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Natural products as starting materials for development of second-generation SERCA inhibitors targeted towards prostate cancer cells

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Abstract—An analysis of the binding of the 8-*O*-*N*-*tert*-butoxycarbonyl-12-aminododecanoyl derivative of 8-*O*-debutanoylthapsigargin to the target molecule, the SERCA pump, has revealed the importance of the length and flexibility of the side chain attached to O-8. Based on the analysis a series of analogues to the 2-unsubstituted analogue trilobolide has been constructed and shown to be equipotent with thapsigargin as SERCA inhibitors. Only the 12-Boc-aminododecaonoyl derivative, however, was found to be apoptotic.

1. Introduction

Currently, no treatment significantly prolongs the survival of men suffering from androgen-independent metastatic prostate cancer.[1] Treatment is complicated by the slow proliferating rate of prostate cancer cells, which makes the cells insensitive towards standard chemotherapeutics.[2–4] Recently, we have shown that the LNCaP prostate cancer cells inoculated in mice were growth-inhibited after intravenous administration of a prodrug of thapsigargin (Tg, Fig. 1; **1**).[5] This approach towards treatment of prostate cancer is based on the potency of Tg as an inhibitor of the endo/sarcoplasmic calcium ATPase (SERCA).[6–8] Inhibition of SERCA will induce an increase in cytosolic calcium concentration, which eventually will cause apoptosis.[2] Because of the ubiquitous presence of SERCA, systemic administration of Tg would result in severe side effects unless the drug is targeted. Targeting can be achieved by taking advantage of the ability of prostate cancer cells to secrete a proteolytic enzyme, prostate specific antigen (PSA), with narrow substrate specificity.[5]

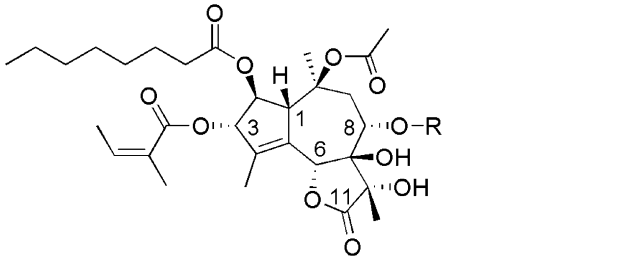

Figure 1. Structure of thapsigargin and analogues carrying a conjugation site.

Coupling of a derivative of Tg to a peptide only cleaved by PSA creates a prodrug, from which cytotoxic Tg

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analogue is only liberated in the vicinity of prostate cancer cells. The absence of amino groups in Tg prevents coupling of a peptide through a peptide bond. Replacement of the butanoyl group with a *N*-*tert*-butoxycarbonyl-12-aminododecanoyl (Boc-12-aminododecaonoyl) residue affords a derivative (**2**) showing similar SERCA inhibitory potency as Tg and possessing an amino group enabling coupling of the peptide promoiety.[9] A priori it could not be expected that replacement of the small butanoyl group with the large 12-Boc-aminododecanoyl group would afford a SERCA inhibitor, in contrast, the long side chain might be expected to interact sterically with transmembrane segments of the protein.

A problem with thapsigargin as the starting point for the development of SERCA inhibitors is the limited solubility in water and other solvents suitable for drug administration. In an attempt to develop second-generation SERCA inhibitors with improved properties, other natural products were investigated as potential lead structures.

As a starting point for finding other inhibitors we decided to elucidate the structure of **2** bound to SERCA. An analysis of this structure would reveal possibilities and limitations for substitution of the butanoyl group without losing the ability to inhibit SERCA.

2. Results and discussion

The overall structure of **2** bound to SERCA in the E2 conformation is similar to Tg bound to SERCA[10] with only a few local adjustments mainly changes of the conformations of the side chains of Leu253 and Ile315 (Fig. 2). The methylene groups of the long 12-Boc-aminododecanoyl group at O-8 are located between the transmembrane helices M3, M4 and M5. The flexibility of the chain enables the 12-aminododecanoyl group to adopt a conformation, which is adjusted between the transmembrane sections of the pump. The necessity of a flexible group is illustrated by the reduced SERCA inhibitory activities of analogues with an aromatic ring in the side chain.[11] The *tert*-butoxycarbonyl (Boc) group is placed in a cavity between the M1, M2 and the M3, M4 helices. In this position, the Boc-group becomes exposed to the solvent and is not affected by sterical constraints from segments in the pump. The appearance of the Boc-group towards the surface of the pump, consequently, explains why **3** is equipotent to Tg as a pump inhibitor, whereas analogues with shorter side chains are less potent.[9] Furthermore in the deprotected 12-amino analogue or an analogue, in which the Boc-group is replaced with a leucinoyl group, this location of the amino group might give the analogue an increased affinity for the pump because of possible hydrogen binding to Glu309.

2.1. Design of new inhibitors

The X-ray structure has revealed that derivatives of Tg inhibit SERCA, if they have a flexible side chain at O-8, and a bulky terminal group is tolerated provided there is sufficient length of the side chain. A GRID analysis of the binding site of Tg in SERCA has revealed that the lipophilic octanoyl residue is only of minor

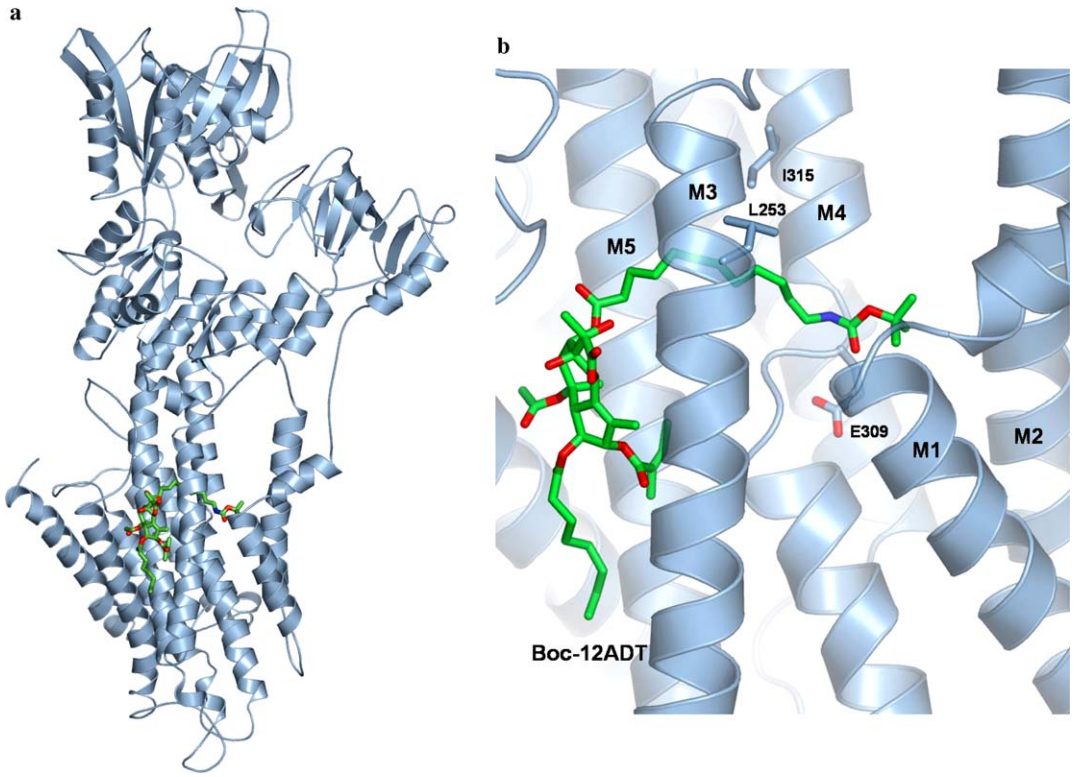


Figure 2. (a) Crystal structure of **2** bound to SERCA. (b) Crystal structure of **2** bound to SERCA the binding site of **2** is expanded. Figure prepared using Pymol (DeLano, 2002).

importance for the binding affinity. As a consequence, trilobolide (**4**) and derivatives hereof would be expected to block SERCA with potencies comparable to that of Tg. An interesting feature by the trilobolide analogues is that these compounds might be synthesized from nor-trilobolide (**5**) or from **4**. The latter has in addition to several *Thapsia* species also been isolated from *Laser trilobum* (L.) Borkh.[12] *L. trilobum* is growing wild in all of central Europe and western Asia.[13] Furthermore, in contrast to the Δ^4 double of Tg this double bond in trilobolide can be hydrogenated, affording a series of new derivatives, which might be derived into second generation of SERCA pump inhibitors.

2.2. Synthesis

Removal of the butanoyl group of **5** was accomplished by treatment with triethylamine in methanol. The higher reactivity of the secondary hydroxy group at O-8 enabled selective acylation to give the compounds **6**–**9** (Scheme 1).

2.3. Pharmacology

The SERCA pump inhibitory activities of the analogues **6**–**9** were tested in the coupled SERCA assay system (Table 1).[14,15,9] All the compounds were found to be almost equipotent to Tg as expected. Unexpectedly, however, it appeared that increasing lipophilicities of all the compounds **6**–**8** decreased their cytoxicities towards TSU cells, except in the case of **9**, the cytotoxicity of which is comparable to that of Tg. This is encouraging, since **9** is the only compound possessing a conjugation site for a peptide moiety.

3. Conclusion

The SERCA inhibitory potency of Tg remains if the butanoyl group at O-8 is replaced with flexible chains and bulky terminal groups are allowed if the chain possesses a sufficient length. Trilobolide and other 2-deoxythapsigargins are alternatives to Tg as starting materials for development of second-generation SERCA inhibitors to be used as prodrugs targeted towards prostate cancer cells.

Table 1. Pharmacological activities of **5**, **6**, **7**, **8** and **9**

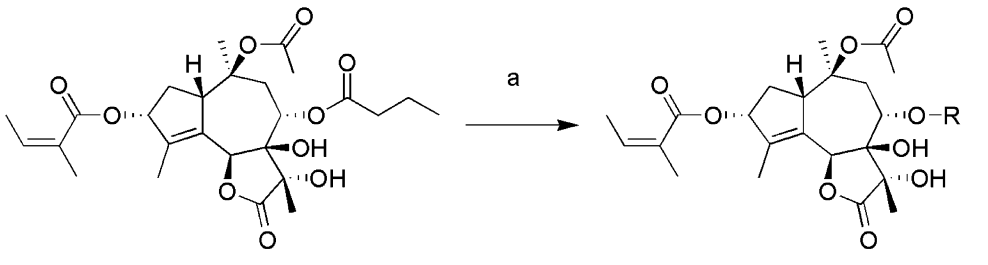
Compound	SERCA activity[a]	TSU-Pr1 activity[b]	C Log *P*[c]
Tg	1	1	6.16
5	1	17	2.94
6	2	20	5.06
7	1	100	6.11
8	1	67	7.17
9	1	3	6.50

[a] Relative potencies are stated (IC_{50} analogue/IC_{50} Tg).
[b] Relative potencies are stated (LC_{50} analogue/LC_{50} Tg) towards the human prostate cancer cell line, TSU-Pr1.
[c] C Log *P* values were calculated by ChemDraw Ultra, from CambridgeSoft.

4. Experimental

4.1. X-ray

Ca^{2+}-ATPase was prepared from sarcoplasmic reticulum vesicles which were isolated from rabbit skeletal muscle and the membrane protein was further extracted and purified with low concentration of deoxycholate according to established procedures.[16] To obtain crystals of the Ca^{2+}-ATPase in the H_nE_2 state (denoted E2), the protein was solubilized with 35 mM octaethyleneglycol dodecylether ($C_{12}E_8$) in 100 mM MOPS (pH 6.8), 20% glycerol, 80 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA and 1 mM AMPPCP in the presence of 125 μM **2** followed by ultracentrifugation for 35 min at 50,000 rpm in a Beckman TLA 110 rotor. The final protein concentration was 12 mg/mL and the supernatant was kept on ice overnight and subjected to a final ultracentrifugation for 15 min at 70,000 rpm right before crystallization setup. Initial crystallization screens were inspired by previously published results.[17] The vapour diffusion method with hanging drops was used for crystallization at 12 °C with 2 + 2 μL volumes of protein solution and reservoir buffer (12% (w/v) PEG2000 mono-methylether, 200 mM $MgSO_4$ and 6% MPD). Single, square crystals appeared after a few days and grew



5

4 R = $COCH(CH_3)CH_2CH_3$
6 R = $CO(CH_2)_6CH_3$
7 R = $CO(CH_2)_8CH_3$
8 R = $CO(CH_2)_{10}CH_3$
9 R = $CO(CH_2)_{11}NHCOOC(CH_3)_3$

Scheme 1. Reagents and conditions: (a) (1) Et_3N, methanol (dry), rt, 5 h; (2) octanoic acid or decanoic acid or dodecanoic acid or 12-*tert*-butoxycarbonyl-aminododecanoic acid, DMAP, DCC, DCM (dry), rt, 3 h, yield over two steps; **6**, 68%; **7**, 63%; **8**, 63%; and **9**, 55%.

to a maximum size of 250 × 250 × 50 μm within 2 weeks. Crystals were stabilised by increasing the glycerol concentration in the reservoir to a final concentration of 20% (w/v) glycerol followed by overnight equilibration at 5 °C. Crystals were mounted directly from the mother liquor in nylon loops (Hampton Research) and flash-cooled in liquid nitrogen. Diffraction data were collected at the Protein Structure Factory beamline BL14.2 of Free University Berlin at BESSY using a wavelength of 1.7 Å to maximise the separation of reflections on a Mar345 detector (Mar Research). Data were processed and merged using the XDS package.[18]

4.2. Structural analysis

The crystallographic data in space group $P4_12_12$ obtained from the E2:**2**:AMPPCP complex were isomorphous to data of the E2:Tg:AMPPCP complex (Jensen A. L., Møller J. V. and Nissen P., unpublished). The structure of E2:**2**:AMPPCP was solved accordingly on the basis of the E2:Tg:AMPPCP structure (Table 2). The Boc-C8 extension on Tg was built using the model building program O,[19] and the resulting structure refined using CNS[20] with the use of the topology and parameter files derived from the PRODRG server.[21] The final model was inspected with PROCHECK.[22]

The overall structure of E2:**2**:AMPPCP is similar to the E2:Tg:AMPPCP structure with only a few local adjustments. The $F^{\mathbf{2}}_{\mathrm{obsd}} - F^{\mathrm{Tg}}_{\mathrm{obsd}}$ difference Fourier map was valuable for the identification of significant, structural differences and showed three positive peaks at 6.33σ, 5.22σ and 5.10σ (background maximum of 4.90σ) which mapped between the transmembrane helices M3–M5 and M1–M2 and in extension from the derivatized side chain of on **2**. This served as a positive localization of the extension on **2**. A negative peak at 6.95σ corresponded to a movement of Leu253 in the M3 helix giving room for the Boc-12-aminododecanoyl extension upon binding. Similarly, the structural refinement indicated that the Ile315 side chain is adjusted.

Another strong peak in the $F^{\mathbf{2}}_{\mathrm{obsd}} - F^{\mathrm{Tg}}_{\mathrm{obsd}}$ difference Fourier map corresponds to local movements of the conserved ^{181}TGES motif in the cytoplasmic A domain and the AMPPCP co-factor. We ascribe these changes to the slightly different crystallization conditions for E2:**2** compared to E2:TG (magnesium sulfate vs sodium acetate as a major salt component of the buffer) since no other movements are observed in interconnecting regions between the inhibitor binding site in the membrane and the TGES motif in cytoplasmic headpiece of the protein.

Electron density maps calculated by σ_A-weighted $2F_{\mathrm{obsd}} - F_{\mathrm{calcd}}$ coefficients show density for the Boc-group located in a cavity between the M1–M2 and the M3–M4 helices.

The structure is deposited with the RCSB Protein Data Bank with accession code 2by4.

Table 2. Data for the X-ray structure E2:**2**:AMPPCP

Data	E2:**2**:AMPPCP
Spacegroup	$P4_12_12$
Cell dimensions (Å)	a = 71.74; b = 71.74; c = 593.7
Wavelength (Å)	1.7
Resolution (Å)	30–3.3 (3.43–3.30)[a]
R_{sym}[b]	15.7 (67.0)
$I/\sigma I$	7.4 (1.6)
Redundancy	5.2 (1.7)
Completeness (%)	82.0 (62.3)
Unique reflections	20425
B-value, Wilson plot (Å^2)[c]	105.4
Model	
Residues	994 amino acids, 2 Mg^{2+}, 1 Na^+, AMPPCP
Resolution	15–3.3 (3.4/3.3)
R/R_{free} (%)[d]	26.6/31.6 (35.7/35.2)
Ramachandran (%)	77.5/22.1/0.3/0.1
Rmsd bonds (Å)	0.0096
Rmsd angles (°)	1.65
Average B-value (Å^2)[e]	80.8

[a] Values in parentheses refer to the highest resolution shell, except when otherwise stated.

[b] $R_{\mathrm{sym}} = \sum_h\sum_i|I_i(h) - \langle I(h)\rangle|/\sum_h\sum_i|I_i(h)$, $I_i(h)$ is the ith measurement.

[c] Determined by TRUNCATE of the CCP4 program package.

[d] R_{free} is the R-factor calculated for a 2.5% subset of reflections excluded from the refinement.

[e] Fractions of residues in most favourable/allowed/generous/disallowed regions of the Ramachandran plot according to PROCHECK.

4.3. Chemistry general

All reactions were performed under a nitrogen atmosphere. Dry glassware was heated in an oven at a temperature above 125 °C for at least 6 h and was quickly assembled and cooled under a stream of dry inert gas. Rotary evaporation was performed under reduced pressure (min. 25 mbar) and at a maximum temperature of 40 °C. High vacuum refers to a pressure of approximately 2 mmHg.

Petrol refers to the fraction of light petroleum ether bp (40–60 °C). Dichloromethane was distilled from calcium hydride. All other reagents and solvents were purified by standard procedures or used as obtained from the supplier. All reactions were followed by thin layer chromatography. Analytical thin layer chromatography was preformed on precoated aluminium sheets Merck Silica Gel 60 F_{254}. Column chromatography was carried out using Merck Kieselgel 60 (230–400 mesh). Compounds were visualized by UV fluorescence (λ = 254 nm), by staining with a 1% naphthoresorcinol solution in ethanolic sulfuric acid (1 M) and visualized by heating. Optical rotations were measured using a Perkin-Elmer 241 Polarimeter. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury 300 instrument at room temperature. Abbreviations used for multiplicities: br, broad signal; s, singlet; d, doublet; t, triplet, and q, quartet, and p, pentet. The signal of the solvent was used as an internal reference. *Marked shifts may be interchanged. Target compounds are characterized by ^{1}H and ^{13}C NMR, optical rotation and HRMS. HRMS was recorded on a JEOL AX505 W instrument. Analyzing COSY, NOESY, HMBC and HMQC assisted

assignment of ^{1}H NMR and ^{13}C NMR signals. Target compounds only showed one spot by TLC and their NMR spectra showed no foreign signals.

4.3.1. 8-*O*-(Octanoyl)-8-*O*-debutanoylnortrilobolide (6). To a stirred solution of nortrilobolide (**5**) (25 mg, 0.05 mmol) in dry methanol (10 mL) triethylamine (49.6 mg, 0.5 mmol) was added at room temperature. After stirring for 5 h at this temperature, the mixture was concentrated under reduced pressure, and the residue was used in the next step without further purification. 8-*O*-Debutanoylnortrilobolide (20.5 mg) was obtained as a colourless gum. To a stirred solution of 8-*O*-debutanoylnortrilobolide (20 mg, 0.05 mmol) in dry dichloromethane (2 mL), octanoic acid (6.6 mg, 0.06 mmol) and DMAP (6.1 mg, 0.06 mmol) were added at room temperature. A solution of DCC (10.3 mg, 0.06 mmol) in dry dichloromethane (0.5 mL) was added after cooling to 0 °C. The mixture was stirred at this temperature for 1 h and then left at room temperature for additional 3 h. The crude product was finally concentrated under reduced pressure and subjected to flash chromatography (petrol/EtOAc, 3:1), providing the target compound **6** (68% over two steps) as a colourless gum. HRMS Calcd for $C_{30}H_{44}O_{10}Na$: 587.2832 Found: 587.2826. $[\alpha]_D^{25}$ −75.4 (*c* 0.8, $CHCl_3$). ^{1}H (300 MHz, $CDCl_3$): δ 6.03 (1H, qq, J = 1.5, 7.2 Hz, angeloyl H-3), 5.63 (1H, s, H-6), 5.55 (1H, t, J = 3.6 Hz, H-8), 5.52–5.46 (1H, m, H-3), 4.36–4.28 (1H, m, H-1), 3.52* (1H, br s, 7-OH), 3.08 (1H, dd, J = 3.3, 14.7 Hz, H-9), 2.68* (1H, br s, 11-OH), 2.51–2.40 (1H, m, H-2), 2.21 (2H, t, J = 7.2 Hz, octanoyl H-2), 2.06 (1H, dd, J = 3.6, 14.7 Hz, H-9′), 1.94 (3H, dq, J = 1.5, 7.2 Hz, angeloyl H-4), 1.90 (3H, s, acetyl H-2), 1.86–1.83 (6H, m, H-15, angeloyl C-2 CH_3), 1.61–1.51 (3H, m, H-2′, octanoyl H-3), 1.41 (3H, s, H-13), 1.25 (3H, s, H-14), 1.24–1.18 (8H, m, octanoyl H-4 to H-7), 0.81 (3H, t, J = 6.3 Hz, octanoyl H-8). ^{13}C (75 MHz, $CDCl_3$): δ 175.7 (C-12), 173.0 (octanoyl C═O), 171.1 (acetyl C═O), 167.8 (angeloyl C═O), 143.5* (C-4) 138.7 (angeloyl C-3), 131.6* (C-5) 127.9 (angeloyl C-2), 86.1 (C-10), 79.9 (C-3), 79.0** (C-7), 78.9** (C-11), 77.5** (C-6), 68.8 (C-8), 51.0 (C-1), 38.8 (C-9), 35.0 (octanoyl C-2), 32.5 (C-2), 32.0 (octanoyl C-6), 29.5*** (octanoyl C-4), 29.3***(octanoyl C-5), 24.9 (octanoyl C-3), 23.0 (octanoyl C-7), 22.9 (C-14), 22.5 (acetyl C-2), 21.1 (angeloyl C-2 *C*H_3), 16.7 (C-13), 16.3 (angeloyl C-4), 14.5 (octanoyl C-8), 13.5 (C-15).

4.3.2. 8-*O*-(Decanoyl)-8-*O*-debutanoylnortrilobolide (7). Compound **7** was prepared as described for **6** using decanoic acid instead of octanoic acid. Product **7** (63% over two steps) was obtained as a colourless gum. HRMS Calcd for $C_{32}H_{48}O_{10}Na$: 615.3145. Found: 615.3191. $[\alpha]_D^{25}$ −66.9 (*c* 0.8, $CHCl_3$). ^{1}H (300 MHz, $CDCl_3$): δ 6.02 (1H, qq, J = 1.5, 7.2 Hz, angeloyl H-3), 5.61 (1H, s, H-6), 5.58 (1H, t, J = 3.6 Hz, H-8), 5.52–5.46 (1H, m, H-3), 4.36–4.28 (1H, m, H-1), 3.58* (1H, br s, 7-OH) 3.08 (1H, dd, J = 3.3, 14.4 Hz, H-9), 2.58* (1H, br s, 11-OH) 2.51–2.40 (1H, m, H-2), 2.21 (2H, t, J = 7.2 Hz, decanoyl H-2), 2.07 (1H, dd, J = 3.6, 14.7 Hz, H-9′), 1.95 (3H, dq, J = 1.5, 7.2 Hz, angeloyl H-4), 1.91 (3H, s, acetyl H-2), 1.86–1.83 (6H, m, H-15, angeloyl C-2 CH_3), 1.61–1.51 (3H, m, H-2′, decanoyl H-3), 1.41 (3H, s, H-13), 1.24 (3H, s, H-14), 1.23–1.17 (12H, m, decanoyl H-4 to H-9), 0.81 (3H, t, J = 6.3 Hz, decanoyl H-10). ^{13}C (75 MHz, $CDCl_3$): δ 175.5 (C-12), 172.8 (decanoyl C═O), 170.9 (acetyl C═O), 167.7 (angeloyl C═O), 143.4* (C-4) 138.7 (angeloyl C-3), 131.5* (C-5) 127,7 (angeloyl C-2), 85.9 (C-10), 79.7 (C-3), 78.9** (C-7), 78.7** (C-11), 77.5** (C-6), 66.7 (C-8), 50.9 (C-1), 37.7 (C-9), 34.9 (decanoyl C-2), 32.5 (C-2), 32.0 (decanoyl C-8), 29.9*** (decanoyl C-4), 29.8*** (decanoyl C-5), 29.5*** (decanoyl C-6), 29.4*** (decanoyl C-7), 24.7 (decanoyl C-3), 22.9 (decanoyl C-9), 22.7 (C-14), 22.4 (acetyl C-2), 21.0 (angeloyl C-2 *C*H_3), 16.7 (C-13), 16.3 (angeloyl C-4), 14.4 (decanoyl C-10), 13.5 (C-15).

4.3.3. 8-*O*-(Dodecanoyl)-8-*O*-debutanoylnortrilobolide (8). Compound **8** was prepared as described for **6** using dodecanoic acid instead of octanoic acid. Product **8** (63% over two steps) was obtained as a colourless gum. HRMS Calcd for $C_{34}H_{52}O_{10}Na$: 643.3458. Found: 643.3466. $[\alpha]_D^{25}$ −142.7 (*c* 0.6, $CHCl_3$). ^{1}H (300 MHz, $CDCl_3$): δ 6.11 (1H, qq, J = 1.5, 7.2 Hz, angeloyl H-3), 5.59 (1H, s, H-6), 5.63 (1H, t, J = 3.6 Hz, H-8), 5.61–5.53 (1H, m, H-3), 4.44–4.37 (1H, m, H-1), 3.67* (1H, br s, 7-OH), 3.15 (1H, dd, J = 3.3, 14.7 Hz, H-9), 2.76* (1H, br s, 11-OH), 2.56–2.47 (1H, m, H-2), 2.27 (2H, t, J = 7.2 Hz, dodecanoyl H-2), 2.12 (1H, dd, J = 3.6, 14.7 Hz, H-9′), 2.01 (3H, dq, J = 1.5, 7.2 Hz, angeloyl H-4), 1.96 (3H, s, acetyl H-2), 1.92–1.88 (6H, m, H-15, angeloyl C-2 CH_3), 1.65–1.55 (3H, m, H-2′, dodecanoyl H-3), 1.47 (3H, s, H-13), 1.29 (3H, s, H-14), 1.27–1.13 (16H, m, dodecanoyl H-4 to H-11), 0.87 (3H, t, J = 6.6 Hz, dodecanoyl H-12). ^{13}C (75 MHz, $CDCl_3$): δ 175.7 (C-12), 173.0 (dodecanoyl C═O), 171.2 (acetyl C═O), 167.8 (angeloyl C═O), 143.4* (C-4) 138.7 (angeloyl C-3), 131.6* (C-5) 127,9 (angeloyl C-2), 86.2 (C-10), 79.9 (C-3), 79.0** (C-7), 78.9** (C-11), 77.5** (C-6), 66.7 (C-8), 51.0 (C-1), 38.7 (C-9), 35.0 (dedecanoyl C-2), 32.5 (C-2), 32.2 (dodecanoyl C-10), 29.9*** (dodecanoyl C-4), 29.8*** (dodecanoyl C-5), 29.7*** (dodecanoyl C-6), 29.6*** (dodecanoyl C-7), 29.5*** (dodecanoyl C-8, C-9), 24.8 (dodecanoyl C-3), 23.0 (dodecanoyl C-11), 22.8 (C-14), 22.5 (acetyl C-2), 21.1 (angeloyl C-2 *C*H_3), 16.6 (C-13), 16.3 (angeloyl C-4), 14.5 (C-12), 13.5 (C-15).

4.3.4. 8-*O*-(12-*tert*-Butoxycarbonylaminododecanoyl)-8-*O*-debutanoylnortrilobolide (9). Compound **9** was prepared as described for **6** using 12-*tert*-butoxycarbonyl-aminododecanoic acid instead of octanoic acid. Product **9** (59% over two steps) was obtained as a colourless gum. HRMS Calcd for $C_{39}H_{61}NO_{12}Na$: 758.4091. Found: 758.4103. $[\alpha]_D^{25}$ −50.5 (*c* 1.8, $CHCl_3$). ^{1}H (300 MHz, $CDCl_3$): δ 6.10 (1H, qq, J = 1.5, 7.2 Hz, angeloyl H-3), 5.70 (1H, s, H-6), 5.64 (1H, t, J = 3.6 Hz, H-8), 5.60–5.53 (1H, m, H-3), 4.40–4.32 (1H, m, H-1), 3.12–3.04 (3H, m, H-9, dodecanoyl H-12), 2.56–2.47 (1H, m, H-2), 2.28 (2H, t, J = 7.2 Hz, dodecanoyl H-2), 2.17 (1H, dd, J = 3.3, 14.4 Hz, H-9′), 2.01 (3H, dq, J = 1.5, 7.2 Hz, angeloyl H-4), 1.96 (3H, s, acetyl C-2), 1.91–1.85 (6H, m, H-15, C-2 CH_3),

1.65–1.55 (5H, m, H-2′, dodecanoyl H-3 H-11), 1.47 (3H, s, H-13), 1.43 (9H, s, Boc CH_3), 1.30 (3H, s, H-14), 1.27–1.23 (14H, m, dodecanoyl H4 to H10). ^{13}C (75 MHz, $CDCl_3$): δ 175.9 (C-12), 172.9 (dodecanoyl C═O), 171.0 (acetyl C═O), 167.8 (angeloyl C═O), 156.2 (Boc C═O), 143.2* (C-4) 138.7 (angeloyl C-3), 131.9* (C-5) 127,9 (angeloyl C-2), 86.2 (C-10), 80.0 (C-3), 79.5 (Boc *tert*-C), 79.0** (C-7), 78.9** (C-11), 77.5** (C-6), 66.7 (C-8), 51.0 (C-1), 41.0 (dodecanoyl C-12), 38.8 (C-9), 35.0 (dodecanoyl C-2), 33.9 (dodecanoyl C-11), 32.5 (C-2), 30.4*** (dodecanoyl C-4), 29.6*** (dodecanoyl C-5), 29.5 (Boc CH_3), 28.8*** (dodecanoyl C-6 C-7), 27.1*** (dodecanoyl C-8), 25.8*** (dodecanoyl C-9), 25.2*** (dodecanoyl C-11) 24.8 (dodecanoyl C-3), 22.9 (C-14), 22.4 (acetyl C-2), 21.1 (angeloyl C-2 CH_3), 16.6 (C-13), 16.3 (angeloyl C-4), 13.5 (C-15).

4.4. Pharmacology

4.4.1. SERCA activity. Isolation of sarcoplasmic reticulum (SR) vesicles containing Ca^{2+}-ATPases from rabbit muscle was done as previously described.[9] The SR vesicles were stored at −80 °C prior to use. The Ca^{2+}-ATPase inhibitory potencies of the analogues were determined as previously described[14,15] with a coupled enzyme assay. The activity of each nortrilobolide analogue and control was determined in triplicate. Plotting the ATPase activity as a function of inhibitor concentration the IC_{50} value was estimated by fitting using the program Origin version 5.0 from Microcal™ Origin™, Microcal Software Inc.

4.4.2. Cytotoxicity. The apoptotic activity of each nortrilobolide analogue against TSU-Pr1 human prostatic cancer cells was determined as previously described.[9] 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.

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