Design and Synthesis of Hydroxyethylene-Based Peptidomimetic Inhibitors of Human β-Secretase

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The hydroxyethylene (HE) transition state isostere was developed as a scaffold to provide potent, small molecule inhibitors of human β-secretase (BACE). The previous work on the statine series proved critical to the discovery of HE structure−activity relationships. Compound **20** with the N-terminal isophthalamide proved to be the most potent HE inhibitor (IC_{50} = 30 nM) toward BACE. Unlike the statine series, we identified HE inhibitors without carboxylic acids on the C terminus, leading to enhanced cell penetration and making them attractive candidates for further drug development in Alzheimer's disease.

Introduction

Two proteases, β- and γ-secretase, are involved in the sequential proteolysis of membrane-anchored amyloid precursor protein (APP), which results in the generation of amyloid β (Aβ) peptide that is thought to be causal for the pathology and subsequent cognitive decline in Alzheimer's disease (AD).[1,2] Agents that inhibit Aβ production via inhibition of these proteolytic pathways would be useful in preventing Aβ plaque deposition in the brain and could be of therapeutic benefit in AD.[3]

β-Secretase (BACE) has been shown by us and others to be a membrane-bound aspartyl protease.[4−6] The cleavage of APP by BACE occurs in the lumina and is considered to be the rate-limiting step in the processing of APP to Aβ peptide. BACE cleavage of APP results in the production of the membrane-bound β-C-terminal fragment, which is then further cleaved to Aβ by γ-secretase. BACE knockout homozygote mice show a complete absence of Aβ production and have no reported side effects.[7] BACE, which is highly expressed in the central nervous system, is thus an attractive therapeutic target for the design of inhibitors of Aβ production. We have previously elaborated on the development of peptidic inhibitors,[8] as well as the synthesis of small molecule statine compounds exhibiting BACE mechanism specific inhibition.[9] In this paper, we describe the evolution of low molecular weight BACE inhibitors containing hydroxyethylene (HE) units as dipeptide isosteres.

Background

Numerous studies have indicated that the number of amide bonds is directly correlated with decreasing metabolic stability and oral bioavailability of compounds, particularly peptidomimetics, which limits their use as drug development candidates.[10] Our previous studies on BACE inhibitors demonstrated that potent, selective inhibitors could be designed, but the high peptidic nature of the compounds limited their utility for drug development. For a variety of reasons, we also decided to move away from the statinelike transition state mimic. Thus, we directed our interest toward other transition state isosteres, potentially exhibiting more promising properties such as increased solubility and enhanced oral bioavailability. Because the HE isostere contains one less secondary amide linkage than the statines, we selected this isostere as a starting point for evaluation for favorable pharmacokinetic properties (Figure 1).

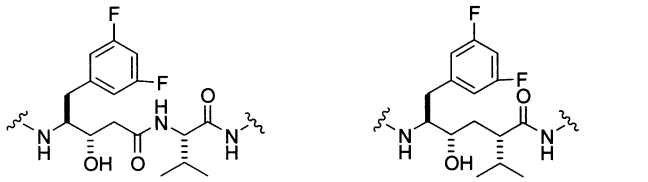

Figure 1. Comparison of statine and HE inhibitors.

Before embarking on small molecule HE peptidomimetics, we had already obtained data, which suggested that potent HEs could be developed. Replacing the statine moiety with the HE central core in the heptapeptide series resulted in a 10-fold increase in BACE inhibitory activity (Figure 2).[8] Tang and co-workers had reported potent HE inhibitors of BACE but containing many amino acid residues and no reported cell activities.[11] However, we needed BACE inhibitors with substantially fewer amino acid residues and lower molecular weights, such that the molecules would be likely to exhibit cellular permeability as well as oral bioavailability and metabolic stability required for drug candidates. We therefore embarked on the following study to produce BACE potent, small molecular weight HEs containing no α-amino acids.

We had noticed that the statine compound **2** was similar in activity to the analogous HE inhibitor **3**. This suggested that since the prime side of the HE molecule differed the most from the statines, the structure−

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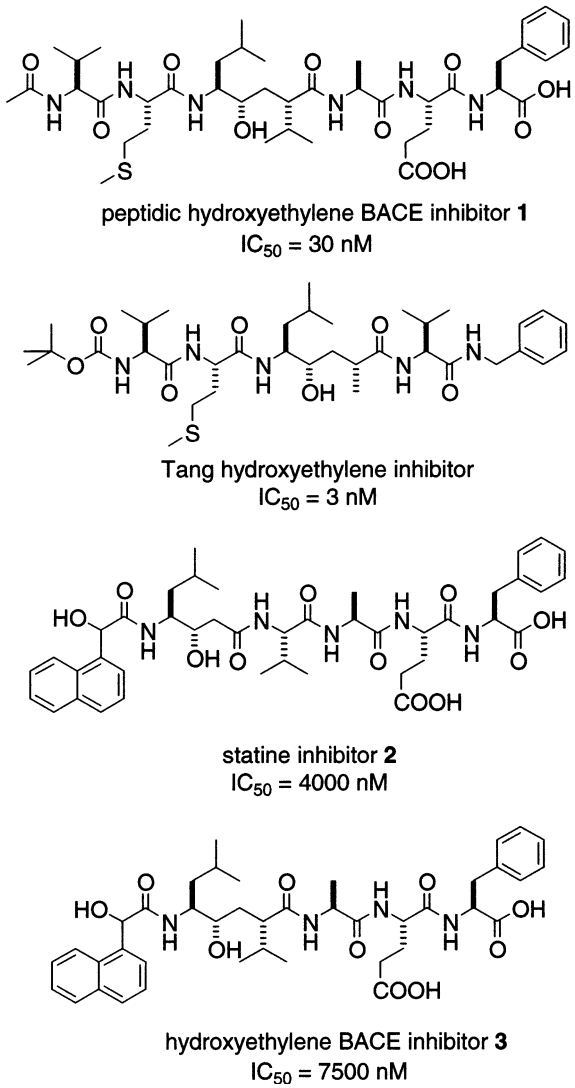


Figure 2. HE and statine BACE inhibitors.

activity relationships (SARs) of the HEs on the C-terminal end should diverge from that observed with the statines. However, the N-terminal SARs would likely be similar between series. On the basis of this hypothesis, we decided to embark on the synthesis of a variety of HEs, with the optimal N terminus discovered in the statine series, differing at the C terminus and at the P_1' substituent. The isophthalamide was found to be an optimal N terminus in the statine series (see below). This group was discovered through application of known asparagine replacements to the P_2-P_3 region of the molecule.[12]

Molecular Modeling

Additional aid in the design of the HEs was provided by molecular modeling. Figure 3 shows a molecular model comparison of HE-based compound **1** to the corresponding statine inhibitor. Models of inhibitors bound to the BACE active site were constructed using the Monte Carlo multiple minimum conformational searching method as implemented in the BatchMin program.[13] Binding modes found using this method were generally similar to that of the inhibitor OM99-2, reported by Tang et al.[14] Models of HE- and statine-based peptidic inhibitors showed nearly identical binding modes for the N-terminal residues. The HE dipeptide isostere, containing the same number of atoms in the backbone chain as a natural dipeptide, permits substratelike registration in the enzyme binding site, with all peptidic amide groups forming hydrogen bonds to the enzyme. The statine dipeptide isostere backbone, however, is shorter by one atom, causing a misregistration of the C-terminal residues. In our models, this led to divergent conformations between the two inhibitor series at the C terminus and an inability of the statines to fill the S_1' subsite.

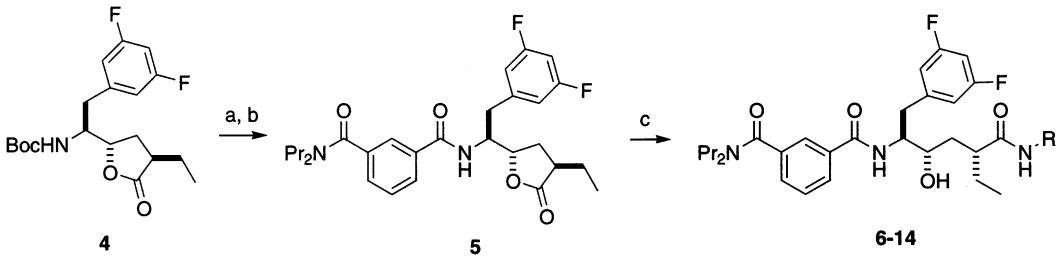
Synthesis

The HE peptide isosteres were synthesized from commercially available Boc-3, 5-difluorophenylalanine (Synthetech), using an amino alcohol-derived chiral auxiliary.[15] An example of the synthetic sequence is illustrated in Scheme 1 with an ethyl P_1' substituent. The key intermediates, such as compound **4**, were readily generated by methods previously described.[15a] Removal of the Boc group and amide coupling to the isophthalamide N terminus afforded intermediate **5**. Finally, the lactone **5** was ring-opened using trimethylaluminum to introduce chemical diversity in the inhibitors **6**−**14**.[16]

Results and Discussion

The C-terminal SARs of the HE compounds are summarized in Table 1. Because carboxylic acid functions proved to be vital for potency in the statine series,[9] various acid linkers were attached. The two carbon-linked COOH in inhibitor **6** exhibited a moderate 2800 nM BACE enzyme inhibitory activity. With the additional methylene spacer found in compound **7**, the activity increased approximately 10-fold to give a 310 nM inhibitor. Further increasing the length of the spacer (compounds **8**−**10**) essentially did not alter the activity of the inhibitor. Restricting the alkyl chain within a ring (compound **11**) led to a 4-fold activity

Scheme 1[a]



[a] CF_3COOH, room temperature. [b]EDC, HOBt, Et_3N, *N*,*N*-dipropylisophthalamic acid, 91%, two steps. [c]$AlMe_3$, RNH_2, 1,2-dichloroethane, reflux.

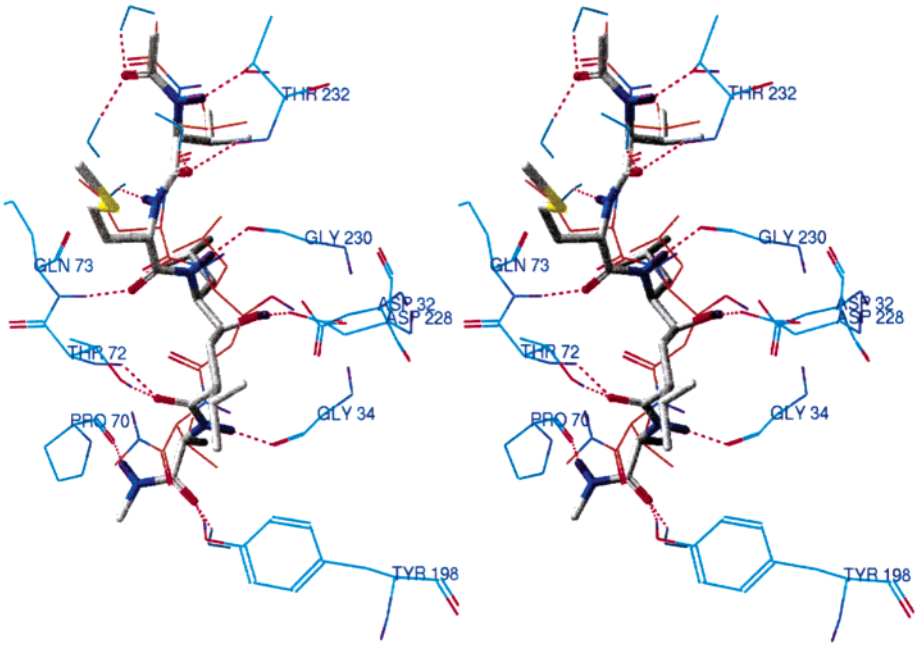
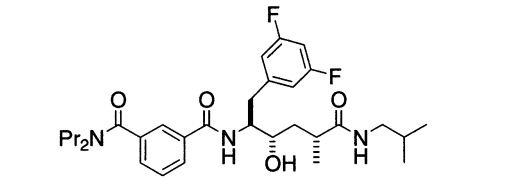
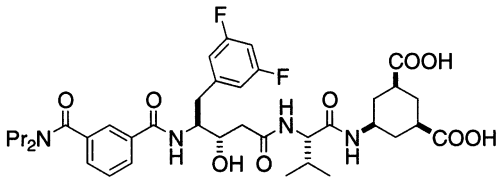


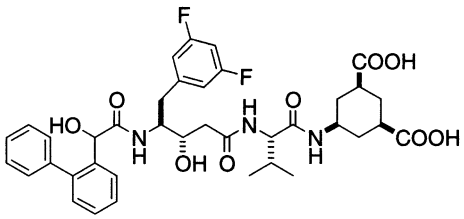
Figure 3. Modeled structure of HE-containing compound **1** (white) bound to BACE. The analogous statine-based inhibitor is shown for comparison (orange). Hydrogen bonds to the inhibitor are indicated by red dashes. The two C-terminal residues of both inhibitors are omitted from the figure. Conformations of the two inhibitors are nearly identical on the N-terminal side of the transition state isosteres but diverge on the C-terminal side. The valinelike side chain of compound **1** fills the S_1' pocket, but the positioning of the first statine prime side amide prevents similar branching into S_1'.



20
BACE IC_{50} = 30 nM
HEK-293 EC_{50} = 3000 nM



21
BACE IC_{50} = 1 nM
HEK-293 EC_{50} = 1000 nM



22
BACE IC_{50} = 20 nM
HEK-293 EC_{50} > 10000 nM

Figure 4. Comparison of HE and statine inhibitors.

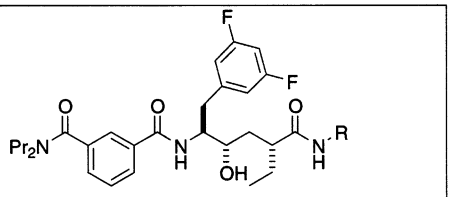
increase as compared with the straight chain (inhibitor **10**) analogue. The formation of the methyl ester (compound **12**) led to little or no loss in activity, in contrast to the approximately 10-fold loss in activity in the statine BACE inhibitors.[9] These data suggest that the carboxylic acid interacts via hydrogen bonding to BACE at the C-terminal end, since the neutral ester was equally active. Brief attempts at replacing the alkyl carboxylates with an ether (compound **13**) or an aryl (compound **14**) C terminus resulted in loss of activity.

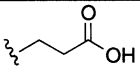
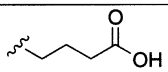
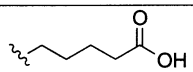
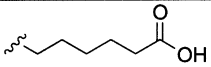
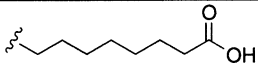
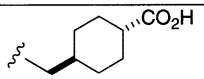
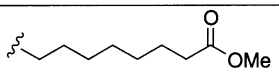
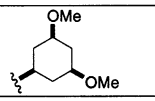
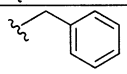
Analogues of compound **11** were then synthesized and allowed for optimization of the P_1' substituent. Alterations at the P_1' substituent led to a SAR consistent with that found in the substrate data (Table 2). The smaller Ala analogue (compound **15**) was consistently the most potent in the BACE inhibition assay. Successively larger substituents (compounds **16**, **18**, and **19**) eroded activity in a systematic fashion. The P_1' Gly analogue (inhibitor **17**) also demonstrated an approximate 10-fold loss in activity as compared with **15**, perhaps due to a loss of conformational preference toward the active form. In contrast to the statines,[9] where Val was optimal in P_1', the most potent HEs contained small P_1' residues.

The lack of a potency difference between the ester and the acids (Table 1) was striking, especially considering the large difference observed in ester and acid forms of statine analogues.[9] This suggested that unlike the statines, the HEs would tolerate nonpolar C termini. Analogue **20** represents the best example of these, where the C-terminal isobutyl substituent is comparable in potency to the carboxylate C-terminal analogue **15**, without the concomitant difficulty in cell permeability usually exhibited by carboxylic acids (Figure 4).[17]

Comparison of the most potent HE inhibitor to the most potent statine inhibitor showed that the HEs were more cell permeable than the statines (Figure 4). Although its inherent enzyme potency is poorer than the best statine analogue, analogue **20** did, in fact, demonstrate a ratio of cell to enzyme activity of 100 (10 times better than two of the most potent statine compounds **21** and **22**). Compound **20** was the culmination of improvements over our initial inhibitor **1** as well

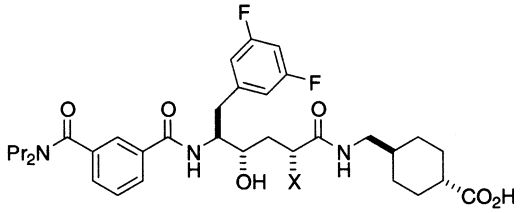
Table 1. C-Terminal SAR of HE BACE Inhibitors



Compound	R	IC_{50} (nM)[a]
6		2800
7		310
8		400
9		240
10		200
11		50
12		410
13		6600
14		1400

[a] Concentration necessary to inhibit 50% of enzyme activity in the MBP-C125 assay;[4] average of two or more runs. Standard deviation of assays is ±5%.

Table 2. P_1' SAR of HE BACE Inhibitors



compd	X	IC_{50} (nM)[a]
11	Et	50
15	Me	26
16	Pr	45
17	H	400
18	*i*-Bu	310
19	Bn	27 000

[a] Concentration necessary to inhibit 50% of enzyme activity in the MBP-C125 assay;[4] average of two or more runs. Standard deviation of assays is ±5%.

as over the Tang inhibitors, all of which were high molecular weight (>700 amu) compounds containing natural amino acid residues and subject to low cell permeability and low metabolic stability.

Conclusion

In our goal to develop Alzheimer's drug candidates through BACE inhibition, we investigated the HE transition state isostere as a scaffold to provide potent, small molecule inhibitors of BACE. With the N-terminal isophthalamide similar to those discovered in the statine series, compound **20** proved to be among the most potent (IC_{50} = 30 nM) toward BACE. Unlike the statine series, nonpolar C termini were tolerated, resulting in inhibitors that were more cell permeable and contained no amino acid residues.

Experimental Section

General. Compounds **1**, **2**, and **22** have been previously described.[8,9] All reagents were obtained from Aldrich, and all solvents were obtained from VWR, unless otherwise indicated. Anhydrous solvents (e.g., tetrahydrofuran (THF), dimethyl formamide (DMF), and dichloromethane (DCM)) were used as received.

Reaction progress was monitored with analytical thin-layer chromatography plates on 0.25 mm Merck F-254 silica gel glass plates. Visualization was achieved using phosphomolybdic acid, potassium permanganate, or ninhydrin spray reagents or UV illumination.

^1H and ^{13}C NMR spectra were obtained at 300 and 75 MHz, respectively, on a Varian or Bruker spectrometer and are reported in parts per million downfield relative to tetramethylsilane or to proton resonances resulting from incomplete deuteration of the NMR solvent (δ scale). Mass spectra were obtained at the University of California at Berkeley, using chemical ionization. Elemental analyses were performed at the University of California at Berkeley or at Desert Analytics, Tucson, Arizona.

Where noted, compounds were determined to be >95% pure by high-performance liquid chromatography (HPLC). Purity of compounds were determined on a Phenomenex Luna C18-(2), 3 μm column, 4.6 mm i.d. × 30 mm length, with [J] 20−70% acetonitrile/water/0.1% trifluoroacetic acid or [K] 50−95% acetonitrile/water/0.1% trifluoroacetic acid mobile phase, 1.5 mL/min elution at 35 °C at 210, 254, or 280 nm wavelength.

MBP-C125 BACE Enzyme Assay. β-Cleavage ELISA assays were carried out in 200 mM sodium acetate, pH 4.8, 0.06% Triton X-100, with 10 μg/mL-1 MBPAPPC125 (or MBP-C125). MBP-C125 is a fusion protein containing the maltose-binding protein at the amino-terminal end connected to the carboxyl-terminal 125 amino acids of APP at the carboxyl-terminal end. Reaction mixtures were incubated at 37 °C for 1−2 h, and the quenched reaction mixtures were then loaded onto 96 well plates coated with a polyclonal antibody raised to MBP. Generated β-cleaved product was detected using biotinylated Sw192 or biotinyated Wt192 as specific reporter antibodies and quantitated against the appropriate MBP-C26 standard.

Peptide Synthesis. The peptide inhibitors were generated in a peptide synthesizer using Boc-protected amino acids for chain assembly. All chemicals, reagents, and Boc amino acids were purchased from Applied Biosystems (ABI; Foster City, CA) with the exception of DCM and DMF, which were from Burdick and Jackson, and Boc-statine, which was purchased from Neosystem. The starting resin, Boc-Phe-OCH_2-Pam resin, was also purchased from ABI. All amino acids were coupled following preactivation to the corresponding 1-hydroxybenzotriazole (HOBT) ester using 1.0 equiv of HOBT and 1.0 equiv of *N*,*N*-dicyclohexylcarbodiimide in DMF. The Boc protecting group on the amino acid α-amine was removed with 50% trifluoroacetic acid in DCM after each coupling step and prior to hydrogen fluoride (HF) cleavage. Amino acid side chain protection was as follows: Glu(Bzl). All other amino acids were used with no further side chain protection including Boc-Statine (Bzl, benzyl; CBZ, carbobenzyloxy; Cl-CBZ, chlorocarbobenzyloxy; OBzl, O-benzyl). The side chain-protected peptide resin was deprotected and cleaved from the resin by reacting

with anhydrous HF at 0 °C for 1 h. This generated the fully deprotected crude peptide as a C-terminal carboxylic acid.

HPLC Purification of Peptides. Following HF treatment, the peptide was extracted from the resin in acetic acid and lyophilized. The crude peptide was then purified using reverse phase HPLC on a Phenomenex Luna C18(2), 5 μm column 4.6 mm i.d. × 25 cm in length [P] or a Vydac C18, 10 μm column 4.6 mm i.d. × 25 cm in length [V]. The solvent systems used with these columns were [A] = 0.1% trifluoroacetic acid/H_2O and [B] = 0.1% trifluoroacetic acid/acetonitrile as the mobile phase. The gradient conditions were as follows: (i) 20−50% [B] in 30 min at 1 mL/min; (ii) 30% [B] hold 5 min, 30−60% [B] in 30 min at 1 mL/min; and (iii) 35% [B] hold 5 min; 35−65% [B] in 30 min at 1 mL/min. The purified peptide was subjected to mass spectrometry and analytical reverse phase HPLC to confirm its composition and purity.

Compound 3. A 1:1 mixture of diastereomers. Gradient conditions (P, 1): R_f = 19.4, 21.0. MNa^+(CI) 786.8. HPLC retention time = 2.787 [J], 1.432 [K].

***N*-[2-(*S*)-(3,5-Difluorophenyl)-1-(*R*)-(4-ethyl-5-oxo-THF-2-yl)ethyl]-*N*′,*N*′-dipropylisophthalamide (5).** R_f = 0.1 (2% MeOH/CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 7.93−7.79 (m, 2 H), 7.55−7.40 (m, 3 H), 6.76 (dd, J = 8.0, 2.0 Hz, 2 H), 6.64 (tt, J = 9.0, 2.2 Hz, 1 H), 4.64 (q, J = 8.4 Hz, 1 H), 4.63−4.48 (m, 1 H), 3.70−3.40 (m, 2 H), 3.11 (t, J = 7.1 Hz, 2 H), 2.96 (dd, J = 13.8, 8.4 Hz, 1 H), 2.87 (dd, J = 13.8, 7.4 Hz, 1 H), 2.52−2.28 (m, 2 H), 2.12−1.95 (m, 1 H), 1.95−1.40 (m, 8 H), 0.95 (t, J = 7.4 Hz, 6 H), 0.72 (t, J = 7.0 Hz, 3 H). MH^+ (CI): 500.8. Anal. ($C_{28}H_{34}F_2N_2O_4$·0.5H_2O) C, H, N.

3-[6-(3,5-Difluorophenyl)-5-(*S*)-(3-dipropylcarbamoyl-benzoylamino)-2-(*R*)-ethyl-4-(*S*)-hydroxyhexanoylamino]-propionic Acid (6). R_f = 0.23 (10% MeOH/CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 7.85−7.60 (m, 3H), 7.52−7.40 (m, 2H), 7.30−7.10 (m, 2H), 6.83 (app d, J = 6.5 Hz, 2H), 6.61 (tt, J = 9.0, 2.2 Hz, 1H), 4.30−4.10 (m, 1H), 3.70−3.22 (m, 5H), 3.22−3.10 (m, 2H), 3.10−2.80 (m, 2H), 2.50−2.20 (m, 3H), 1.80−1.30 (m, 7H), 0.99 (t, J = 7.3 Hz, 3H), 0.84 (t, J = 7.3 Hz, 3H), 0.73 (t, J = 7.3 Hz, 3H). MH^+ (CI): 590.3. HPLC retention time = 2.392 [J], 0.685 [K].

4-[6-(3,5-Difluorophenyl)-5-(*S*)-(3-dipropylcarbamoyl-benzoylamino)-2-(*R*)-ethyl-4-(*S*)-hydroxyhexanoylamino]-butyric Acid (7). R_f = 0.075 (5% MeOH/CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 7.90−7.70 (m, 2 H), 7.55−7.25 (m, 3 H), 7.10−6.75 (m, 1 H), 6.81 (d, J = 6.3 Hz, 2 H), 6.59 (t, J = 9.0 Hz, 1 H), 4.80 (br s, 1 H, OH), 4.24 (app d, J = 6.6 Hz, 1 H), 3.71 (d, J = 8.6 Hz, 1 H), 3.60−3.40 (m, 2 H), 3.40−3.20 (m, 1 H), 3.13 (t, J = 7.0 Hz, 2 H), 3.10−2.85 (m, 3 H), 2.45−2.25 (m, 1 H), 2.25−2.10 (m, 2 H), 2.00−1.80 (m, 1 H), 1.90−1.10 (m, 10 H), 0.97 (t, J = 7.2 Hz, 3 H), 0.82 (t, J = 7.1 Hz, 3 H), 0.72 (t, J = 7.2 Hz, 3 H). MH^+ (CI): 603.7. HPLC retention time = 2.406 [J], 0.716 [K].

5-[6-(3,5-Difluorophenyl)-5-(*S*)-(3-dipropylcarbamoyl-benzoylamino)-2-(*R*)-ethyl-4-(*S*)-hydroxyhexanoylamino]-pentanoic Acid (8). ^{1}H NMR ($CDCl_3$): δ 7.90−7.70 (m, 2 H), 7.55−7.30 (m, 3 H), 6.82 (d, J = 6.1 Hz, 2 H), 6.59 (tt, J = 9.0, 2.1 Hz, 1 H), 4.90 (br s, 1 H, OH), 4.28 (tt, J = 12.2, 7.1 Hz, 1 H), 3.80−2.80 (m, 9 H), 2.36 (t, J = 6.4 Hz, 2 H), 2.30−2.00 (m, 2 H), 2.00−1.20 (m, 12 H), 0.98 (t, J = 7.3 Hz, 3 H), 0.87 (t, J = 7.1 Hz, 3 H), 0.73 (t, J = 7.2 Hz, 3 H). MH^+ (CI): 618.3. HPLC retention time = 2.460 [J], 0.751 [K].

6-[6-(3,5-Difluorophenyl)-5-(*S*)-(3-dipropylcarbamoyl-benzoylamino)-2-(*R*)-ethyl-4-(*S*)-hydroxyhexanoylamino]-hexanoic Acid (9). R_f = 0.15 (5% MeOH/CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 7.90−7.65 (m, 2 H), 7.55−7.30 (m, 2 H), 6.81 (app d, J = 6.3 Hz, 2 H), 6.60 (tt, J = 9.0, 2.2 Hz, 1 H), 6.20−5.90 (m, 1 H), 4.35−4.10 (m, 1 H), 3.80−3.40 (m, 4 H), 3.30−2.80 (m, 5 H), 2.60−2.30 (m, 2 H), 2.20 (t, J = 6.3 Hz, 2 H), 2.00−1.80 (m, 1 H), 1.80−1.10 (m, 14 H), 0.98 (t, J = 7.3 Hz, 3 H), 0.88 (t, J = 7.2 Hz, 3 H), 0.73 (t, J = 7.2 Hz, 3 H). ^{13}C NMR ($CDCl_3$): δ 176.7, 176.2, 171.6, 167.7, 162.8 (dd, J = 48.0, 12.8 Hz, 1 C), 142.2 (t, J = 9.2 Hz, 1 C), 136.3, 135.0, 128.9, 128.7, 128.5, 125.8, 112.5−112.0 (m, 1 C), 101.8 (t, J = 25.2 Hz, 1 C), 69.2, 55.8, 50.9 46.8, 45.6, 38.9, 37.5, 37.3, 33.8, 28.6, 26.0, 25.4, 24.1, 21.8, 20.6, 12.1, 11.4, 10.9. MH^+ (CI): 632.2. HPLC retention time = 2.525 [J], 1.076 [K].

8-[6-(3,5-Difluorophenyl)-5-(*S*)-(3-dipropylcarbamoyl-benzoylamino)-2-(*R*)-ethyl-4-(*S*)-hydroxyhexanoylamino]-octanoic Acid (10). R_f = 0.4 (10% MeOH/CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 7.90−7.65 (m, 2 H), 7.55−7.30 (m, 3 H), 6.83 (d, J = 6.1 Hz, 2 H), 6.61 (tt, J = 9.0, 2.2 Hz, 1 H), 6.39 (t, J = 5.5 Hz, 1 H), 5.60 (br s, 1 H), 4.19 (dd, J = 5.2, 3.0 Hz, 1 H), 3.69 (dt, J = 9.7, 3.3 Hz, 1 H), 3.60−3.25 (m, 3 H), 3.25−3.00 (m, 3 H), 3.00 (d, J = 7.4 Hz, 2 H), 2.45−2.25 (m, 1 H), 2.25 (t, J = 7.2 Hz, 2 H), 1.80−1.20 (m, 19 H), 0.98 (t, J = 7.2 Hz, 3 H), 0.85 (t, J = 7.1 Hz, 3 H), 0.72 (t, J = 7.2 Hz, 3 H). ^{13}C NMR ($CDCl_3$): δ 177.1, 176.3, 171.4, 167.9, 162.9 (dd, J = 248, 13 Hz, 2 C), 142.5 (t, J = 9.1 Hz, 1 C), 136.6, 134.7, 129.1, 128.6, 128.4, 125.7, 112.2 (dd, J = 16.8, 7.3 Hz, 2 C), 101.8 (t, J = 25 Hz, 1 C), 69.4, 56.0, 50.9, 46.7, 45.5, 39.1, 38.9, 37.7, 37.4, 34.0, 29.1, 28.6, 28.3, 26.0, 25.2, 24.3, 21.87, 20.6, 12.2, 11.4, 10.9. MH^+ (CI): 660.4. HPLC retention time = 2.707 [J], 1.324 [K].

4-{[6-(3,5-Difluoro-phenyl)-5-(*S*)-(3-dipropylcarbamoyl-benzoylamino)-2-(*R*)-ethyl-4-(*S*)-hydroxy-hexanoylamino]-methyl}cyclohexanecarboxylic Acid (11). R_f = 0.3 (10% MeOH/CH_2Cl_2). ^{1}H NMR (CD_3OD): δ 8.16 (d, J = 9.1 Hz, 0.2 H), 7.95 (t, J = 5.7 Hz, 0.8 H), 7.90−7.75 (m, 1 H), 7.68 (s, 1 H), 7.60−7.45 (m, 2 H), 6.89 (dd, J = 8.4, 2.1 Hz, 2 H), 6.72 (tt, J = 10.3, 2.2 Hz, 1 H), 4.40−4.22 (m, 1 H), 3.64 (dt, J = 9.9, 2.9 Hz, 1 H), 3.47 (t, J = 7.2 Hz, 2 H) 3.17 (t, J = 7.0 Hz, 2 H), 3.10−2.80 (m, 4 H), 2.44 (sep, J = 4.7 Hz, 1 H), 2.30−2.10 (m, 1 H), 1.96 (d, J = 11.5 Hz, 2 H), 1.90−1.20 (m, 14 H), 1.10−0.80 (m, 3 H), 0.99 (t, J = 7.3 Hz, 3 H), 0.89 (t, J = 7.3 Hz, 3 H), 0.70 (t, J = 7.3 Hz, 3 H). ^{13}C NMR (CD_3OD): δ 179.9, 178.2, 173.1, 169.4, 164.3 (dd, J = 246.7, 13.0 Hz, 2 C), 144.9 (t, J = 9.2 Hz, 1 C), 138.5, 136.1, 130.5, 130.0, 129.3, 126.3, 113.1 (dd, J = 17.0, 7.5 Hz, 2 C), 102.5 (t, J = 25.8 Hz, 1 C), 71.4, 57.1, 52.2, 48.0, 46.4, 44.5, 38.6, 37.7, 37.5, 31.1, 29.9, 27.4, 22.9, 21.7, 12.4, 11.7, 11.3. MH^+ (CI): 658.4. HPLC retention time = 2.560 [J], 0.897 [K].

8-[6-(3,5-Difluoro-phenyl)-5-(*S*)-(3-dipropylcarbamoyl-benzoylamino)-2-(*R*)-ethyl-4-(*S*)-hydroxy-hexanoylamino]octanoic Acid Methyl Ester (12). R_f = 0.4 (5% ^{i}PrOH/$CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.85−7.65 (m, 2 H), 7.55−7.35 (m, 2 H), 6.89 (d, J = 9.0 Hz, 1 H), 6.80 (dd, J = 8.1, 2.0 Hz, 2 H), 6.63 (tt, J = 9.0, 2.3 Hz, 1 H), 5.98 (t, J = 5.6 Hz, 1 H), 5.19 (s, 1 H), 4.17 (q, J = 7.6 Hz, 1 H), 3.85−3.70 (m, 1 H), 3.66 (s, 3 H), 3.46 (t, J = 7.1 Hz, 2 H), 3.40−2.90 (m, 6 H), 2.40−2.20 (m, 1 H), 2.29 (t, J = 7.5 Hz, 2 H), 1.90−1.15 (m, 18 H), 0.99 (t, J = 7.0 Hz, 3 H), 0.86 (t, J = 7.3 Hz, 3 H), 0.74 (t, J = 6.9 Hz, 3 H). ^{13}C NMR ($CDCl_3$): δ 176.5, 174.3, 170.9, 167.0, 162.9 (dd, J = 248.2, 12.9 Hz, 2 C), 142.4 (t, J = 9.1 Hz, 1 C), 137.6, 134.7, 129.3, 127.8, 127.6, 125.1, 112.1 (dd, J = 16.8, 7.5 Hz, 2 C), 101.9 (t, J = 25.1 Hz, 1 C), 67.6, 55.6, 51.5, 50.8, 46.5, 46.0, 39.5, 38.3, 36.6, 34.0, 29.3, 28.9, 28.7, 26.5, 24.72, 24.69, 21.9, 20.7, 12.2, 11.4, 11.0. MH^+ (CI) 674.4. HPLC retention time = 3.052 [J], 1.815 [K].

(1*S*,2*S*,4*R*)-*N*-[1-(3,5-Difluorobenzyl)-4-(*syn*,*syn*)-(3,5-dimethoxycyclohexylcarbamoyl)-2-hydroxyhexyl]-*N*′,*N*′-dipropylisophthalamide (13). R_f = 0.15 (5% ^{i}PrOH/$CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 7.85−7.75 (m, 1 H), 7.71 (s, 1 H), 7.55−7.40 (m, 2 H), 6.84 (d, J = 9.0 Hz, 1 H), 6.75 (app d, J = 6.2 Hz, 1 H), 6.62 (tt, J = 9.0, 2.2 Hz, 1 H), 6.43 (d, J = 8.4 Hz, 1 H), 5.00 (d, J = 3.6 Hz, 1 H), 4.17 (q, J = 8.2 Hz, 1 H), 4.00−3.80 (m, 1 H), 3.80−3.60 (m, 1 H), 3.60−3.40 (m, 2 H), 3.40−3.20 (m, 2 H), 3.34 (s, 6 H), 3.20−3.05 (m, 2 H), 3.05−2.85 (m, 2 H), 2.40−1.95 (m, 4 H), 1.85−1.50 (m, 8 H), 1.50−1.30 (m, 2 H), 1.30−1.10 (m, 2 H), 0.99 (t, J = 7.0 Hz, 3 H), 0.86 (t, J = 7.3 Hz, 3 H), 0.75 (t, J = 6.9 Hz, 3 H). MH^+ (CI): 660.4. Anal. ($C_{36}H_{51}F_2N_3O_6$) C, H, N.

(1*S*,2*S*,4*R*)-*N*-[4-Benzylcarbamoyl-1-(3,5-difluorobenzyl)-2-hydroxy-hexyl]-*N*′,*N*′-dipropyl-isophthalamide (14). R_f = 0.3 (5% MeOH/CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ 7.75−7.60 (m, 2 H), 7.45−7.12 (m, 7 H), 7.08 (d, J = 9.0 Hz, 1 H), 6.85−6.65 (m, 3 H), 6.61 (tt, J = 9.0, 2.2 Hz, 1 H), 5.10 (br s, 1 H), 4.44 (dd, J = 14.7, 6.1 Hz, 1 H), 4.30−4.10 (m, 1 H), 4.19 (dd, J = 14.7, 5.1 Hz, 1 H), 3.67 (br s, 1 H), 3.37 (t, J = 7.3 Hz, 2 H), 3.10 (t, J = 7.3 Hz, 2 H), 2.97 (dd, J = 13.6, 7.7 Hz, 1 H), 2.88

(dd, J = 13.6, 7.4 Hz, 1 H), 2.40−2.20 (m, 1 H), 1.80−1.20 (m, 8 H), 0.95 (t, J = 7.1 Hz, 3 H), 0.83 (t, J = 7.4 Hz, 3 H), 0.71 (t, J = 7.1 Hz, 3 H). ^{13}C NMR ($CDCl_3$): δ 176.2, 171.1, 167.0, 162.8 (dd, J = 248.0, 12.8 Hz, 2 C), 142.4 (t, J = 9.1 Hz, 1 C), 138.1, 137.1, 134.8, 129.1, 128.8, 128.6, 128.0, 127.7, 127.4, 125.0, 112.1 (dd, J = 16.6, 7.5 Hz, 2 C), 101.8 (t, J = 25.1 Hz, 1 C), 68.1, 55.3, 50.8, 46.6, 45.4, 43.5, 38.1, 37.2, 25.1, 21.8, 20.6, 12.1, 11.4, 10.9. MH^+ (CI): 608.3. Anal. ($C_{35}H_{43}F_2N_3O_4$) C, H, N.

4-(*anti*)-{[6-(3,5-Difluoro-phenyl)-5-(*S*)-(3-dipropylcarbamoyl-benzoylamino)-4-(*S*)-hydroxy-2-(*R*)-methyl-hexanoylamino]methyl}cyclohexanecarboxylic Acid (15). ^{1}H NMR (CD_3OD): δ 8.32 (d, 1H), 7.9−7.6 (m, 3H), 3.5−3.4 (m, 1H), 6.9−6.6 (m, 3H), 4.54 (m, 1H), 3.78 (m, 1H), 3.50 (m, 2H), 3.20 (m, 2H), 3.1−2.9 (m, 4H), 2.6−2.1 (m, 2H), 2.0−1.3 (m, 4H), 1.3 (d, 3H), 0.99 (m, 3H), 0.78 (m, 3H). MNa^+ (CI): 666.4. Anal. ($C_{35}H_{47}F_2N_3O_6$) C, H, N.

4-(*anti*)-{[6-(3,5-Difluorophenyl)-5-(*S*)-(3-dipropylcarbamoyl-benzoylamino)-4-(*S*)-hydroxy-2-(*R*)-propyl-hexanoylamino]methyl}cyclohexanecarboxylic Acid (16). ^{1}H NMR (CD_3OD): δ 8.22 (d, 1H), 8.0−7.6 (m, 4H), 6.9−6.7 (m, 3H), 4.44 (m, 1H), 3.6−2.9 (m, 10H), 2.50 (m, 1H), 2.20 (m, 1H), 2.0−1.3 (m, 22H), 1.0−0.7 (m, 13H). MNa^+ (CI): 694.4. Anal. ($C_{37}H_{51}F_2N_3O_6 \cdot H_2O$) C, H, N.

4-(*anti*)-{[6-(3,5-Difluoro-phenyl)-5-(*S*)-(3-dipropylcarbamoyl-benzoylamino)-4-(*S*)-hydroxy-hexanoylamino]-methyl}cyclohexanecarboxylic Acid (17). ^{1}H NMR (CD_3OD): δ 7.98 (t, J = 5.7 Hz, 1H), 7.84 (dt, J = 7.1, 1.8 Hz, 1H), 7.70 (s, 1H), 7.60−7.45 (m, 2H), 6.92 (app dd, J = 8.3, 1.8 Hz, 2H), 6.74 (tt, J = 9.2, 2.2 Hz, 1H), 4.45−4.30 (m, 1H), 1.00 (t, J = 7.4 Hz, 3H), 0.71 (t, J = 7.3 Hz, 3H). ^{13}C NMR (CD_3OD): δ 180.0, 175.9, 173.1, 169.4, 164.3 (dd, J = 246.6, 13.1 Hz, 2C), 144.9 (t, J = 9.3 Hz, 1C), 138.5, 136.1, 130.5, 130.0, 129.3, 126.3, 113.2 (dd, J = 17.0, 7.5 Hz, 2C), 102.5 (t, J = 25.8 Hz, 1C), 72.9, 56.3, 52.3, 46.4, 44.5, 38.6, 38.1, 33.6, 31.3, 31.0, 29.9, 22.9, 21.7, 11.7, 11.3. MH^+ (CI): 686.3. Anal. ($C_{34}H_{45}F_2N_3O_6 \cdot 0.5H_2O$) C, H, N.

4-(*anti*)-{[6-(3,5-Difluoro-phenyl)-5-(*S*)-(3-dipropylcarbamoyl-benzoylamino)-4-(*S*)-hydroxy-2-(*R*)-isobutyl-hexanoylamino]methyl}cyclohexanecarboxylic Acid (18). ^{1}H NMR (CD_3OD): δ 7.97 (t, J = 5.7 Hz, 1H), 7.83 (dt, J = 7.1, 1.8 Hz, 1H), 7.70 (s, 1H), 7.70−7.55 (m, 2H), 6.90 (app dd, J = 8.3, 1.8 Hz, 2H), 6.74 (tt, J = 9.2, 2.2 Hz, 1H), 4.40−4.20 (m, 1H), 3.64 (tt, J = 9.4, 3.0 Hz, 1H), 3.48 (t, J = 7.5 Hz, 2H), 3.18 (t, J = 7.5 Hz, 2H), 3.20−2.85 (m, 4H), 2.63 (sept, J = 4.8 Hz, 1H), 2.18 (tt, J = 12.1, 3.4 Hz, 1H), 1.95 (d, J = 9.0 Hz, 2H), 1.85−1.65 (m, 5H), 1.65−1.25 (m, 9H), 1.20−1.10 (m, 1H), 1.00 (t, J = 7.4 Hz, 3H), 0.90 (dd, J = 11.4, 6.4 Hz, 6H), 0.72 (t, J = 7.3 Hz, 3H). ^{13}C NMR (CD_3OD): δ 180.0, 178.7, 173.3, 169.7, 163.8 (dd, J = 248.0, 12.8 Hz, 2 C), 145.0, 138.6, 130.6, 130.1, 129.4, 126.4, 113.1 (dd, J = 16.6, 7.5 Hz, 2 C), 102.6 (t, J = 25.1 Hz, 1 C), 71.1, 56.9, 52.2, 46.5, 44.4, 43.4, 42.7, 38.5, 38.3, 37.5, 31.0, 29.8, 27.2, 23.6, 22.8, 22.4, 21.7, 11.6, 11.2. MH^+ (CI): 630.3. Anal. ($C_{38}H_{53}F_2N_3O_6$) C, H, N.

4-(*anti*)-{[2-(*R*)-Benzyl-6-(3,5-difluorophenyl)-5-(*S*)-(3-dipropylcarbamoyl-benzoylamino)-4-(*S*)-hydroxy-hexanoylamino]methyl}cyclohexanecarboxylic Acid (19). ^{1}H NMR (CD_3OD): δ 8.20 (d, 1H), 7.7−7.6 (m, 6H), 7.4−7.2 (m, 6H), 6.9−6.7 (m, 3H), 4.4 (br s, 1H), 3.6 (m, 1H), 3.5−2.7 (m, 15H), 2.2−1.6 (m, 13H), 1.3−1.2 (m, 4H), 1.00 (m, 3H), 0.70 (m, 5H). ^{13}C NMR (CD_3OD): δ 180.1, 177.4, 177.33, 173.30, 169.7, 169.6, 166.25, 166.1, 163.0, 162.8, 145.0, 144.9, 141.0, 138.6, 136.3, 130.3, 130.3, 130.1, 129.5, 129.4, 127.5, 126.4, 113.4, 113.3, 113.2, 113.1, 102.9, 102.6, 102.2, 101.5, 71.3, 57.1, 57.1, 52.2, 48.0, 46.4, 46.3, 44.3, 40.5, 38.3, 37.6, 30.7, 30.6, 29.8, 29.7, 22.8, 21.7,11.0, 11.2. MNa^+ (CI): 743. Anal. ($C_{41}H_{51}F_2N_3O_6 \cdot 0.5H_2O$) C, H, N.

***N*-[1-(3,5-Difluorobenzyl)-2-hydroxy-4-isobutylcarbamoylpentyl]-*N*′,*N*′-dipropylisophthalamide (20).** ^{1}H NMR ($CDCl_3$): δ 7.80−7.65 (m, 2H), 7.50−7.35 (m, 2H), 6.87 (d, J = 9.1 Hz, 1H), 6.81 (app d, J = 9.1 Hz, 1H), 6.63 (tt, J = 9.0, 2.2 Hz, 1H), 5.98 (t, J = 5.9 Hz, 1H), 5.11 (d, J = 3.7 Hz, 1H), 4.20 (q, J = 7.9 Hz, 1H), 3.77 (app d, J = 9.2 Hz, 1H), 3.46 (t, J = 7.4 Hz, 2H), 3.25−3.08 (m, 2H), 3.08−2.90 (m, 4H), 2.65−2.45 (m, 1H), 1.80−1.60 (m, 7H), 1.60−1.40 (m, 3H), 1.13 (d, J = 7.1 Hz, 3H), 0.98 (t, J = 7.2 Hz, 3H), 0.85 (dd, J = 6.7, 2.0 Hz, 6H), 0.73 (t, J = 7.0 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 177.2, 170.8, 167.0, 162.9 (dd, J = 248.3, 12.9 Hz, 2C), 142.3 (t, J = 9.1 Hz, 1C), 137.6, 134.7, 129.4, 128.8, 127.6, 125.1, 112.1 (dd, J = 16.7, 7.4 Hz, 2C), 101.9 (t, J = 25.4 Hz, 1C), 67.6, 55.1, 50.7, 46.8, 46.5, 38.3, 38.2, 38.0, 28.4, 21.9, 20.7, 20.0, 17.0, 11.4, 11.0. MH^+ 560.3. Anal. ($C_{31}H_{43}F_2N_3O_4$) C, H, N.

5-{2-[5-(3,5-Difluorophenyl)-4-(3-dipropylcarbamoyl-benzoylamino)-3-hydroxypentanoylamino]-3-methylbutyryl-amino}cyclohexane-1,3-dicarboxylic Acid (21). ^{1}H NMR (CD_3OD): δ 8.2 (m, 1H, amide), 7.85 (d, 1H, aryl), 7.75 (s, 1H, aryl), 7.5 (m, 2H, aryl), 6.9 (d, 2H, aryl), 6.65 (m, 1H, aryl), 4.4 (m, 1H), 4.1 (m, 2H), 3.8 (m, 1H), 3.65 (m, 2H), 3.2 (m, 2H), 3.0 (m, 2H), 2.5 (m, 4H), 2.1 (m, 4H), 1.7 (m, 2H), 1.5 (m, 2H), 1.35 (m, 4H), 1.0 (m, 9H, CH_3 ×3), 0.7 (m, 3H, CH_3). ^{13}C NMR (CD_3OD): δ 172.0, 168.1, 167.3, 167.2, 163.5, 160.2, 160.1, 157.0, 156.8, 138.8, 132.5, 130.2, 124.6, 124.1, 123.6, 120.6, 107.4, 96.6, 64.8, 54.5, 50.1, 46.2, 46.3, 36.4, 36.3, 35.5, 32.0, 29.3, 29.2, 25.7, 25.6, 16.8, 15.7, 13.6, 12.4, 5.6, 5.2. MH^+ (CI): 745.2. Anal. ($C_{38}H_{52}F_2N_4O_{10} \cdot H_2O$) C, H, N.

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(17) The detailed protocol for the human embryonic kidney cellular assay can be found in ref 3.

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