Design, Synthesis, and Pharmacological Evaluation of Thapsigargin Analogues for Targeting Apoptosis to Prostatic Cancer Cells

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A series of thapsigargin (TG) analogues, containing an amino acid applicable for conjugation to a peptide specifically cleaved by prostate-specific antigen (PSA), has been prepared to develop the drug-moiety of prodrugs for treatment of prostatic cancer. The analogues were synthesized by converting TG into *O*-8-debutanoylthapsigargin (DBTG) and esterifying *O*-8 of DBTG with various amino acid linkers. The compounds were evaluated for their ability to elevate the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in TSU-Pr1 cells, their ability to inhibit the rabbit skeletal muscle SERCA pump, and their ability to induce apoptosis in TSU-Pr1 human prostatic cancer cells. The activity of analogues, in which DBTG were esterified with ω-amino acids [HOOC-$(CH_2)_nNH_2$, $n = 5-7$, 10, 11], increased with the linker length. Analogues with 3-[4-(L-leucinoylamino)phenyl]propanoyl, 6-(L-leucinoylamino)hexanoyl, and 12-(L-serinoylamino)dodecanoyl were considerably less active than TG, and analogues with 12-(L-alaninoylamino)dodecanoyl and 12-(L-phenylalaninoylamino)dodecanoyl were almost as active as TG. The 12-(L-leucinoylamino)dodecanoyl gave an analogue equipotent with TG, making this compound promising as the drug-moiety of a PSA sensitive prodrug of TG.

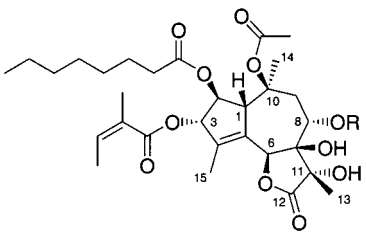
Introduction

Thapsigargin (TG, **1**) (Chart 1) is a sesquiterpene-γ-lactone isolated from seeds and roots of the umbelliferous plant, *Thapsia garganica* L.[1,2] TG selectively inhibits the ubiquitous sarcoplasmic and endoplasmic reticulum Ca^{2+}-dependent ATPases (SERCA's) with an apparent dissociation constant of 2.2 pM or less.[3,4] TG induced inhibition of the SERCA pump leads to depletion of the ER Ca^{2+} pool and a capacitance influx of extracellular Ca^{2+} resulting in a sustained elevation (i.e., 200−400 nM) of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$).[5] This sustained elevation of $[Ca^{2+}]_i$ subsequently leads to DNA fragmentation and programmed cell death (apoptosis) of treated cells.

TG induces apoptosis in rat (AT-3) and human (TSU-Pr1, PC-3, DU-145) androgen-independent prostatic cancer cell lines with LC_{50} values for cell death in the 10−100 nM range.[5] This TG induced apoptosis does not require the cells to be in proliferative cell cycle but can be induced in primary human prostatic cancer cell cultures in which about 98% of the cells are out of cycle in G_0.[6] These studies have identified the SERCA pump as a new therapeutic target for activating apoptosis of androgen-independent prostatic cancer cells.

TG's ability to kill proliferatively quiescent G_0 cells by inhibiting the ubiquitous SERCA's means that it will be difficult to administer TG systemically as a therapeutic agent without significant host toxicity. One approach to specifically target TG cytotoxicity to prostatic cancer cells is to take advantage of the unique secretion of prostate-specific antigen (PSA) by these cells. PSA is a serine protease with chymotrypsin-like substrate specificity that is enzymatically active only in the extracellular fluid of prostatic cancer cells, whereas it is enzymatically inactivated in blood serum.[7−9] Previously a highly specific and efficient PSA substrate with the sequence His−Ser−Ser−Lys−Leu−Gln-(HSSKLQ) was identified.[9] If TG is converted to *O*-8-debutanoylthapsigargin (DBTG, **2**) (Chart 1) and DBTG is esterified in the *O*-8 position with an amino carboxylic acid linker, the resulting TG analogue can be coupled to the C-terminal glutamine (Q) of this peptide to form a peptide bond that is hydrolyzable by enzymatically active PSA (Figure 1). Such a TG analogue prodrug will be cleaved only in the extracellular fluid of PSA-

Chart 1[a]



TG (**1**), R = $CO(CH_2)_2CH_3$

DBTG (**2**), R = H

[a] Structures of thapsigargin (TG, **1**) and *O*-8-debutanoylthapsigargin (DBTG, **2**).

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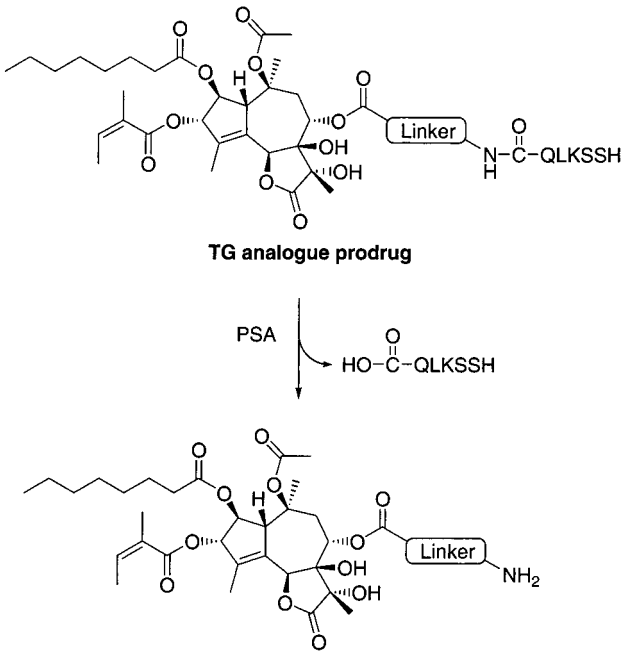


Figure 1. Prostate-specific antigen (PSA) releases the active TG analogue from the TG analogue prodrug by hydrolysis of the peptide promoiety (HSSKLQ).

secreting prostatic cancer cells thus specifically targeting the TG analogue cytotoxicity to prostatic cancer cells.

In a previous study,[10] we reported on the synthesis and apoptotic activity of a series of *O*-8 substituted aromatic amine TG analogues (**3c**,**e**,**f** and **4a**−**e**) (Chart 2). Analogues **4c**−**e** were the most potent, being 1.5, 1.7, and 1.7 times less potent inhibitors of the rabbit skeletal muscle SERCA pump than TG, respectively, and 9, 4, and 8 times less cytotoxic against TSU-Pr1 human prostatic cancer cells, respectively.

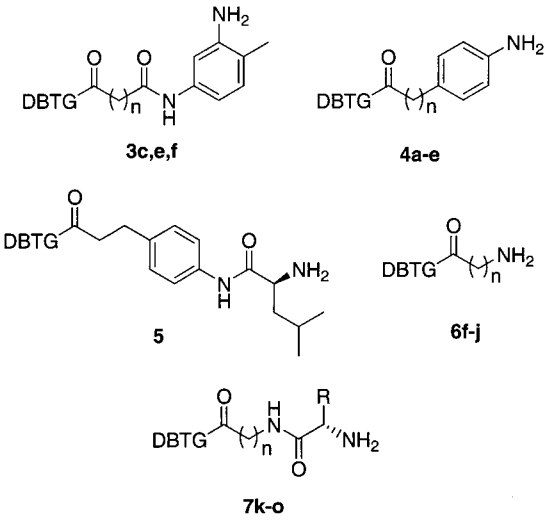
The goal of this paper was to investigate the structure−activity relationship of TG analogues, in which the *O*-8 butyric acid has been substituted with an *ω*-amino acid (analogues **6f**−**j**) (Chart 2) or an α-amino acid conjugated *ω*-amino acid (analogues **5** and **7k**-**o**) (Chart 2).

All target compounds were evaluated for their ability to elevate $[Ca^{2+}]_i$ in TSU-Pr1 cells, their ability to inhibit the rabbit skeletal muscle SERCA pump, and their ability to kill TSU-Pr1 human prostatic cancer cells.

Chemistry

DBTG (**2**) was prepared from TG (**1**) by selective trans-esterification of the *O*-8-butanoyl ester in methanol using triethylamine as catalyst.[11,12] Analogue **5** was synthesized as shown in Scheme 1. 4-Amino-*trans*-cinnamic acid hydrochloride was hydrogenated and the carboxyl group protected as the methyl ester to give **8**. N_α-Boc-L-leucine was coupled[13] to the aromatic amine to give **9**. Deprotection of the carboxylic acid gave **10**, coupling to **2** gave **11**, and deprotection of the amine gave analogue **5**. Analogues **6f**−**j** were synthesized as outlined in Scheme 2. Coupling of the *N*-Boc protected aliphatic amino acids **12f**−**j** with **2** gave **13f**−**j**, and deprotection of the amino group gave **6f**−**j**. Analogues **7k**−**m** were synthesized as shown in Scheme 3. N_α-Boc-L-leucine was coupled to methyl esters **14f**,**j** to give **15k**,**l**, and N_α-Boc-L-alanine was coupled to methyl ester **14j** to give **15m**. Deprotection of the carboxylic acid gave **16k**−**m**, coupling to **2** gave **17k**−**m**, and deprotection of the amine gave **7k**−**m**. Analogues **7n**,**o** were synthesized as outlined in Scheme 4. N_α-Boc-L-serine and N_α-Boc-L-phenylalanine were coupled to analogue **6j** to give **18n**,**o**, and deprotection of the amine gave **7n**,**o**.

Chart 2[a]



a: n = 0
b: n = 1
c: n = 2
d: n = 2, *trans* C=C
e: n = 3
f: n = 5
g: n = 6
h: n = 7
i: n = 10
j: n = 11
k: n = 5, R = $CH_2(CH_3)_2$
l: n = 11, R = $CH_2(CH_3)_2$
m: n = 11, R = CH_3
n: n = 11, R = CH_2OH
o: n = 11, R = CH_2Ph

[a] TG analogues with amine-containing linkers esterified to the *O*-8 hydroxyl of **2**.

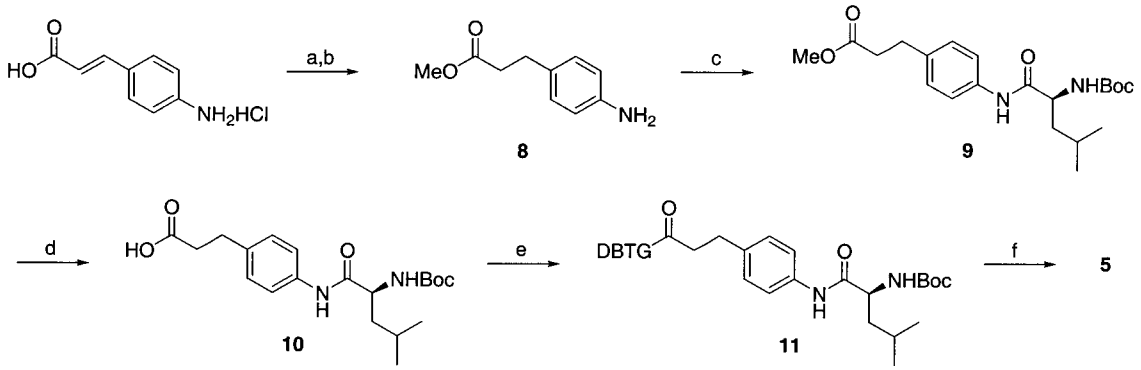
Pharmacology

SERCA containing microsomes were isolated from rabbit skeletal muscle by differential centrifugation of the muscle homogenate.[14,15]

The SERCA activity was measured with a coupled enzyme assay as the rate of ATP hydrolysis.[16,17] The activity at each dose (nmol/mg of SR protein) of TG analogue was expressed as percentage of the uninhibited control activity and was determined in triplicate. Inhibition curves were corrected for Ca^{2+}-independent (TG insensitive) ATPase activity by subtracting the residual activity at high inhibitor concentrations, which typically represented 10% of the total activity. The amount of TG analogue required to inhibit 50% of the maximal Ca^{2+}-dependent ATPase activity in 1 mg of SR protein was expressed as ID_{50} values, and was determined by 4-parameter curve-fitting (Table 1).

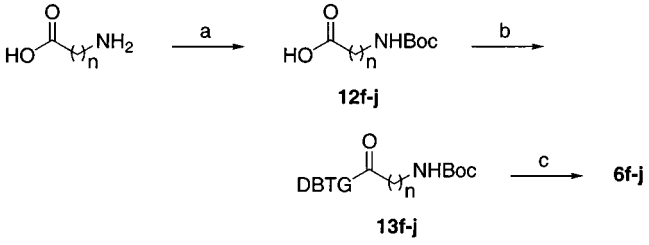
The apoptotic activity of each TG analogue against TSU-Pr1 human prostatic cancer cells was determined as previously described.[18] The apoptotic activity was expressed as the concentration of analogue LC_{50} (μM) capable of inducing 50% loss of clonogenic survival as compared to untreated controls (Table 1).

Scheme 1[a]



[a] Reagents: (a) Pd/C, H_2, 2-metoxyethanol; (b) MeOH, $SOCl_2$; (c) N_α-*tert*-butoxycarbonyl-L-leucine, hexachloroacetone, Ph_3P, pyridine, THF; (d) 2 M NaOH (aq), MeOH; (e) **2**, DCC, DMAP, CH_2Cl_2; (f) TFA, CH_2Cl_2.

Scheme 2[a]



[a] Reagents: (a) 5 M NaOH (aq), $(Boc)_2O$, *tert*-BuOH; (b) **2**, DCC, DMAP, CH_2Cl_2; (c) TFA, CH_2Cl_2.

The increase in cytosolic calcium concentration ($[Ca^{2+}]_i$) in TSU-Pr1 cells induced by TG analogues at effective cytotoxic concentrations was determined as previously described (Table 2).[10,19]

Results and Discussion

Previous published results concerning PSA activated doxorubicin prodrugs[20] promoted us to conjugate analogue **4c** with L-leucine to give analogue **5**. The conjugated analogue showed decreased SERCA inhibition and apoptotic activity (Table 1). The decreased activity of **5** was attributed to a decreased lipophilicity due to protonization of the α-amine at physiological pH. Previously, a positive correlation between lipophilicity and histamine-releasing activity was found in a series of *O*-2 and *O*-8 substituted TG analogues.[21]

The previously prepared aromatic analogues **3c**,**e**,**f** were 328, 81, and 4.4 times less potent SERCA inhibitors than TG, respectively, and >800, 533, and 103 times less cytotoxic, respectively.[10] Apparently, the potency also in this case increases with increasing lipophilicity. A similar structure−activity relationship was found in the series of aliphatic analogues **6f**−**j** (Table 1).

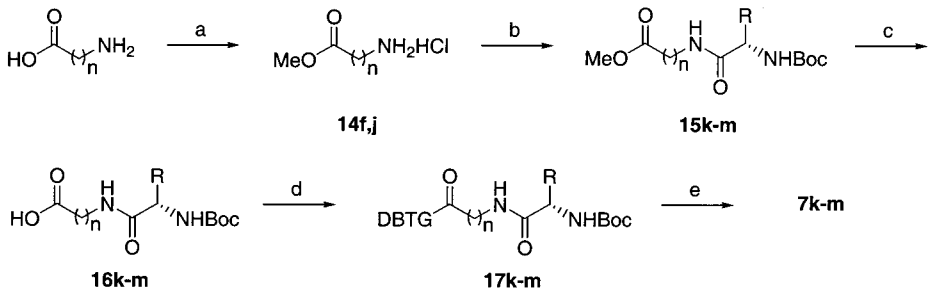
Table 1. ID_{50} (nmol/mg of SR protein) Values for Inhibiting 50% of Maximal Rabbit Skeletal Muscle SERCA Activity and LC_{50} (μM) Values for 50% Loss of Clonogenic Survival of Human Prostate Cancer TSU-Pr1 Cells[a]

compd	ID_{50} (nmol/mg SR protein)	activity relative to TG[b]	LC_{50} (μM)	activity relative to TG[c]
TG	13.4 ± 1.4	1	0.03 ± 0.004	1
5	466 ± 22	35	0.88 ± 0.04	29
6f	1332 ± 83	99	>20	>667
6g	1206 ± 57	90	10.92 ± 2.28	364
6h	223 ± 15	17	3.85 ± 0.21	128
6i	40 ± 3	3.0	0.75 ± 0.03	25
6j	35 ± 4	2.6	1.16 ± 0.16	39
7k	3842 ± 315	287	>20	>667
7l	45 ± 3	3.4	0.03 ± 0.01	1.0
7m	16.5 ± 1.6	1.2	0.28 ± 0.06	9.3
7n	10.3 ± 0.5	0.8	0.89 ± 0.04	30
7o	n.d.	-	0.21 ± 0.02	7.0

[a] Results expressed as mean ± standard deviation of triplicate measurements. [b] ID_{50} analogue/ID_{50} TG. [c] LC_{50} analogue/LC_{50} TG.

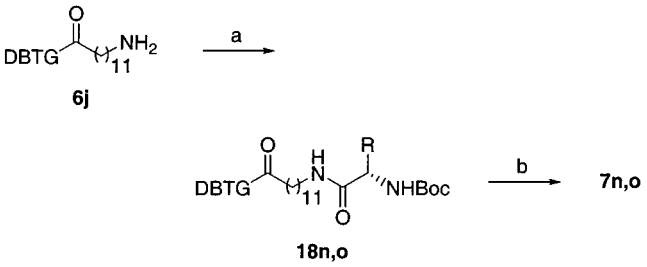
Conjugation of **6f** with L-leucine to give **7k** decreased the SERCA inhibitory activity, and the apoptotic activity was still poor (Table 1). In contrast, conjugation of **6j** with L-leucine to give **7l** only marginally changed the SERCA inhibition, and surprisingly increased the apoptotic activity affording an analogue equipotent with TG. Replacement of L-leucine in **7l** with L-alanine and L-phenylalanine (**7m**,**o**, respectively) only to a limited extent influenced the activity, whereas introduction of the more hydrophilic L-serine (**7n**) afforded a less apoptotic analogue.

Scheme 3[a]



[a] Reagents: (a) MeOH, $SOCl_2$; (b) N_α-*tert*-butoxycarbonyl-L-leucine or N_α-*tert*-butoxycarbonyl-L-alanine, DIPEA, DCC, CH_2Cl_2; (c) 2 M NaOH (aq), MeOH; (d) **2**, DCC, DMAP, CH_2Cl_2; (e) TFA, CH_2Cl_2.

Scheme 4[a]



[a] Reagents: (a) N_α-*tert*-butoxycarbonyl-L-serine or N_α-*tert*-butoxycarbonyl-L-phenylalanine, DCC, HOBT, DMF; (b) TFA, CH_2Cl_2.

Table 2. Increase in Cytosolic Ca^{2+} Concentration ($[Ca^{2+}]_i$) in TSU-Pr1 Cells at Effective Cytotoxic Concentrations[a]

compd	1000 nM $[Ca^{2+}]_i$ (nM)	100 nM $[Ca^{2+}]_i$ (nM)
5	451 ± 54	91 ± 16
6f	284 ± 51	NE[b]
6g	209 ± 6	NE[b]
6h	217 ± 20	NE[b]
6i	420 ± 3	242 ± 7
6j	369 ± 2	235 ± 12
7k	84 ± 7	40 ± 3
7l	414 ± 44	173 ± 28
7m	410 ± 45	338 ± 10
7n	348 ± 20	93 ± 10
7o	446 ± 33	406 ± 15

[a] $[Ca^{2+}]_i$ was monitored for 20 min. Baseline $[Ca^{2+}]_i$ was 35 ± 4 nM. Results are expressed as the mean ± standard error of triplicate measurements. [b] NE = no significant elevation of $[Ca^{2+}]_i$ above baseline level.

The ability of the analogues to elevate $[Ca^{2+}]_i$ in TSU-Pr1 cells followed the relative SERCA inhibitory and apoptotic activities (Table 2).

In conclusion, a series of TG analogues containing an α-amino acid applicable for conjugation to PSA-specific peptides has been prepared. In general, the potency follows the relative lipophilicity of the analogues. The high activity of **7l** makes this analogue especially interesting as a drug moiety in a prodrug of TG.

Experimental Section

Chemistry. TG (**1**) was isolated from the seeds of *Thapsia garganica* L. as previously described.[1] Reagents and precursors were supplied by Aldrich and were used without further purification. Thin-layer chromatography was done using precoated aluminum sheets with Silica gel 60 F_{254} or RP-18 F_{254} (Merck). Compounds were visualized by inspection under UV (λ = 254 nm) or after spraying with naphthoresorcinol solution (0.2% w/v in ethanol diluted 1:1 with 2 M H_2SO_4) or ninhydrin solution (0.05% w/v in ethanol) followed by heating. Normal phase column chromatography (NPCC) was done with Silica gel 60, 40−63 μm (Merck) using mixtures of EtOAc−toluene−AcOH, 20:10:0.3 (A), heptane−acetone−AcOH, 7:3:0.1 (B), or toluene−acetone, 19:1 (C), and 1:1 (D). Reverse phase column chromatography (RPCC) was done with LiChroprep RP-18, 40−63 μm (Merck) using mixtures of MeOH-water, 4:1 (E), 5:1 (F), 6:1 (G), 7:1 (H), and 9:1 (I) or MeOH−water−AcOH, 9:1:0.1 (J). Melting points were measured with capillary tubes in an oil bath and were corrected. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a GEMINI 2000 BB, 300 MHz spectrometer. Chemical shift values (δ) are expressed in ppm relative to tetramethylsilane as internal standard. The following abbreviations are used for multiplicity of NMR signals: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, m = multiplet. NMR signals corresponding to exchangeable protons are omitted. Signals from α-amino acids are assigned with Greek letters α, β, and γ. Signals originating from the TG nucleus of all the *O*-8 acylated compounds were in the following range: ^{1}H NMR ($CDCl_3$) δ 0.86−0.87 (t, J = 7−8 Hz, 3H, octanoyl H-8), 1.26−1.28 (m, 8H, octanoyl H-4 to H-7), 1.37−1.44 (br s, 3H, H-14), 1.39−1.50 (s, 3H, H-13), 1.58−1.60 (m, 2H, octanoyl H-3), 1.83−1.87 (br s, 3H, H-15), 1.88−1.90 (s, 3H, acetyl H-2), 1.90−1.92 (m, 3H, angeloyl C-2 CH_3), 1.99−2.00 (d, J = 7−8 Hz, 3H, angeloyl H-4), 2.28−2.30 (m, 2H, octanoyl H-2), 2.28−2.42 (dd, J = 3−4 and 13−15 Hz, 1H, H-9′), 2.89−3.03 (dd, J = 3−4 and 13−15 Hz, 1H, H-9), 4.18−4.33 (br s, 1H, H-1), 5.46−5.51 (m, 1H, H-2), 5.61−5.71 (m, 3H, H-3, H-6 and H-8), 6.10−6.12 (q, J = 7−8 Hz, 1H, angeloyl H-3). Some times H-8 appeared at a slightly lower ppm value than H-3 and H-6 and some times H-9′ was overlapped by signals from the acyl residues. ^{13}C NMR ($CDCl_3$) (labeled assignments are interchangeable) δ 12.8−13.4 (C-15), 14.1−14.4 (octanoyl C-8), 15.7−16.1 (C-13), 15.8−16.3 (angeloyl C-4), 19.6−20.8 (angeloyl C-2 CH_3), 22.2−22.8 (acetyl C-2), 22.5−22.8 (C-14), 23.6−24.4 (octanoyl C-7), 24.3−25.2 (octanoyl C-3), 29.0−29.5 (octanoyl C-4 to C-6), 34.6−36.1 (octanoyl C-2), 38.3−40.7 (C-9), 57.5−59.4 (C-1), 66.2−66.5 (C-8)[a], 77.0−77.8 (C-6)[a], 77.9−78.8 (C-2)[a], 78.5−78.9 (C-7)[b], 78.6−79.0 (C-11)[b], 84.3−85.7 (C-3)[a], 84.7−85.7 (C-10), 127.7−127.9 (angeloyl C-2), 130.1−131.2 (C-4)[c], 138.8−139.2 (angeloyl C-3), 141.1−142.1 (C-5)[c], 165.1−167.4 (C=O, angeloyl), 170.9−171.8 (C=O, acetyl), 172.9−173.1 (C=O, octanoyl), 174.5−177.6 (C=O, C-12). The identity of target compounds was confirmed with NMR and HRMS. The target compounds were pure according to TLC, and their NMR spectra showed no foreign signals other than minor solvent residuals.

8-*O*-Debutanoylthapsigargin (2). Triethylamine (2.5 mL) was added to a solution of **1** (0.80 mmol) in dry MeOH (50 mL) at room temperature. After 6 h at room temperature, the mixture was concentrated in vacuo. The residue was concentrated two times from toluene (50 mL) in vacuo to give **2** (yield 100%) as a white amorphous solid: ^{1}H NMR ($CDCl_3$) δ 0.87 (t, J = 7.0 Hz, 3H, octanoyl H-8), 1.28 (br s, 8H, octanoyl H-4 to H-7), 1.44 (s, 3H, H-14), 1.49 (s, 3H, H-13), 1.60 (m, 2H, octanoyl H-3), 1.84 (s, 3H, H-15), 1.90 (m, 6H, acetyl H-2 and angeloyl C-2 CH_3), 1.99 (d, J = 7.0 Hz, 3H, angeloyl H-4), 2.29 (m, 2H, octanoyl H-2), 2.47 (dd, J = 3.3 and 14.1 Hz, 1H, H-9′), 2.84 (d, J = 14.1 Hz, 1H, H-9), 3.57 (br s, 1H, H-8), 4.36 (s, 1H, H-1), 5.45 (m, 1H, H-2), 5.69 (s, 1H, H-3), 5.80 (s, 1H, H-6), 6.12 (q, J = 7.2 Hz, 1H, angeloyl H-3).

3-(4-Aminophenyl)propionic Acid Methyl Ester (8). Triethylamine (10.0 mmol) was added dropwise to a suspension of 4-amino-*trans*-cinnamic acid hydrochloride (10.0 mmol) in 2-methoxyethanol (12 mL) at room temperature. The suspension was filtered, and Pd-C 10% (120 mg) was added to the filtrate. The mixture was hydrogenated (4 atm) for 3 h at room temperature. The mixture was filtered through a column of Celite, and the filtrate was concentrated in vacuo to give 3-(4-aminophenyl)propionic acid (1.7 g) as a yellowish crystalline solid. This compound was esterified without further purification. Thionyl chloride (2.5 mL) was added dropwise to dry MeOH (10 mL) at −10 °C, and the solution was left for 10 min. 3-(4-Aminophenyl)propionic acid (1.7 g) was added to the solution, and the mixture was left overnight at room temperature. The solvent was removed in vacuo, and the residue was dissolved in EtOAc (200 mL). The solution was washed with 5% $NaHCO_3$ (200 mL), 10% NaCl (100 mL), and water (100 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated in vacuo to give **8** (yield 80%) as a crystalline yellow solid: ^{1}H NMR ($CDCl_3$) δ 2.55 (t, J = 7.0 Hz, 2H, H-2), 2.85 (t, J = 7.0 Hz, 2H, H-3), 3.65 (s, 3H, OCH_3), 6.60 (d, J = 9.0 Hz, 2H, Ar H-3 and H-5), 6.95 (d, J = 9.0 Hz, 2H, Ar H-2 and H-6).

3-(4-[N_α-*tert*-Butoxycarbonyl-L-leucinoylamino]phenyl)-propanoic Acid Methyl Ester (9). A solution of triphenylphosphine (1.00 mmol) in dry THF (1.0 mL) was under argon dropwise added to a solution of N_α-*tert*-butoxycarbonyl-L-leucine (1.00 mmol) and hexachloroacetone (0.50 mmol) in dry THF (2.0 mL) at −78 °C, and the solution was left for 30 min. A solution of compound **8** (1.00 mmol) in dry THF (1.0 mL)

and a solution of pyridine (6.00 mmol) in dry THF (6.0 mL) was added to the reaction mixture at −78 °C, and the mixture was left at room temperature for 1 h and filtered. The filtrate was concentrated in vacuo, and the residue was dissolved in CH_2Cl_2 (100 mL). The solution was washed twice with 1 M HCl (50 mL), twice with 5% $NaHCO_3$ (50 mL), and twice with 10% NaCl (50 mL) and concentrated in vacuo. Purification of the residue by NPCC (eluent A) afforded **9** (50%) as a yellowish crystalline solid: ^{1}H NMR ($CDCl_3$) δ 0.95 (m, 6H, Leu CH_3 and CH'_3), 1.43 (s, 9H, Boc CH_3), 1.62 (m, 1H, γ-H), 1.72 (m, 2H, β-H), 2.58 (t, J = 7.8 Hz, 2H, H-2), 2.88 (t, J = 7.8 Hz, 2H, H-3), 3.66 (s, 3H, OCH_3), 4.35 (m, 1H, α-H), 7.07 (d, J = 8.1 Hz, 2H, Ar H-2 and H-2′), 7.41 (d, J = 8.1 Hz, 2H, Ar H-3 and H-3′); ^{13}C NMR ($CDCl_3$) δ 22.3 (Leu CH_3), 23.6 (Leu CH'_3), 25.2 (γ-C), 28.5 (Boc CH_3), 31.0 (C-3), 36.5 (C-2), 41.7 (β-C), 52.1 (OCH_3), 54.5 (α-C), 81.2 (Boc *tert*-C), 120.3 (Ar C-3 and C-5), 129.0 (Ar C-2 and C-6), 132.7 (Ar C-1), 136.7 (Ar C-4), 156.4 (C═O, carbamate), 171.2 (C═O, C-1), 173.4 (C═O, amide).

3-(4-[N_α-*tert*-Butoxycarbonyl-L-leucinoylamino]phenyl)-propanoic Acid (10). 2 M NaOH (10 mL) was added at room temperature to a solution of **9** (4.89 mmol) in MeOH (65 mL), and the mixture was left for 10 min at room temperature. The MeOH was removed in vacuo, and the aqueous residue was acidified to pH 2 with 2 M H_2SO_4. The aqueous solution was extracted twice with EtOAc (60 mL), and the combined organic phases were washed with 10% w/v NaCl (60 mL) and water (60 mL). The organic phase was dried ($MgSO_4$), filtered, and concentrated in vacuo to give **10** (99%) as a yellowish crystalline solid: mp 64.5−66.5 °C; ^{1}H NMR ($CDCl_3$) δ 0.93 (d, J = 6.3 Hz, 3H, Leu CH_3), 0.96 (d, J = 6.3 Hz, 3H, Leu CH'_3), 1.38 (s, 9H, Boc CH_3), 1.66 (m, 1H, γ-H), 1.74 (m, 2H, β-H), 2.63 (t, J = 7.2 Hz, 2H, H-2), 2.89 (t, J = 7.2 Hz, 2H, H-3), 4.48 (m, 1H, α-H), 7.05 (d, J = 8.0 Hz, 2H, Ar H-2 and H-6), 7.43 (d, J = 8.0 Hz, 2H, Ar H-3 and H-5); ^{13}C NMR ($CDCl_3$) δ 21.9 (Leu CH_3), 23.0 (Leu CH'_3), 24.8 (γ-C), 28.4 (Boc CH_3), 30.2 (C-3), 35.8 (C-2), 41.4 (β-C), 54.0 (α-C), 80.5 (Boc *tert*-C), 120.4 (Ar C-3 and C-5), 128.9 (Ar C-2 and C-6), 136.4 (Ar C-1), 156.9 (C═O, carbamate), 176.5 (C═O, amide), 178.2 (C═O, acid); MS (FAB+) m/z 379 ([M+H]$^+$, 33%), 323 (100%).

8-*O*-(3-[4-{N_α-*tert*-Butoxycarbonyl-L-leucinoylamino}-phenyl]propanoyl)-8-*O*-debutanoylthapsigargin (11). To a mixture of **10** (0.26 mmol), **2** (0.26 mmol), and DMAP (0.03 mmol) in dry CH_2Cl_2 (2.0 mL) was added a solution of DCC (0.29 mmol) in dry CH_2Cl_2 (0.75 mL) at 0 °C. The mixture was left for 1 h at 0 °C and then 7.5 h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by RPCC (eluent F) yielded **11** (50%) as a white amorphous solid: 3-(4-[N_α-*tert*-butoxycarbonyl-L-leucinoylamino]phenyl)propanoyl ^{1}H NMR ($CDCl_3$) δ 0.93 (d, J = 6.3 Hz, 3H, Leu CH_3), 0.97 (d, J = 6.3 Hz, 3H, Leu CH'_3) 1.40 (s, 9H, Boc CH_3), 1.62 (m, 1H, γ-H), 1.74 (m, 2H, β-H), 2.56 (t, J = 7.0 Hz, 2H, H-2), 2.83 (t, J = 7.0 Hz, 2H, H-3), 4.34 (m, 1H, α-H), 7.00 (d, J = 8.0 Hz, 2H, Ar H-2 and H-2′), 7.35 (d, J = 8.0 Hz, 2H, Ar H-3 and H-3′); ^{13}C NMR ($CDCl_3$) δ 23.0 (Leu CH'_3 and CH_3), 24.8 (γ-C), 28.4 (Boc CH_3), 29.8 (C-3), 36.2 (C-2), 40.9 (β-C), 53.9 (α-C), 80.6 (Boc *tert*-C), 120.5 (Ar C-3 and C-5), 128.7 (Ar C-2 and C-6), 136.3 (Ar C-4), 156.7 (C═O, carbamate), 172.0 (C═O, C-1), 176.5 (C═O, amide); HRMS (FAB+) m/z 963.4902 ([M+Na]$^+$, $C_{50}H_{73}N_2O_{15}Na$ requires 963.4830).

8-*O*-(3-[4-{L-Leucinoylamino}phenyl]propanoyl)-8-*O*-debutanoylthapsigargin (5). TFA (1.00 mL) was added to a solution of **11** (0.12 mmol) in dry CH_2Cl_2 (2.0 mL) at room temperature. The mixture was stirred for 1 h at room temperature and concentrated in vacuo to give **5** (yield 100%) as a yellowish amorphous solid: 3-(4-[L-leucinoylamino]phenyl)-propanoyl ^{1}H NMR ($CDCl_3$) δ 0.86 (m, 6H, Leu CH_3 and CH'_3), 1.59 (m, 1H, γ-H), 1.74 (m, 2H, β-H), 2.58 (br s, 2H, H-2), 2.83 (br s, 2H, H-3), 4.21 (m, 1H, α-H), 7.02 (br s, 2H, Ar H-2 and H-6), 7.22 (br s, 2H, Ar H-3 and H-5); ^{13}C NMR ($CDCl_3$) δ 23.0 (Leu CH'_3 and CH_3), 24.8 (γ-C), 29.8 (C-3), 36.2 (C-2), 40.9 (β-C), 53.9 (α-C), 120.5 (Ar C-3 and C-5), 128.7 (Ar C-2 and C-6), 136.3 (Ar C-4), 172.0 (C═O, C-1), 176.5 (C═O, amide); HRMS (FAB+) m/z 841.4551 ([M+H]$^+$, $C_{45}H_{65}N_2O_{13}$ requires 841.4487).

6-*tert*-Butoxycarbonylaminohexanoic Acid (12f). A solution of sodium hydroxide (1.47 mmol) in water (0.3 mL) was added to a solution of 6-aminohexanoic acid (1.50 mmol) in *tert*-butyl alcohol (3.0 mL), and the solution was left for 10 min at room temperature. Di-*tert*-butyl-dicarbonate (1.65 mmol) dissolved in *tert*-butyl alcohol (2.8 mL) was added to the solution, and the mixture was left overnight at room temperature. The mixture was concentrated in vacuo, and the residue was suspended in water (2.4 mL). The suspension was cooled on ice and acidified (pH 2) with 2 M H_2SO_4. The aqueous suspension was quickly extracted three times with EtOAc (3.6 mL), and the combined organic phases were washed three times with water (2.4 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. Purification of the residue by NPCC (eluent B) afforded **12f** (yield 61%) as white crystals: mp 38−39 °C; ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 2H, H-4), 1.40 (s, 9H, Boc CH_3), 1.50 (m, 2H, H-3 or H-5), 1.57 (m, 2H, H-3 or H-5), 2.28 (t, J = 7.4 Hz, 2H, H-2), 3.01 (m, 2H, H-6).

Compounds **12g**−**j** were prepared as described for **12f** using 7-aminoheptanoic acid, 8-aminooctanoic acid, 11-aminoundecanoic acid, and 12-aminododecanoic acid, respectively, as starting materials.

7-*tert*-Butoxycarbonylaminoheptanoic Acid (12g). NPCC (eluent A) afforded **12g** (yield 82%) as white crystals: mp 55−56 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (m, 4H, H-4 and H-5), 1.45 (s, 9H, Boc CH_3), 1.48 (m, 2H, H-3 or H-6), 1.64 (m, 2H, H-3 or H-6), 2.35 (t, J = 7.5 Hz, 2H, H-2), 3.10 (m, 2H, H-7).

8-*tert*-Butoxycarbonylaminooctanoic Acid (12h). NPCC (eluent A) afforded **12h** (yield 79%) as white crystals: mp 56−57 °C; ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 6H, H-4 to H-6), 1.45 (s, 9H, Boc CH_3), 1.46 (m, 2H, H-3 or H-7), 1.63 (m, 2H, H-3 or H-7), 2.34 (t, J = 7.4 Hz, 2H, H-2), 3.10 (m, 2H, H-8).

11-*tert*-Butoxycarbonylaminoundecanoic Acid (12i). NPCC (eluent A) afforded **12i** (yield 54%) as white crystals: mp 67−68 °C; ^{1}H NMR ($CDCl_3$) δ 1.28 (br s, 12H, H-4 to H-9), 1.45 (br s, 9H, Boc CH_3), 1.53 (m, 2H, H-3 or H-10), 1.63 (m, 2H, H-3 or H-10), 2.34 (t, J = 7.5 Hz, 2H, H-2), 3.10 (m, 2H, H-11).

12-*tert*-Butoxycarbonylaminododecanoic Acid (12j). NPCC (eluent B) afforded **12j** (yield 33%) as white crystals: mp 83.5−84.5 °C; ^{1}H NMR ($CDCl_3$) δ 1.27 (br s, 14H, H-4 to H-10), 1.45 (br s, 9H, Boc CH_3), 1.50 (m, 2H, H-3 or H-11), 1.63 (m, 2H, H-3 or H-11), 2.35 (t, J = 7.4 Hz, 2H, H-2), 3.10 (m, 2H, H-12).

8-*O*-(6-*tert*-Butoxycarbonylaminohexanoyl)-8-*O*-debutanoylthapsigargin (13f). Compound **2** (0.18 mmol), **12f** (0.20 mmol), and DMAP (0.20 mmol) was dissolved in dry CH_2Cl_2 (1.5 mL) at room temperature. After cooling on ice, a solution of DCC (0.20 mmol) in dry CH_2Cl_2 (0.5 mL) was added. The mixture was kept on ice for 1 h and then left for 5 h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by RPCC (eluent E) afforded **13f** (yield 54%) as a white amorphous solid: 6-*tert*-Butoxycarbonylaminohexanoyl ^{1}H NMR ($CDCl_3$) δ 1.28 (m, 2H, H-4), 1.43 (s, 9H, Boc CH_3), 1.61 (m, 4H, H-3 and H-5), 2.32 (m, 2H, H-2), 3.09 (m, 2H, H-6); ^{13}C NMR ($CDCl_3$) δ 25.2 (C-3), 25.8 (C-4), 28.7 (Boc CH_3), 29.5 (C-5), 34.8 (C-2), 40.7 (C-6), 80.1 (Boc *tert*-C), 172.7 (C═O, C-1); HRMS (FAB+) m/z 816.4139 ([M+Na]$^+$, $C_{41}H_{63}NO_{14}Na$ requires 816.4146).

Compounds **13g**−**j** were prepared as described for **13f**, using compounds **12g**−**j**, respectively, as starting materials.

8-*O*-(7-*tert*-Butoxycarbonylaminoheptanoyl)-8-*O*-debutanoylthapsigargin (13g). RPCC (eluent F) afforded **13g** (yield 41%) as a white amorphous solid: 7-*tert*-Butoxycarbonylaminoheptanoyl ^{1}H NMR ($CDCl_3$) δ 1.28 (m, 4H, H-4 to H-5), 1.44 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-6), 2.29 (m, 2H, H-2), 3.07 (m, 2H, H-7); ^{13}C NMR ($CDCl_3$) δ 25.0 (C-3), 28.6 (Boc CH_3), 29.0 (C-4 and C-5), 32.0 (C-6), 34.4 (C-2), 40.0 (C-7), 80.0 (Boc *tert*-C), 172.9 (C═O, C-1); HRMS (FAB+) m/z 830.4419 ([M+Na]$^+$, $C_{42}H_{65}NO_{14}Na$ requires 830.4303).

8-*O*-(8-*tert*-Butoxycarbonylaminooctanoyl)-8-*O*-debutanoylthapsigargin (13h). RPCC (eluent F) afforded **13h** (yield 52%) as a white amorphous solid: 8-*tert*-Butoxycarbonylaminooctanoyl ^{1}H NMR ($CDCl_3$) δ 1.30 (m, 6H, H-4 to H-6), 1.43 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-7), 2.29 (m, 2H, H-2), 3.07 (m, 2H, H-8); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 28.4 (Boc CH_3), 28.9 (C-4 to C-6), 31.6 (C-7), 34.3 (C-2), 40.3 (C-8), 79.8 (Boc *tert*-C), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 844.4340 ([M+Na]$^+$, $C_{43}H_{67}NO_{14}Na$ requires 844.4459).

8-*O*-(11-*tert*-Butoxycarbonylaminoundecanoyl)-8-*O*-debutanoylthapsigargin (13i). RPCC (eluent F) afforded **13i** (yield 64%) as a white amorphous solid: 11-*tert*-Butoxycarbonylaminoundecanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 12H, H-4 to H-9), 1.44 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-10), 2.29 (m, 2H, H-2), 3.08 (m, 2H, H-11); ^{13}C NMR ($CDCl_3$) δ 25.0 (C-3), 28.6 (Boc CH_3), 29.1 (C-4 to C-8), 31.8 (C-10), 34.8 (C-2), 172.8 (C=O, C-1); HRMS (FAB+) *m/z* 886.5028 ([M+Na]$^+$, $C_{46}H_{73}NO_{14}Na$ requires 886.4929).

8-*O*-(12-*tert*-Butoxycarbonylaminododecanoyl)-8-*O*-debutanoylthapsigargin (13j). RPCC (eluent I) afforded **13j** (yield 77%) as a white amorphous solid: 12-*tert*-Butoxycarbonylaminododecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (m, 14H, H-4 to H-10), 1.44 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-11), 2.28 (m, 2H, H-2), 3.09 (m, 2H, H-12); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 26.7 (C-10), 28.5 (Boc CH_3), 29.1 (C-4 to C-9), 31.7 (C-11), 34.6 (C-2), 41.0 (C-12), 172.8 (C=O, C-1); HRMS (FAB+) *m/z* 900.5084 ([M+Na]$^+$, $C_{47}H_{75}NO_{14}Na$ requires 900.5085).

8-*O*-(6-Aminohexanoyl)-8-*O*-debutanoylthapsigargin (6f). TFA (0.5 mL) was added to a solution of **13f** (0.05 mmol) in dry CH_2Cl_2 (3.0 mL) at room temperature. The mixture was left for 45 min at room temperature. Evaporation in vacuo afforded **6f** (yield 100%) as an amorphous yellowish solid: 6-Aminohexanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 2H, H-4), 1.60 (m, 4H, H-3 and H-5), 2.31 (m, 2H, H-2), 2.97 (m, 2H, H-6); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 31.8 (C-5), 34.3 (C-2), 173.0 (C=O, C-1); HRMS (FAB+) *m/z* 694.3809 ([M+H]$^+$, $C_{36}H_{56}NO_{12}$ requires 694.3802).

Compounds **6g**−**j** were prepared as described for **6f**, using compounds **13g**−**j**, respectively, as starting materials.

8-*O*-(7-Aminoheptanoyl)-8-*O*-debutanoylthapsigargin (6g). Amorphous yelowish solid (yield 100%): 7-Aminoheptanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 4H, H-4 to H-5), 1.60 (m, 4H, H-3 and H-6), 2.30 (m, 2H, H-2), 2.99 (m, 2H, H-7); ^{13}C NMR ($CDCl_3$) δ 25.0 (C-3), 29.0 (C-4 to C-5), 31.9 (C-6), 34.4 (C-2), 40.3 (C-7), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 708.3965 ([M+H]$^+$, $C_{37}H_{58}NO_{12}$ requires 708.3959).

8-*O*-(8-Aminooctanoyl)-8-*O*-debutanoylthapsigargin (6h). Amorphous yellowish solid (yield 100%): 8-Aminooctanoyl ^{1}H NMR ($CDCl_3$) δ 1.28 (m, 6H, H-4 to H-6), 1.60 (m, 4H, H-3 and H-7), 2.28 (m, 2H, H-2), 3.00 (m, 2H, H-8); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 29.0 (C-4 to C-6), 31.6 (C-7), 34.8 (C-2), 40.3 (C-8), 173.1 (C=O, C-1); HRMS (FAB+) *m/z* 722.4113 ([M+H]$^+$, $C_{38}H_{60}NO_{12}$ requires 722.4116).

8-*O*-(11-Aminoundecanoyl)-8-*O*-debutanoylthapsigargin (6i). Amorphous yellowish solid (yield 100%): 11-Aminoundecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (m, 12H, H-4 to H-9), 1.59 (m, 4H, H-3 and H-10), 2.29 (m, 2H, H-2), 2.97 (m, 2H, H-11); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 28.8 (C-4 to C-9), 31.6 (C-10), 34.4 (C-2), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 764.4655 ([M+H]$^+$, $C_{41}H_{66}NO_{12}$ requires 764.4585).

8-*O*-(12-Aminododecanoyl)-8-*O*-debutanoylthapsigargin (6j). Amorphous yellowish solid (yield 100%): 12-Aminododecanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 14H, H-4 to H-10), 1.60 (m, 4H, H-3 and H-11), 2.30 (m, 2H, H-2), 3.00 (m, 2H, H-12); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 29.1 (C-4 to C-10), 31.7 (C-11), 34.3 (C-2), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 778.4700 ([M+H]$^+$, $C_{42}H_{68}NO_{12}$ requires 778.4742).

6-Aminohexanoic Acid Methyl Ester Hydrochloride (14f). Thionyl chloride (4.0 mL) was slowly added to dry MeOH (30 mL) at −10 °C. After 10 min at −10 °C to the solution was added 6-aminohexanoic acid (15.25 mmol), and the mixture was left overnight at room temperature. The solution was concentrated in vacuo, and the residue was dissolved in MeOH (15 mL). To the solution was added Et_2O (60 mL) to precipitate the methyl ester hydrochloride. Filtration afforded **14f** (yield 84%) as white crystals: mp 118−122 °C; ^{1}H NMR (CD_3OD) δ 1.44 (m, 2H, H-4), 1.69 (m, 4H, H-3, and H-5), 2.37 (t, J = 7.5 Hz, 2H, H-2), 2.93 (t, J = 7.5 Hz, 2H, H-6), 3.66 (s, 3H, OCH_3); ^{13}C NMR (CD_3OD) δ 25.4 (C-3), 26.9 (C-4), 28.3 (C-2), 34.5 (C-5), 40.7 (C-6), 52.2 (OCH_3), 175.9 (C=O, C-1).

12-Aminododecanoic Acid Methyl Ester Hydrochloride (14j). Thionyl chloride (4.0 mL) was slowly added to dry MeOH (75 mL) at −10 °C. After 10 min at −10 °C, to the solution was added 12-aminododecanoic acid (13.93 mmol), and the mixture was left overnight at room temperature. The solution was concentrated in vacuo, and the residue was dissolved in MeOH (50 mL). To the solution was added Et_2O (80 mL) to precipitate the methyl ester hydrochloride. Filtration afforded **14j** (yield 93%) as white crystals: mp 160−161 °C; ^{1}H NMR (CD_3OD) δ 1.34 (m, 14H, H-4 to H-10), 1.62 (m, 4H, H-3 and H-11), 2.31 (t, J = 7.5 Hz, 2H, H-2), 2.91 (t, J = 7.5 Hz, 2H, H-12), 3.65 (s, 3H, OCH_3); ^{13}C NMR (CD_3OD) δ 26.1 (C-3), 27.5 (C-10), 28.6, 30.2, 30.3, 30.4, 30.5, 30.6, 30.6 (C-2 and C-4 to C-9), 34.9 (C-11), 40.9 (C-12), 52.1 (OCH_3), 176.3 (C=O, C-1).

6-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)hexanoic Acid Methyl Ester (15k). N_α-*tert*-Butoxycarbonyl-L-leucine (5.50 mmol), **14f** (5.50 mmol), and DIPEA (5.50 mmol) was dissolved in dry CH_2Cl_2 (16.5 mL) at room temperature. To the mixture cooled on ice was added a solution of DCC (6.00 mmol) in dry CH_2Cl_2 (6.0 mL). After 3 h at room temperature, the mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by NPCC (eluent C) afforded **15k** (yield 50%) as a yellowish oil: ^{1}H NMR ($CDCl_3$) δ 0.93 (d, J = 6.5 Hz, 3H, Leu CH_3), 0.94 (d, J = 6.5 Hz, 3H, Leu CH'_3), 1.34 (m, 2H, H-4), 1.44 (s, 9H, Boc CH_3), 1.51 (m, 2H, H-5), 1.64 (m, 5H, H-3, β-H and γ-H), 2.31 (t, J = 7.5 Hz, 2H, H-2), 3.24 (m, 2H, H-6), 3.67 (s, 3H, OCH_3), 4.09 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.1 (Leu CH_3), 23.0 (Leu CH'_3), 24.5 (γ-C), 24.8 (C-3), 26.3 (C-4), 28.4 (Boc CH_3), 29.2 (C-5), 33.9 (C-2), 39.2 (β-C), 41.4 (C-6), 51.6 (OCH_3), 53.2 (α-C), 80.0 (Boc *tert*-C), 156.1 (C=O, carbamate), 173.0 (C=O, C-1), 174.3 (C=O, amide); HRMS (FAB+) *m/z* 359.2522 ([M+H]$^+$, $C_{18}H_{35}N_2O_5$ requires 359.2546).

Compounds **15l**,**m** were prepared as described for **15k**, using N_α-*tert*-butoxycarbonyl-L-leucine and N_α-*tert*-butoxycarbonyl-L-alanine, respectively, together with compound **14j** as starting materials.

12-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)dodecanoic Acid Methyl Ester (15l). NPCC (eluent C) afforded **15l** (yield 53%) as white crystals: mp 63−64 °C; ^{1}H NMR ($CDCl_3$) δ 0.93 (m, 6H, Leu CH_3 and CH'_3), 1.26 (br s, 14H, H-4 to H-10), 1.44 (s, 9H, Boc CH_3), 1.48 (m, 3H, β-H and γ-H), 1.63 (m, 4H, H-3 and H-11), 2.31 (t, J = 7.5 Hz, 2H, H-2), 3.23 (m, 2H, H-12), 3.67 (s, 3H, OCH_3), 4.08 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.2 (Leu CH_3), 22.9 (Leu CH'_3), 24.8 (γ-C), 25.0 (C-3), 26.9 (C-10), 28.4 (Boc CH_3), 29.2, 29.3, 29.5 (C-4 to C-9 and C-11), 34.2 (C-2), 39.5 (β-C), 41.4 (C-12), 51.6 (OCH_3), 53.2 (α-C), 80.1 (Boc *tert*-C), 156.1 (C=O, carbamate), 172.8 (C=O, C-1), 174.7 (C=O, amide); HRMS (FAB+) *m/z* 443.3517 ([M+H]$^+$, $C_{24}H_{47}N_2O_5$ requires 443.3485).

12-(N_α-*tert*-Butoxycarbonyl-L-alaninoylamino)dodecanoic Acid Methyl Ester (15m). NPCC (eluent C) afforded **15m** (yield 55%) as white crystals: ^{1}H NMR (CD_3OD) δ 1.30 (br s, 14H, H-4 to H-10), 1.44 (s, 9H, Boc CH_3), 1.49 (m, 3H, Ala CH_3), 1.59 (m, 4H, H-3 and H-11), 2.31 (t, J = 7.5 Hz, 2H, H-2), 3.17 (m, 2H, H-12), 3.65 (s, 3H, OCH_3), 3.99 (m, 1H, α-H); ^{13}C NMR (CD_3OD) δ 18.6 (β-C), 26.1 (C-3), 28.0 (C-10), 28.8 (Boc CH_3), 30.3, 30.5, 30.7 (C-4 to C-9 and C-11), 34.9 (C-2), 40.4 (C-12), 51.8 (OCH_3), 52.1 (α-C), 80.7 (Boc *tert*-C), 158.2 (C=O, carbamate), 176.3 (C=O, amide); HRMS (FAB+) *m/z* 401.3036 ([M+H]$^+$, $C_{21}H_{41}N_2O_5$ requires 401.3015).

6-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)hexanoic Acid (16k). 2 M NaOH (10 mL) was added to a solution of **15k** (0.5 mmol) in MeOH (20 mL), and the mixture was left for 40 min at room temperature. The MeOH was removed in vacuo, and the aqueous residue was cooled on ice and acidified to pH 2 with 2 M H_2SO_4. The aqueous solution was extracted

three times with EtOAc (50 mL) and the combined organic phases were washed with 10% w/v NaCl (25 mL) and water (25 mL). The organic phase was dried ($MgSO_4$) and filtered. Concentration in vacuo afforded **16k** (yield 90%) as white crystals: mp 100.5−102.5 °C; ^{1}H NMR ($CDCl_3$) δ 0.91 (d, J = 4.5 Hz, 3H, Leu CH_3), 0.93 (d, J = 4.5 Hz, 3H, Leu CH'_3), 1.37 (m, 2H, H-4), 1.43 (s, 9H, Boc CH_3), 1.51 (m, 3H, β-H and γ-H), 1.65 (m, 4H, H-3 and H-5), 2.34 (t, J = 7.5 Hz, 2H, H-2), 3.24 (m, 2H, H-6), 4.15 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.1 (Leu CH_3), 22.8 (Leu CH'_3), 24.4 (γ-C), 24.8 (C-3), 26.2 (C-4), 28.4 (Boc CH_3), 29.0 (C-5), 33.9 (C-2), 39.3 (β-C), 41.3 (C-6), 53.2 (α-C), 80.3 (Boc *tert*-C), 156.4 (C═O, carbamate), 173.3 (C═O, amide), 177.9 (C═O, C-1); HRMS (FAB+) *m/z* 345.2430 ([M+H]$^+$, $C_{17}H_{33}N_2O_5$ requires 345.2389).

Compounds **16l**,**m** were prepared as described for **16k**, using compounds **15l**,**m**, respectively, as starting materials.

12-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)dodecanoic Acid (16l). Yellowish oil (yield 95%): ^{1}H NMR ($CDCl_3$) δ 0.93 (m, 6H, Leu CH_3 and CH'_3), 1.27 (br s, 14H, H-4 to H-10), 1.43 (s, 9H, Boc CH_3), 1.48 (m, 3H, β-H and γ-H), 1.62 (m, 4H, H-3 and H-11), 2.35 (t, J = 7.5 Hz, 2H, H-2), 3.23 (m, 2H, H-12), 4.13 (br s, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.1 (Leu CH_3), 22.8 (Leu CH'_3), 24.7 (γ-C), 24.8 (C-3), 26.6 (C-10), 28.3 (Boc CH_3), 28.7, 28.9, 29.1, 29.3 (C-4 to C-9 and C-11), 34.0 (C-2), 39.5 (β-C), 41.3 (C-12), 53.1 (α-C), 80.3 (Boc *tert*-C), 156.4 (C═O, carbamate), 173.1 (C═O, amide), 178.3 (C═O, C-1); HRMS (FAB+) *m/z* 429.3356 ([M+H]$^+$, $C_{23}H_{45}N_2O_5$ requires 429.3328).

12-(N_α-*tert*-Butoxycarbonyl-L-alaninoylamino)dodecanoic Acid (16m). Amorphous solid (yield 93%): ^{1}H NMR (CD_3OD) δ 1.30 (br s, 14H, H-4 to H-10), 1.44 (s, 9H, Boc CH_3), 1.49 (m, 3H, Ala CH_3), 1.59 (m, 4H, H-3 and H-11), 2.27 (t, J = 7.5 Hz, 2H, H-2), 3.18 (m, 2H, H-12), 4.00 (m, 1H, α-H); ^{13}C NMR (CD_3OD) δ 18.8 (β-C), 26.3 (C-3), 28.2 (C-10), 28.9 (Boc CH_3), 30.7, 30.9, 31.1 (C-4 to C-9 and C-11), 35.2 (C-2), 40.6 (C-12), 52.0 (α-C), 80.8 (Boc *tert*-C), 175.8 (C═O, amide), 177.8 (C═O, C-1); HRMS (FAB+) *m/z* 387.2807 ([M+H]$^+$, $C_{20}H_{39}N_2O_5$ requires 387.2859).

8-*O*-(6-[N_α-*tert*-Butoxycarbonyl-L-leucinoylamino]hexanoyl)-8-*O*-debutanoylthapsigargin (17k). Compound **2** (0.36 mmol), **16k** (0.36 mmol), and DMAP (0.04 mmol) was dissolved in dry CH_2Cl_2 (2.0 mL) at room temperature. To the mixture cooled on ice was added a solution of DCC (0.40 mmol) in dry CH_2Cl_2 (1.0 mL). The mixture was left on ice for 1 h and then left for 3.5 h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by RPCC (eluent E) afforded **17k** (yield 69%) as a white amorphous solid: 6-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)hexanoyl ^{1}H NMR ($CDCl_3$) δ 0.92 (m, 6H, Leu CH_3 and CH'_3), 1.28 (br s, 2H, H-4), 1.42 (br s, 9H, Boc CH_3), 1.61 (m, 4H, H-3 and H-5), 2.30 (m, 2H, H-2), 3.20 (m, 2H, H-6), 4.06 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.6 (Leu CH_3 and CH'_3), 24.8 (γ-C), 24.9 (C-3), 28.4 (Boc CH_3), 29.1 (C-4), 31.7 (C-5), 34.3 (C-2), 38.4 (β-C), 41.2 (C-6), 53.1 (α-C), 80.1 (Boc *tert*-C) 156.3 (C═O, carbamate), 172.9 (C═O, C-1), 173.6 (C═O, amide); HRMS (FAB+) *m/z* 907.5177 ([M+H]$^+$, $C_{47}H_{75}N_2O_{15}$ requires 907.5167).

Compounds **17l**,**m** were prepared as described for **17k**, using compounds **16l**,**m**, respectively, as starting materials.

8-*O*-(12-[N_α-*tert*-Butoxycarbonyl-L-leucinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (17l). RPCC (eluent D) afforded **17l** (yield 94%) as a white amorphous solid: 12-(N_α-*tert*-Butoxycarbonyl-L-leucinoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 0.92 (m, 6H, Leu CH_3 and CH'_3), 1.26 (br s, 14H, H-4 to H-10), 1.42 (br s, 9H, Boc CH_3), 1.61 (m, 4H, H-3 and H-11), 2.28 (m, 3H, H-2), 3.20 (m, 2H, H-12), 4.05 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.6 (Leu CH_3 and CH'_3), 24.8 (γ-C), 24.9 (C-3), 26.7 (C-10), 28.4 (Boc CH_3), 29.0−29.3 (C-4 to C-9), 31.7 (C-11), 34.3 (C-2), 38.4 (β-C), 41.1 (C-12), 53.1 (α-C), 80.1 (Boc *tert*-C), 156.2 (C═O, carbamate), 172.9 (C═O, C-1), 173.0 (C═O, amide); HRMS (FAB+) *m/z* 1013.5938 ([M+Na]$^+$, $C_{53}H_{86}N_2O_{15}Na$ requires 1013.5926).

8-*O*-(12-[N_α-*tert*-Butoxycarbonyl-L-alaninoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (17m). RPCC (eluent F) afforded **17m** (yield 78%) as a white amorphous solid: 12-(N_α-*tert*-Butoxycarbonyl-L-alaninoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (br s, 14H, H-4 to H-10), 1.43 (s, 9H, Boc CH_3), 1.47 (m, 3H, Ala CH_3), 1.60 (m, 4H, H-3 and H-11), 2.28 (m, 2H, H-2), 3.22 (m, 2H, H-12), 4.12 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 18.2 (β-C), 24.9 (C-3), 26.6 (C-10), 28.3 (Boc CH_3), 28.8, 29.1, 29.5 (C-4 to C-9), 31.7 (C-11), 34.5 (C-2), 50.0 (α-C), 80.6 (Boc *tert*-C), 173.0 (C═O, C-1 and amide); HRMS (FAB+) *m/z* 949.5622 ([M+H]$^+$, $C_{50}H_{81}N_2O_{15}$ requires 949.5637).

8-*O*-(6-[L-Leucinoylamino]hexanoyl)-8-*O*-debutanoylthapsigargin (7k). TFA (1.2 mL) was added to a solution of **17k** (0.20 mmol) in dry CH_2Cl_2 (3.0 mL) at room temperature. The mixture was left for 45 min at room temperature. Evaporation in vacuo afforded **7k** (yield 100%) as an amorphous yellowish solid: 6-(L-Leucinoylamino)hexanoyl ^{1}H NMR ($CDCl_3$) δ 0.93 (m, 6H, Leu CH_3 and CH'_3), 1.28 (m, 2H, H-4), 1.60 (m, 4H, H-3 and H-5), 2.29 (m, 2H, H-2), 3.20 (m, 2H, H-6), 3.62 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.6 (Leu CH_3 and CH'_3), 24.8 (γ-C), 24.9 (C-3), 29.0 (C-4), 31.7 (C-5), 34.3 (C-2), 38.3 (β-C), 44.3 (C-6), 53.6 (α-C), 170.8 (C═O, C-1), 172.9 (C═O, amide); HRMS (FAB+) *m/z* 807.4624 ([M+H]$^+$, $C_{42}H_{67}N_2O_{13}$ requires 807.4643).

Compounds **7l**,**m** were prepared as described for **7k**, using compounds **17l**,**m**, respectively, as starting materials.

8-*O*-(12-[L-Leucinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (7l). Amorphous yellowish solid (yield 100%): 12-(L-Leucinoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 0.95 (m, 6H, Leu CH_3 and CH'_3), 1.25 (br s, 14H, H-4 to H-10), 1.61 (m, 4H, H-3 and H-11), 2.33 (m, 2H, H-2), 3.25 (m, 2H, H-12), 4.19 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 22.6 (Leu CH_3 and CH'_3), 24.6 (C-3), 24.8 (γ-C), 26.5 (C-10), 28.8−29.1 (C-4 to C-9), 31.7 (C-11), 34.4 (C-2), 38.1 (β-C), 40.5 (C-12), 53.3 (α-C), 173.1 (C═O, C-1), 174.5 (C═O, amide); HRMS (FAB+) *m/z* 891.5641 ([M+H]$^+$, $C_{48}H_{79}N_2O_{13}$ requires 891.5582).

8-*O*-(12-[L-Alaninoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (7m). Amorphous yellowish solid (yield 100%): 12-(L-Alaninoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.24 (m, 14H, H-4 to H-10), 1.53 (m, 3H, Ala CH_3), 1.57 (m, 4H, H-3 and H-11), 2.30 (m, 2H, H-2), 3.20 (br s, 2H, H-12), 4.22 (br s, 1H, α-H); ^{13}C NMR ($CDCl_3$) δ 17.4 (β-C), 24.9 (C-3), 26.8 (C-10), 28.8, 29.3, 29.6 (C-4 to C-9), 31.8 (C-11), 34.6 (C-2), 50.4 (α-C), 174.1 (C═O, amide); HRMS (FAB+) *m/z* 849.5057 ([M+H]$^+$, $C_{45}H_{73}N_2O_{13}$ requires 849.5112).

8-*O*-(12-[N_α-*tert*-Butoxycarbonyl-L-serinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (18n). *N*-*tert*-Butoxycarbonyl-L-serine (0.18 mmol), **6j** (0.18 mmol), and HOBT (0.18 mmol) were dissolved in dry DMF (2.0 mL) at room temperature. To the mixture cooled on ice was added a solution of DCC (0.18 mmol) in dry DMF (1.0 mL). The mixture was left on ice for 1 h and then left for 3.5 h at room temperature. The mixture was filtered, and the filtrate was concentrated in vacuo. Purification of the residue by RPCC (eluent J) afforded **18n** (yield 72%) as a white amorphous solid: 12-(N_α-*tert*-Butoxycarbonyl-L-serinoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (br s, 14H, H-4 to H-10), 1.45 (s, 9H, Boc CH_3), 1.60 (m, 4H, H-3 and H-11), 2.29 (m, 2H, H-2), 3.24 (t, J = 6.2 Hz, 2H, H-12), 3.64 (m, 1H, β-H′), 4.05 (dd, J = 3.0 and 11.1 Hz, 1H, β-H), 4.11 (m, 1H, α-H); ^{13}C NMR ($CDCl_3$) 24.9 (C-3), 26.6 (C-10), 28.3 (Boc CH_3), 28.8−29.3 (C-4 to C-9), 31.7 (C-11), 34.3 (C-2), 39.6 (C-12), 62.8 (β-C), 80.8 (Boc *tert*-C), 156.6 (C═O, carbamate), 171.6 (C═O, C-1), 173.2 (C═O, amide); HRMS (FAB+) *m/z* 965.5593 ([M+H]$^+$, $C_{50}H_{81}N_2O_{16}$ requires 965.5586).

Compound **18o** was prepared as described for **18n**, using N_α-*tert*-butoxycarbonyl-L-phenylalanine as starting material.

8-*O*-(12-[N_α-*tert*-Butoxycarbonyl-L-phenylalaninoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (18o). RPCC (eluent J) afforded **18o** (yield 73%) as a white amorphous solid: 12-(N_α-*tert*-Butoxycarbonyl-L-phenylalaninoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (br s, 14H, H-4 to H-10), 1.38 (br s, 9H, Boc CH_3), 1.58 (m, 6H, H-3 and H-11), 2.28 (m, 3H, H-2), 3.01 (m, 2H, β-H), 3.13 (m, 2H, H-12), 4.25 (m, 1H, α-H), 7.18−7.29 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 26.7 (C-10), 28.3 (Boc CH_3), 29.0−29.3 (C-4 to C-9), 31.7

(C-11), 34.3 (C-2), 38.4 (β-C), 41.1 (C-12), 62.0 (α-C), 81.4 (Boc *tert*-C), 127.0 (Phe C-4), 128.8 (Phe C-2, C-2′), 129.5 (Phe C-3, C-3′), 137.0 (Phe C-1), 158.3 (C=O, carbamate), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 1025.606 ($[M+H]^+$, $C_{56}H_{85}N_2O_{15}$ requires 1025.595).

Compounds **7n**,**o** were prepared as described for **7k**, using compounds **18n**,**o**, respectively, as starting materials.

8-*O*-(12-[L-Serinoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (7n). Amorphous yellowish solid (yield 100%): 12-(L-Serinoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.26 (br s, 14H, H-4 to H-10), 1.58 (m, 4H, H-3 and H-11), 2.29 (m, 2H, H-2), 3.17 (br s, 2H, H-12), 3.74 (dd, J = 13.7 and 6.8 Hz, 1H, β-H′), 3.88 (br s, 1H, α-H), 4.01 (br s, 1H, β-H); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 26.6 (C-10), 28.8−29.1 (C-4 to C-9), 31.7 (C-11), 34.3 (C-2), 55.4 (α-C), 59.6 (β-C), 173.0 (C=O, C-1), 174.4 (C=O, amide); HRMS (FAB+) *m/z* 865.5010 ($[M+H]^+$, $C_{45}H_{73}N_2O_{14}$ requires 865.5062).

8-*O*-(12-[L-Phenylalaninoylamino]dodecanoyl)-8-*O*-debutanoylthapsigargin (7o). Amorphous yellowish solid (yield 100%): 12-(L-Phenylalaninoylamino)dodecanoyl ^{1}H NMR ($CDCl_3$) δ 1.27 (m, 14H, H-4 to H-10), 1.60 (m, 6H, H-3 and H-11), 2.28 (m, 2H, H-2), 2.68 (dd, J = 9.3 and 13.7 Hz, 1H, β-H), 3.23 (m, 3H, β-H′ and H-12), 3.58 (dd, J = 9.3 and 4.2 Hz, 1H, α-H), 7.20−7.34 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ 24.9 (C-3), 26.7 (C-10), 29.0−29.3 (C-4 to C-9), 31.7 (C-11), 34.3 (C-2), 38.4 (β-C), 41.1 (C-12), 61.1 (α-C), 127.0 (Phe C-4), 128.9 (Phe C-2, C-2′), 129.5 (Phe C-3, C-3′), 138.1 (Phe C-1), 172.9 (C=O, C-1); HRMS (FAB+) *m/z* 925.539 ($[M+H]^+$, $C_{51}H_{77}N_2O_{13}$ requires 925.543).

Isolation of Sarcoplasmic Reticulum (SR). Frozen rabbit muscle was purchased from Pel-Freez Biologicals (Rogers, AR) and MOPS, sucrose, EDTA, and KCl were purchased from SIGMA. Homogenization was done with a commercial blender (Waring, USA). Centrifugation was done with a Sorvall RC-5B superspeed centrifuge (DuPont, USA) and a L7 ultracentrifuge (Beckman Coulter, USA). The temperature was kept at 0−4 °C during the preparation. Frozen rabbit muscle (180 g) was blended 15 s every 5 min during 1 h with 510 mL of a solution containing 10 mM MOPS, pH 7.0, 10% sucrose and 0.1 mM EDTA. The pH was kept between 6.5 and 7.0 by adding 10% NaOH. The homogenate was centrifuged at 15000*g* for 20 min. The supernatant was filtered through a path of cheesecloth, and centrifuged at 40000*g* for 90 min. The pellet was suspended with a Dounce glass homogenizer in 60 mL of a solution containing 10 mM MOPS, pH 7.0, and 0.6 M KCl. After incubating for 40 min at 4 °C, the suspension was centrifuged at 15000*g* for 20 min. The 10% top of the supernatant and the pellet were discarded. The supernatant was collected and centrifuged at 40000*g* for 90 min. The pellet was suspended with a Dounce glass homogenizer in 40 mL of microsome storage solution containing 10 mM MOPS, pH 7.0 and 30% sucrose. The microsomes were stored at −80 °C. The sarcoplasmic reticulum (SR) protein concentration (1.1 mg/mL) was determined with the Micro BCA Protein Assay Reagent kit supplied by Pierce (Rockford, IL) using bovine serum albumin (BSA) as standard.

Measurement of ATPase Activity. KCl, Trizma-HCl, $MgCl_2$, EGTA, $CaCl_2$, β-NADH, phosphoenolpyruvate (PEP), A23187, phosphoenolpyruvate kinase (PK), lactate dehydrogenase (LDH), and ATP were supplied by Sigma. The ATPase activity was measured with a Spectramax Plus^{384} microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA) as the rate of ATP hydrolysis essentially as previously described.[66,128] Buffer A: 0.1 M KCl, 20 mM Trizma-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.7 mM $CaCl_2$. Solution 1: 1.2 mM β-NADH, 1.5 mM PEP, 4.5 μM A23187, 22.5 U/mL PK, 54 U/mL LDH and 30 μg/mL SR protein in buffer A. Solution 2: Control or inhibitor dilutions in buffer A (concentrations corrected for a 1:3 dilution). Solution 3: 0.72 mM ATP in buffer A. 100 μL of solution 1 was mixed with 100 μL of solution 2 and 100 μL of solution 3 was added to start the reaction. After 5 min of incubation, the OD_{340} was measured kinetically at room temperature (n = 3) for at least 10 min. Typically, a 1 mM DMSO solution of inhibitor was diluted 1:100 in buffer A before making serial dilutions in buffer A. The amount of DMSO present did not influence the measured ATPase activity. The total ATPase activity was 7.0 μmol of ATP (mg of SR protein)$^{-1}$ min^{-1}.

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