 (m, 1H), 6.19 (d, $J$ = 8.3 Hz, 0.5H, NH), 6.31 (d, $J$ = 7.3 Hz, 0.5H, NH), 7.07−7.18 (m, 2H), 7.22−7.36 (m, 3H), 7.73 (d, $J$ = 8.8 Hz, 1H), 7.76 (d, $J$ = 8.8 Hz, 1H), 7.97 (d, $J$ = 8.8 Hz, 1H), 8.00 (d, $J$ = 8.8 Hz, 1H), 8.49 (d, $J$ = 9.3 Hz, 0.5H, NH), 8.60 (d, $J$ = 9.3 Hz, 0.5H, NH); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 14.06, 14.09, 16.3, 16.4, 22.3, 22.4, 22.6, 22.8, 27.2, 27.7, 29.2, 29.3, 37.5, 37.7, 43.6, 43.9, 52.6, 52.8, 61.77, 61.84, 62.06, 62.13, 114.7, 118.2, 127.3, 127.4, 127.7, 128.6, 128.8, 129.0, 129.2, 132.37, 132.42, 135.4, 135.5, 138.5, 138.7, 165.47, 165.53, 171.2, 171.4, 177.7, 177.8; MS (ESI) *m*/*z* 464 $(M + H)^+$, 486 $(M + Na)^+$. Anal. ($C_{27}H_{33}N_3O_4$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)heptanoyl]-L-phenylalanine ethyl ester (9):** 63% yield from **5f**; white foam; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.80−0.88 (m, 3H), 1.19 (s, 3H), 1.22 (s, 3H), 1.30 (t, $J$ = 7.3 Hz, 1.5H), 1.31 (t, $J$ = 7.3 Hz, 1.5H), 1.05−1.42 (m, 5H), 1.47−1.67 (m, 1H), 3.07−3.27 (m, 2H), 3.92−4.04 (m, 1H), 4.23 (q, $J$ = 7.3 Hz, 2H), 4.77−4.90 (m, 1H), 6.10 (d, $J$ = 8.3 Hz, 0.5H, NH), 6.19 (d, $J$ = 7.3 Hz, 0.5H, NH), 7.07−7.14 (m, 2H), 7.20−7.31 (m, 3H), 7.70 (d, $J$ = 8.8 Hz, 1H), 7.74 (d, $J$ = 8.8 Hz, 1H), 7.81 (d, $J$ = 9.8 Hz, 0.5H, NH), 7.88 (d, $J$ = 8.8 Hz, 1H), 7.94 (d, $J$ = 8.8 Hz, 1H), 7.86−7.96 (m, 0.5H, NH); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 14.0, 14.1, 22.5, 23.0, 25.1, 25.8, 28.6, 28.7, 30.6, 30.7, 37.5, 37.6, 45.1, 45.4, 52.67, 52.74, 57.5, 57.6, 61.8, 61.9, 114.7, 114.8, 118.2, 127.3, 127.4, 127.7, 128.6, 128.7, 129.0, 129.2, 132.3, 132.4, 135.4, 138.4, 138.6, 165.1, 165.3, 171.3, 171.8, 176.6, 177.1; MS (ESI) *m*/*z* 478 $(M + H)^+$, 500 $(M + Na)^+$. Anal. ($C_{28}H_{35}N_3O_4 \cdot 0.25H_2O$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)-5-methylhexanoyl]-L-phenylalanine ethyl ester (10):** 59% yield from **5g**; white foam; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.86 (d, $J$ = 6.8 Hz, 3H), 0.93 (d, $J$ = 4.9 Hz, 1.5H), 0.95 (d, $J$ = 4.9 Hz, 1.5H), 1.19 (s, 3H), 1.22 (s, 3H), 1.15−1.37 (m, 2H), 1.30 (t, $J$ = 7.3 Hz, 1.5H), 1.31 (t, $J$ = 7.3 Hz, 1.5H), 1.49−1.67 (m, 1H), 3.08−3.25 (m, 2H), 4.02−4.13 (m, 1H), 4.23 (q, $J$ = 7.3 Hz, 2H), 4.77−4.91 (m, 1H), 6.08 (d, $J$ = 8.3 Hz, 0.5H, NH), 6.18 (d, $J$ = 7.3 Hz, 0.5H, NH), 7.07−7.13 (m, 2H), 7.20−7.32 (m, 3H), 7.70 (d, $J$ = 8.8 Hz, 1H), 7.74 (d, $J$ = 8.8 Hz, 1H), 7.69−7.83 (m, 1H, NH), 7.87 (d, $J$ = 8.8 Hz, 1H), 7.93 (d, $J$ = 8.8 Hz, 1H); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 14.1, 21.5, 23.0, 23.9, 24.0, 25.0, 25.1, 25.7, 37.5, 37.6, 40.1, 40.3, 45.3, 45.5, 52.66, 52.74, 55.8, 61.8, 62.0, 114.7, 114.8, 118.2, 127.3, 127.5, 127.7, 128.6, 128.7, 129.0, 129.2, 132.3, 132.4, 135.4, 135.5, 138.3, 138.6, 165.0, 165.2, 171.3, 171.9, 176.6, 177.1; MS (ESI) *m*/*z* 478 $(M + H)^+$, 500 $(M + Na)^+$. Anal. ($C_{28}H_{35}N_3O_4 \cdot H_2O$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)octanoyl]-L-phenylalanine ethyl ester (11):** 88% yield from **5h**; white

foam; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.83 (br t, $J$ = 6.8 Hz, 1.5H), 0.85 (br t, $J$ = 6.8 Hz, 1.5H), 1.19 (s, 3H), 1.22 (s, 3H), 1.30 (t, $J$ = 7.3 Hz, 1.5H), 1.31 (t, $J$ = 7.3 Hz, 1.5H), 1.08−1.38 (m, 7H), 1.47−1.67 (m, 1H), 3.07−3.25 (m, 2H), 3.92−4.04 (m, 1H), 4.23 (q, $J$ = 7.3 Hz, 2H), 4.78−4.90 (m, 1H), 6.10 (d, $J$ = 8.3 Hz, 0.5H, NH), 6.20 (d, $J$ = 7.8 Hz, 0.5H, NH), 7.08−7.14 (m, 2H), 7.20−7.34 (m, 3H), 7.70 (d, $J$ = 8.8 Hz, 1H), 7.74 (d, $J$ = 8.8 Hz, 1H), 7.88 (d, $J$ = 8.8 Hz, 1H), 7.94 (d, $J$ = 8.8 Hz, 1H), 7.78−7.97 (m, 1H, NH); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 14.0, 14.1, 22.5, 22.97, 23.00, 25.1, 25.8, 26.1, 26.2, 30.8, 31.0, 31.6, 37.5, 37.6, 45.1, 45.4, 52.67, 52.74, 57.58, 57.64, 61.8, 61.9, 114.68, 114.74, 118.2, 127.3, 127.4, 127.7, 128.6, 128.7, 129.0, 129.2, 132.3, 132.4, 135.5, 138.4, 138.6, 165.1, 165.3, 171.3, 171.8, 176.6, 177.1; MS (ESI) $m/z$ 492 $(M + H)^+$, 514 $(M + Na)^+$. Anal. ($C_{29}H_{37}N_3O_4$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)decanoyl]-L-phenylalanine ethyl ester (12):** 30% yield from **5i**; white solid; mp 107−109 °C; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.81−0.88 (m, 3H), 1.22 (br s, 3H), 1.24 (br s, 3H), 1.30 (br t, $J$ = 6.8 Hz, 1.5H), 1.31 (br t, $J$ = 6.8 Hz, 1.5H), 1.16−1.39 (m, 11H), 1.48−1.67 (m, 1H), 3.08−3.27 (m, 2H), 3.97 (m, 1H), 4.23 (q, $J$ = 6.8 Hz, 2H), 4.76−4.91 (m, 1H), 6.10 (d, $J$ = 7.8 Hz, 0.5H, NH), 6.19 (d, $J$ = 7.8 Hz, 0.5H, NH), 7.07−7.30 (m, 5H), 7.68−7.98 (m, 5H); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 14.0, 14.1, 22.56, 22.59, 22.97, 23.00, 25.1, 25.8, 26.5, 26.6, 29.1, 29.4, 30.9, 31.0, 31.7, 37.5, 37.6, 45.1, 45.4, 52.67, 52.74, 57.6, 57.7, 61.8, 61.9, 114.7, 118.2, 127.3, 127.4, 127.7, 128.6, 128.7, 129.0, 129.2, 132.3, 132.4, 135.5, 138.4, 138.6, 165.1, 165.3, 171.5, 171.8, 176.6, 177.1; MS (ESI) $m/z$ 520 $(M + H)^+$, 542 $(M + Na)^+$. Anal. ($C_{31}H_{41}N_3O_4$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)undecanoyl]-L-phenylalanine ethyl ester (13):** 49% yield from **5j**; white solid; mp 114 °C; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.84 (br t, $J$ = 6.8 Hz, 1.5H), 0.85 (br t, $J$ = 6.8 Hz, 1.5H), 1.19 (s, 3H), 1.21 (s, 3H), 1.30 (t, $J$ = 7.3 Hz, 1.5H), 1.31 (t, $J$ = 7.3 Hz, 1.5H), 1.04−1.43 (m, 13H), 1.47−1.68 (m, 1H), 3.07−3.24 (m, 2H), 3.91−4.05 (m, 1H), 4.23 (q, $J$ = 7.3 Hz, 2H), 4.77−4.90 (m, 1H), 6.10 (d, $J$ = 7.3 Hz, 0.5H, NH), 6.19 (d, $J$ = 7.3 Hz, 0.5H, NH), 7.07−7.14 (m, 2H), 7.20−7.34 (m, 3H), 7.70 (d, $J$ = 8.3 Hz, 1H), 7.77 (d, $J$ = 8.3 Hz, 1H), 7.88 (d, $J$ = 8.3 Hz, 1H), 7.94 (d, $J$ = 8.3 Hz, 1H), 7.75−7.98 (m, 1H, NH); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 14.08, 14.12, 22.6, 23.0, 25.1, 25.9, 26.5, 26.6, 29.2, 29.4, 29.5, 30.9, 31.0, 31.8, 37.5, 37.6, 45.1, 45.4, 52.67, 52.74, 57.6, 57.7, 61.8, 61.9, 114.68, 114.74, 118.2, 127.3, 127.4, 127.7, 128.6, 128.7, 129.0, 129.2, 132.3, 132.4, 135.5, 138.4, 138.6, 165.1, 165.3, 171.3, 171.8, 176.6, 177.1; MS (ESI) $m/z$ 534 $(M + H)^+$, 556 $(M + Na)^+$. Anal. ($C_{32}H_{43}N_3O_4$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)tridecanoyl]-L-phenylalanine ethyl ester (14):** 87% yield from **5k**; waxy solid; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.86 (br t, $J$ = 6.8 Hz, 3H), 1.19 (s, 3H), 1.12−1.37 (m, 20H), 1.30 (t, $J$ = 7.3 Hz, 1.5H), 1.31 (t, $J$ = 7.3 Hz, 1.5H), 1.47−1.67 (m, 1H), 3.07−3.25 (m, 2H), 3.90−4.03 (m, 1H), 4.23 (q, $J$ = 7.3 Hz, 2H), 4.77−4.90 (m, 1H), 6.10 (d, $J$ = 7.3 Hz, 0.5H, NH), 6.19 (d, $J$ = 8.3 Hz, 0.5H, NH), 7.07−7.13 (m, 2H), 7.19−7.33 (m, 3H), 7.70 (d, $J$ = 8.8 Hz, 1H), 7.74 (d, $J$ = 8.8 Hz, 1H), 7.88 (d, $J$ = 8.8 Hz, 1H), 7.94 (d, $J$ = 8.8 Hz, 1H), 7.77−7.97 (m, 1H, NH); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 14.09, 14.12, 22.7, 22.97, 23.00, 25.1, 25.8, 26.5, 26.6, 29.3, 29.5, 29.6, 30.9, 31.0, 31.9, 37.5, 37.6, 45.1, 45.4, 52.67, 52.74, 57.6, 57.7, 61.8, 61.9, 114.68, 114.74, 118.2, 127.3, 127.4, 127.7, 128.6, 128.7, 129.0, 129.2, 132.3, 132.4, 135.5, 138.4, 138.6, 165.1, 165.3, 171.3, 171.8, 176.6, 177.1; MS (ESI) $m/z$ 562 $(M + H)^+$, 584 $(M + Na)^+$. Anal. ($C_{34}H_{47}N_3O_4 \cdot H_2O$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)pentadecanoyl]-L-phenylalanine ethyl ester (15):** 71% yield from **5l**; waxy solid; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.87 (br t, $J$ = 6.8 Hz, 3H), 1.19 (br s, 3H), 1.05−1.43 (m, 24H), 1.30 (t, $J$ = 7.3 Hz, 1.5H), 1.31 (t, $J$ = 7.3 Hz, 1.5H), 1.47−1.68 (m, 1H), 3.07−3.26 (m, 2H), 3.91−4.04 (m, 1H), 4.23 (q, $J$ = 7.3 Hz, 2H), 4.77−4.90 (m, 1H), 6.10 (d, $J$ = 8.3 Hz, 0.5H, NH), 6.19 (d, $J$ = 7.3 Hz, 0.5H, NH), 7.07−7.13 (m, 2H), 7.19−7.31 (m, 3H), 7.70 (d, $J$ = 8.8 Hz, 1H), 7.74 (d, $J$ = 8.8 Hz, 1H), 7.81 (d, $J$ = 9.8 Hz, 0.5H, NH), 7.88 (d, $J$ = 8.8 Hz, 1H), 7.94 (d, $J$ = 8.8 Hz, 1H), 7.85−7.95 (m, 0.5H, NH); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 14.1, 22.7, 23.0, 25.1, 25.8, 26.5, 26.6, 29.3, 29.5, 29.6, 30.8, 31.0, 31.9, 37.5, 37.6, 45.1, 45.4, 52.67, 52.74, 57.6, 57.7, 61.8, 61.9, 114.7, 114.8, 118.2, 127.3, 127.4, 127.7, 128.6, 128.7, 129.0, 129.2, 132.3, 132.4, 135.4, 138.4, 138.6, 165.1, 165.3, 171.2, 171.8, 176.6, 177.1; MS (ESI) $m/z$ 612 $(M + Na)^+$. Anal. ($C_{36}H_{51}N_3O_4$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-benzoyl)amino)nonanoyl]-L-phenylalanine ethyl ester (16):** 57% yield from **5m**; colorless oil; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.79−0.88 (m, 3H), 1.21 (s, 3H), 1.22 (s, 3H), 1.29 (t, $J$ = 7.3 Hz, 3H), 1.10−1.40 (m, 9H), 1.45−1.67 (m, 1H), 3.07−3.25 (m, 2H), 3.96−4.09 (m, 1H), 4.22 (q, $J$ = 7.3 Hz, 2H), 4.79−4.91 (m, 1H), 6.12 (d, $J$ = 7.8 Hz, 0.5H, NH), 6.19 (d, $J$ = 7.8 Hz, 0.5H, NH), 7.07−7.15 (m, 2H), 7.20−7.32 (m, 3H), 7.37−7.51 (m, 3H), 7.59 (d, $J$ = 9.8 Hz, 0.5H, NH), 7.65 (d, $J$ = 9.8 Hz, 0.5H, NH), 7.78−7.87 (m, 2H); MS (ESI) $m/z$ 481 $(M + H)^+$, 503 $(M + Na)^+$. Anal. ($C_{29}H_{40}N_2O_4 \cdot 0.5H_2O$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(butyloxycarbonyl)amino)nonanoyl]-L-phenylalanine ethyl ester (21):** 98% yield from **5n**; white solid; mp 82−84 °C; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.86 (t, $J$ = 6.8 Hz, 1.5H), 0.87 (t, $J$ = 6.8 Hz, 1.5H), 1.14 (br s, 3H), 1.15 (br s, 3H), 0.92−1.48 (m, 10H), 1.26 (t, $J$ = 6.8 Hz, 3H), 1.41 (s, 4.5H), 1.44 (s, 4.5H), 3.00−3.23 (m, 2H), 3.36−3.48 (m, 1H), 4.19 (q, $J$ = 6.8 Hz, 2H), 4.76−4.87 (m, 1H), 5.11 (d, $J$ = 10.3 Hz, 0.5H, NH), 5.33 (d, $J$ = 10.3 Hz, 0.5H, NH), 6.00−6.13 (m, 1H, NH), 7.09−7.15 (m, 2H), 7.21−7.34 (m, 3H); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 14.1, 22.5, 22.6, 23.3, 23.8, 23.9, 26.5, 26.6, 28.36, 28.40, 29.0, 29.1, 31.1, 31.8, 37.7, 37.8, 45.8, 45.9, 52.8, 57.9, 58.1, 61.5, 61.6, 78.6, 78.7, 127.16, 127.19, 128.6, 129.1, 129.2, 135.8, 135.9, 156.4, 171.6, 171.7, 176.2, 176.3; MS (ESI) $m/z$ 477 $(M + H)^+$, 499 $(M + Na)^+$. Anal. ($C_{27}H_{44}N_2O_5$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)nonanoyl]-L-phenylalanine methyl ester (22):** 61% yield from **5b**; colorless oil; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.83 (t, $J$ = 6.8 Hz, 1.5H), 0.85 (t, $J$ = 6.8 Hz, 1.5H), 1.19 (s, 3H), 1.22 (s, 3H), 1.05−1.34 (m, 9H), 1.45−1.65 (m, 1H), 3.05−3.26 (m, 2H), 3.79 (s, 3H), 3.91−4.06 (m, 1H), 4.80−4.92 (m, 1H), 6.10 (d, $J$ = 8.4 Hz, 0.5H, NH), 6.18 (d, $J$ = 7.3 Hz, 0.5H, NH), 7.06−7.13 (m, 2H), 7.20−7.32 (m, 3H), 7.71 (d, $J$ = 8.8 Hz, 1H), 7.74 (d, $J$ = 8.3 Hz, 1H), 7.88 (d, $J$ = 8.8 Hz, 1H), 7.94 (d, $J$ = 8.8 Hz, 1H), 7.73−7.95 (m, 1H, NH); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 14.0, 22.5, 22.6, 23.0, 25.1, 25.8, 26.4, 26.5, 29.2, 30.9, 31.0, 31.6, 37.4, 37.6, 45.1, 45.4, 52.55, 52.66, 52.71, 57.58, 57.64, 114.7, 114.8, 118.2, 127.4, 127.5, 127.9, 128.7, 128.8, 129.0, 129.1, 132.3, 132.4, 135.4, 138.4, 138.6, 165.1, 165.3, 171.7, 172.2, 176.7, 177.2; MS (ESI) $m/z$ 492 $(M + H)^+$, 514 $(M + Na)^+$. Anal. ($C_{29}H_{37}N_3O_4 \cdot 0.5H_2O$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)nonanoyl]-L-phenylalanine *n*-propyl ester (23):** 99% yield from **5b**; colorless oil; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.83 (t, $J$ = 6.8 Hz, 1.5H), 0.85 (t, $J$ = 6.8 Hz, 1.5H), 0.95 (t, $J$ = 7.3 Hz, 1.5H), 0.96 (t, $J$ = 7.3 Hz, 1.5H), 1.19 (s, 3H), 1.21 (s, 3H), 1.04−1.47 (m, 9H), 1.48−1.68 (m, 1H), 1.62−1.75 (m, 2H), 3.07−3.26 (m, 2H), 3.92−4.03 (m, 1H), 4.13 (t, $J$ = 6.8 Hz, 2H), 4.79−4.92 (m, 1H), 6.11 (d, $J$ = 7.3 Hz, 0.5H, NH), 6.20 (d, $J$ = 7.8 Hz, 0.5H, NH), 7.07−7.14 (m, 2H), 7.19−7.33 (m, 3H), 7.70 (d, $J$ = 8.8 Hz, 1H), 7.74 (d, $J$ = 8.8 Hz, 1H), 7.88 (d, $J$ = 8.8 Hz, 1H), 7.94 (d, $J$ = 8.8 Hz, 1H), 7.81 (br d, $J$ = 9.8 Hz, 0.5H, NH), 7.87−7.96 (m, 0.5H, NH); $^{13}$C NMR (67.5 MHz, $CDCl_3$) $\delta$ 10.3, 14.0, 21.8, 22.5, 22.6, 22.96, 22.99, 25.1, 25.8, 26.4, 26.5, 29.1, 30.8, 31.0, 31.6, 37.5, 37.7, 45.1, 45.4, 52.67, 52.72, 57.58, 57.64, 67.3, 67.5, 114.67, 114.73, 118.2, 127.3, 127.4, 127.7, 128.6, 128.7, 129.0, 129.2, 132.3, 132.4, 135.4, 138.4, 138.6, 165.1, 165.2, 171.3, 171.9, 176.6, 177.1; MS (ESI) $m/z$ 520 $(M + H)^+$, 542 $(M + Na)^+$. Anal. ($C_{31}H_{41}N_3O_4 \cdot 2H_2O$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)nonanoyl]-L-phenylalanine isopropyl ester (24):** 84% yield from **5b**; colorless oil; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.83 (t, $J$ = 6.8 Hz, 1.5H), 0.85 (t, $J$ = 6.8 Hz, 1.5H), 1.19 (s, 3H), 1.22 (s, 3H), 1.07−1.48 (m, 15H), 1.50−1.70 (m, 1H), 3.07−3.24 (m,

2H), 3.91−4.05 (m, 1H), 4.74−4.87 (m, 1H), 5.00−5.12 (m, 1H), 6.10 (d, $J$ = 7.8 Hz, 0.5H, NH), 6.20 (d, $J$ = 7.3 Hz, 0.5H, NH), 7.08−7.15 (m, 2H), 7.19−7.31 (m, 3H), 7.70 (d, $J$ = 8.8 Hz, 1H), 7.74 (d, $J$ = 8.8 Hz, 1H), 7.83 (d, $J$ = 9.8 Hz, 0.5H, NH), 7.88 (d, $J$ = 8.8 Hz, 1H), 7.94 (d, $J$ = 8.8 Hz, 1H), 7.88−7.97 (m, 0.5H, NH); MS (ESI) $m/z$ 520 $(M + H)^+$, 542 $(M + Na)^+$. Anal. ($C_{31}H_{41}N_3O_4 \cdot H_2O$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)-nonanoyl]-L-phenylalanine benzyl ester (25):** 92% yield from **5b**; colorless oil; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.84 (t, $J$ = 6.8 Hz, 1.5H), 0.85 (t, $J$ = 6.8 Hz, 1.5H), 1.18 (s, 3H), 1.19 (s, 3H), 1.16−1.40 (m, 9H), 1.49−1.68 (m, 1H), 3.06−3.24 (m, 2H), 3.91−4.02 (m, 1H), 4.82−4.96 (m, 1H), 5.10−5.30 (m, 2H), 6.08 (d, $J$ = 7.8 Hz, 0.5H, NH), 6.18 (d, $J$ = 7.8 Hz, 0.5H, NH), 6.93−7.00 (m, 2H), 7.15−7.26 (m, 2H), 7.32−7.42 (m, 6H), 7.68 (d, $J$ = 8.3 Hz, 1H), 7.73 (d, $J$ = 8.8 Hz, 1H), 7.73−7.79 (m, 0.5H, NH), 7.85 (d, $J$ = 8.3 Hz, 1H), 7.93 (d, $J$ = 8.8 Hz, 1H), 7.84−7.92 (m, 0.5H, NH); MS (ESI) $m/z$ 566 $(M - H)^+$. Anal. ($C_{35}H_{41}N_3O_4 \cdot 2H_2O$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(4-cyanobenzoyl)amino)-nonanoyl]-L-phenylalanine *tert*-butyl ester (26):** 55% yield from **5b**; white solid; mp 174−176 °C; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.83 (br t, $J$ = 6.8 Hz, 3H), 1.19 (s, 3H), 1.22 (s, 3H), 1.15−1.36 (m, 9H), 1.46 (s, 4.5H), 1.47 (s, 4.5H), 1.54−1.68 (m, 1H), 3.04−3.21 (m, 2H), 3.92−4.04 (m, 1H), 4.66−4.81 (m, 1H), 6.10 (d, $J$ = 7.8 Hz, 0.5H, NH), 6.22 (d, $J$ = 7.8 Hz, 0.5H, NH), 7.10−7.33 (m, 5H), 7.69 (d, $J$ = 8.3 Hz, 1H), 7.74 (d, $J$ = 8.3 Hz, 1H), 7.89 (d, $J$ = 8.3 Hz, 1H), 7.94 (d, $J$ = 8.3 Hz, 1H), 7.80−8.00 (m, 1H, NH); MS (ESI) $m/z$ 535 $(M + H)^+$, 556 $(M + Na)^+$. Anal. ($C_{32}H_{43}N_3O_4$) C, H, N.

**General Procedure D for Preparation of Target Compounds. *N*-[2,2-Dimethyl-3-(*N*-(3,4-difluorobenzoyl)amino)nonanoyl]-L-phenylalanine Ethyl Ester (18).** To **21** (0.11 g, 0.23 mmol) was added 4 N HCl−dioxane (10 mL). The reaction mixture was stirred for 1 h under cooling with ice. After removal of the solvent in vacuo at room temperature, the resulting residue was washed three times with hexane and dissolved in $CH_2Cl_2$ (10 mL) under cooling with ice. After the mixture was neutralized by triethylamine, 3,4-difluorobenzoic acid (40 mg, 0.23 mmol), HOBt (40 mg, 0.26 mmol), and WSCD·HCl (60 mg, 0.3 mmol) were added, and the mixture was stirred overnight. After removal of the solvent by evaporation, the resulting residue was dissolved in EtOAc (30 mL), washed with 5% citric acid (3 × 30 mL), 5% $NaHCO_3$ (3 × 30 mL), and saturated NaCl (3 × 30 mL), dried over $Na_2SO_4$, and concentrated in vacuo. The residue was purified by column chromatography on silica (1.8 × 20 cm) using hexanes−EtOAc (6:1 to 1:1) as an eluent to give an oil of **18** (36 mg, 30%): colorless oil; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.83 (t, $J$ = 6.8 Hz, 1.5H), 0.85 (t, $J$ = 6.8 Hz, 1.5H), 1.18 (s, 3H), 1.20 (s, 3H), 1.30 (t, $J$ = 7.3 Hz, 1.5H), 1.31 (t, $J$ = 7.3 Hz, 1.5H), 1.04−1.38 (m, 9H), 1.47−1.66 (m, 1H), 3.07−3.25 (m, 2H), 3.89−4.01 (m, 1H), 4.18−4.29 (m, 2H), 4.76−4.91 (m, 1H), 6.06 (d, $J$ = 8.3 Hz, 0.5H, NH), 6.17 (d, $J$ = 8.3 Hz, 0.5H, NH), 7.07−7.34 (m, 5H), 7.50−7.75 (m, 3H); MS (ESI) $m/z$ 517 $(M + H)^+$. Anal. ($C_{29}H_{38}F_2N_2O_4 \cdot 0.5H_2O$) C, H, N.

Compounds **17**, **19**, and **20** were prepared according to procedure D described for the preparation of **18** starting from **21**. In these preparations, 3,4-dichlorobenzoyl chloride, 2-furoyl chloride, and 2-fluoro-4-cyanobenzoic acid were used instead of 3,4-difluorobenzoic acid, respectively.

***N*-[2,2-Dimethyl-3-(*N*-(3,4-dichlorobenzoyl)amino)-nonanoyl]-L-phenylalanine ethyl ester (17):** 96% yield from **21**; colorless oil; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.83 (t, $J$ = 6.8 Hz, 1.5H), 0.85 (t, $J$ = 6.8 Hz, 1.5H), 1.17 (s, 3H), 1.19 (s, 3H), 1.30 (t, $J$ = 7.3 Hz, 1.5H), 1.32 (t, $J$ = 7.3 Hz, 1.5H), 1.05−1.38 (m, 9H), 1.50−1.67 (m, 1H), 3.07−3.25 (m, 2H), 3.90−4.02 (m, 1H), 4.18−4.31 (m, 2H), 4.78−4.91 (m, 1H), 6.03 (d, $J$ = 8.3 Hz, 0.5H, NH), 6.16 (d, $J$ = 8.3 Hz, 0.5H, NH), 7.06−7.33 (m, 5H), 7.47 (d, $J$ = 8.3 Hz, 0.5H), 7.51 (d, $J$ = 8.3 Hz, 0.5H), 7.57−7.66 (m, 2H), 7.73 (d, $J$ = 9.8 Hz, 0.5H, NH), 7.90 (d, $J$ = 2.4 Hz, 0.5H), 7.95 (d, $J$ = 2.4 Hz, 0.5H); MS (ESI) $m/z$ 549 $(M + H)^+$.

***N*-[2,2-Dimethyl-3-(*N*-(2-fluoro-4-cyanobenzoyl)amino)nonanoyl]-L-phenylalanine ethyl ester (19):** 60% yield from **21**; colorless oil; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.86 (br t, $J$ = 6.8 Hz, 3H), 1.19−1.40 (m, 15H), 1.27 (br t, $J$ = 7.3 Hz, 3H), 1.42−1.60 (m, 1H), 3.04−3.22 (m, 2H), 3.94−4.06 (m, 1H), 4.19 (q, $J$ = 7.3 Hz, 2H), 4.80−4.91 (m, 1H), 6.11 (d, $J$ = 8.3 Hz, 0.5H, NH), 6.20 (d, $J$ = 7.8 Hz, 0.5H, NH), 7.07−7.15 (m, 2H), 7.22−7.32 (m, 3H), 7.37−7.45 (m, 1H), 7.50−7.59 (m, 1H), 7.95−8.20 (m, 2H); MS (ESI) $m/z$ 524 $(M + H)^+$, 546 $(M + Na)^+$. Anal. ($C_{30}H_{38}FN_3O_4 \cdot 0.5H_2O$) C, H, N.

***N*-[2,2-Dimethyl-3-(*N*-(2-furoyl)amino)nonanoyl]-L-phenylalanine ethyl ester (20):** 51% yield from **21**; pale-yellow oil; $^1$H NMR (270 MHz, $CDCl_3$) $\delta$ 0.83 (br t, $J$ = 6.8 Hz, 1.5H), 0.85 (br t, $J$ = 6.8 Hz, 1.5H), 1.19 (br s, 3H), 1.22 (br s, 3H), 1.28 (br t, $J$ = 7.3 Hz, 3H), 1.06−1.38 (m, 9H), 1.44−1.65 (m, 1H), 3.04−3.25 (m, 2H), 3.90−4.03 (m, 1H), 4.21 (q, $J$ = 7.3 Hz, 2H), 4.80−4.92 (m, 1H), 6.14 (d, $J$ = 7.8 Hz, 0.5H, NH), 6.20 (d, $J$ = 7.8 Hz, 0.5H, NH), 6.46−6.56 (m, 1H), 7.07−7.33 (m, 6H), 7.44−7.65 (m, 2H); MS (ESI) $m/z$ 472 $(M + H)^+$, 493 $(M + Na)^+$. Anal. ($C_{27}H_{38}N_2O_5 \cdot 0.25H_2O$) C, H, N.

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**Supporting Information Available:** Figures 3 and 4 and $^1$H and $^{13}$C NMR spectral data for all synthetic intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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