Structure–Activity Relationships of Analogues of Thapsigargin Modified at O-11 and O-12

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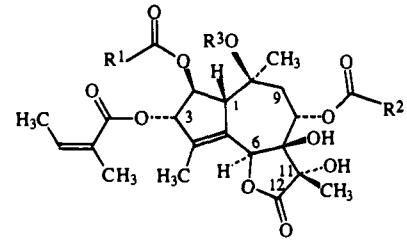
A number of analogues of thapsigargin have been synthesized by alkylating or acylating O-11 and O-12 in the lactol obtained by reducing thapsigargicin. Introduction of α-disposed substituents decreased the Ca^{2+}–ATPase inhibitory potency of the analogue, whereas the enzyme was more tolerant toward β-disposed substituents, indicating that the α-face of the lactone ring is in close contact with the binding site when the inhibitor is bound to the enzyme.

Introduction

The Ca^{2+}–ATPases of the plasma membrane (the PMCA family) and the sarco/endoplasmic membranes (the SERCA family) are crucially involved in the fine tuning of the cytosolic Ca^{2+} concentration and hence in the general control of cellular functions. The enzymes belong to the P-type of cation-transporting enzymes which after phosphorylation of an aspartic residue translocate cations across the membrane.[1] The amino acid sequences of a number of the Ca^{2+}–ATPases[2,3] have been elucidated, and the structures at 14 Å resolution have been determined by electron microscopy.[4] On the basis of the amino acid sequences, site-specific mutagenesis,[5,6] chimeric enzymes consisting of parts from the Ca^{2+}–ATPase and the Na^+,K^+–ATPase,[7,8] and labeling experiments,[9] the phosphorylation site and the binding sites for Ca^{2+} and ATP in the SERCA–ATPases have been deduced. The detailed mechanism of action, including an understanding of the conformational changes involved in the Ca^{2+} translocation, however, is only poorly understood. The sesquiterpene lactone thapsigargin (**1**) selectively[10] and with a subnanomolar dissociation constant[11] binds to the enzyme, forming a catalytically inactive dead-end complex.[12] The loss of conformational flexibility by binding of thapsigargin indicates that the binding site is affected when the enzyme changes conformation. An improved knowledge of the topography of the binding site, thus, might help to understand the displacements of the segments during the cation translocation. Studies on the binding of thapsigargin to chimeric Ca^{2+}–ATPase/Na^+,K^+–ATPase molecules led to the conclusion that the binding site is located between amino acid residue 200 and 348, and in the light of the lipophilicity of the molecule, the binding site was assumed to be located within the transmembrane segments M3 or M4.[13] Amino acid residues in the transmembrane segments M4, M5, M6, and M8 are crucial for the binding and translocation of Ca^{2+}.[14]

The binding site for thapsigargin can be characterized through structure–activity relationships. Previous attempts to estimate the excluded and essential volume of the binding site have disclosed that hydrolysis of the acetyl group at O-10 to give deacetylthapsigargin (**2**) or epimerization of either of the chiral centers C-3 or C-8 removes the potent ATPase-inhibiting activity.[15] Exchange of the angeloyl residue with an octanoyl or acetylation of both hydroxy groups reduced the potency by a factor of about 10. Acetylation of one of the hydroxy groups or reduction of the lactone carbonyl into a methylene group[16] only to a limited extent affected the inhibitory potency when studied on the isolated enzyme, whereas acetylation of either of the hydroxy groups significantly reduces the effect of thapsigargin as a secretagogue.[17]



1 $R^1 = (CH_2)_6CH_3$, $R^2=(CH_2)_2CH_3$, $R^3 = OCCH_3$

2 $R^1 = (CH_2)_6CH_3$, $R^2 = (CH_2)_2CH_3$, $R^3 = H$

3 $R^1 = (CH_2)_4CH_3$, $R^2 = (CH_2)_2CH_3$, $R^3 = OCCH_3$

15 $R^1 = R^2 = (CH_2)_6CH_3$, $R^3 = H$

The mixture of anomeric lactols (**4** + **5**) obtained by reducing thapsigargicin (**3**) inhibits the Ca^{2+}–ATPase almost as potently as thapsigargin (Table 1). The presence of the two hydroxy groups at C-12 and C-11 in the lactol gives a possibility for introduction of different substituents in these positions. The present paper presents syntheses and Ca^{2+}–ATPase inhibitory activities of such analogues. In addition we have tested if analogues possessing a poor ATPase–inhibiting activity bind to the ATPase without locking the enzyme into the "dead-end" conformation. Such poor inhibitors might prevent binding of thapsigargin and other negative effectors to the enzyme. The consequence of this binding would be that the cells were protected against damages caused by the increased Ca^{2+} concentration caused by these negative effectors.

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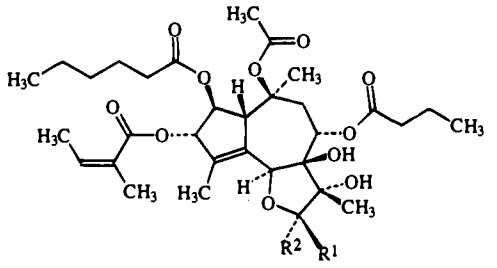
Chemistry

The key lactol was obtained as a mixture of the two epimeric hemiacetals **4** and **5** by reduction of **3** with sodium bis(2-methoxyethoxy)ethoxyaluminum hydride, which was found to be superior to sodium borohydride.[17] A general procedure for formation of an alkyl acetal is an acid-catalyzed transacetalation between the hemiacetal and an orthoformic ester of the alcohol.[18] At-



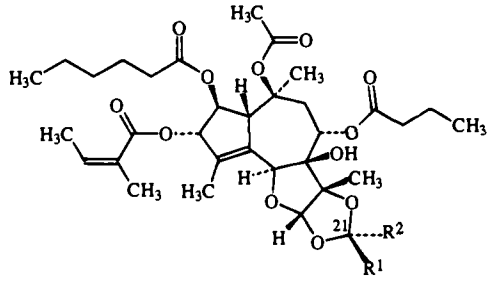
4 R^1 = H, R^2 = OH

5 R^1 = OH, R^2 = H

8 $R^1 = OCH_2CH_3$, R^2 = H

10 R^1 = H, $R^2 = OOCCH_3$

tempts to prepare **8** in this way afforded **8** together with the two epimeric orthoesters **6** and **7**. The stereochemistry of the orthoester carbon was verified by NOESY spectroscopy. In compound **8** an NOE interaction between H-12 and H-6 was observed. The stereochemistry at C-12 in **6** and **7** is defined by the stereochemistry at C-11 since the two five-membered rings have to be cis fused. In **7** a nuclear Overhauser enhancement between the methylene hydrogens of the ethoxy group and H-6 was demonstrated, whereas this interaction was missing in **6** in which, however, H-6 interfered with the hydrogen at the new asymmetric center. Compound **9** was prepared by treatment of **4** and **5** with acetone in the presence of 2,2-dimethoxypropane and Dowex 50 H^+.



6 $R^1 = OCH_2CH_3$, R^2 = H

7 R^1 = H, $R^2 = OCH_2CH_3$

9 $R^1 = R^2 = CH_3$

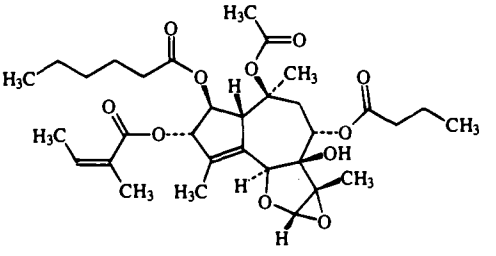
Attempts to prepare orthoacetates by allowing the hemiacetals **4** and **5** to react with triethyl orthoacetate beside **8** only afforded the acetate **10**. The stereochemistry at C-12 in **10** was established by NOESY spectroscopy. The α-disposal of the acetate group is in accordance with a reaction path, in which **10** is formed by rearrangement of an intermediate orthoacetate.

Table 1. IC_{50} Values for Inhibition of Rabbit White Muscle Microsomal Ca^{2+}–ATPase of a Number of Thapsigargicin Analogues Modified at the γ-Lactone Ring[a]

analogue	IC_{50} ± SD (nM)	R^b	no. of experiments
1	9.6 ± 0.25	1	9
3	9.8 ± 0.4	1.02	6
8	11.2 ± 1.5	1.17	3
4 + **5**	15.6 ± 2.0	1.63	4
10	18.4 ± 1.3	1.92	6
6	24.1 ± 0.6	2.51	3
11	51.7 ± 4.5	5.39	4
14	119.9 ± 7.7	12.5	3
9	157.0 ± 9.2	16.4	3
7	387.5 ± 49.2	40.4	3
12	5.59×10^3 ± 0.53	582	4
13	60.6×10^3 ± 0.4	6312	4

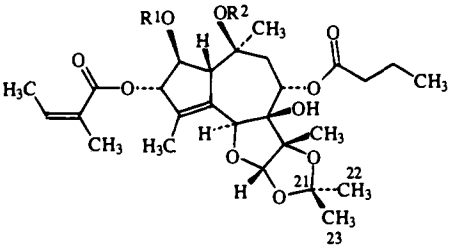
[a] Sarcoplasmic vesicles isolated from rabbit white muscles are incubated with different concentrations of the inhibitor, and the decrease in rate of formation of inorganic phosphate by hydrolysis of ATP in the presence of Ca^{2+} and Mg^{2+} is measured determining phosphate as a phosphate–malachite green complex. Eight different concentrations affording an inhibition of the enzyme between 10 and 90% were used, and the IC_{50} value was estimated after the date had been fit to eq 1. [b] *R* equals the ratio between the IC_{50} value of the analogue and the IC_{50} value of thapsigargin. A high *R* value designates a poor inhibitor.

Reaction of **4** and **5** with *N,N*-dimethylformamide diethyl acetal afforded the epoxide **11**[19] and some methyl acetal.



11

The starting material for the 10-deacetyl acetonide **12** was **9**. By saponification, **9** afforded **13**. The high sensitivity of the butyric ester group in **1** and **3** toward bases has been explained by assuming an anchimeric assistance from the 11-hydroxy group. The missing cleavage of the butyric ester group in **9** confirms that the 11-hydroxy group assists the hydrolysis of the butyric ester in **1** and **3** by anchimeric assistance since masking of this group changes the reaction path. The



12 $R^1 = OC(CH_2)_4CH_3$, R^2 = H

13 R^1 = H, R^2 = H

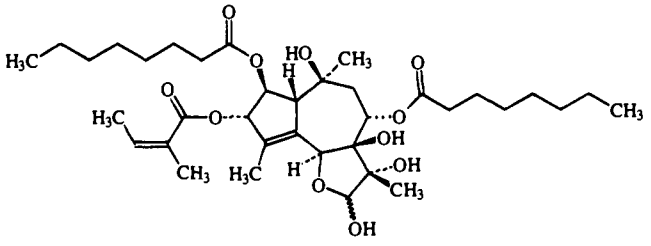
apparently parallel hydrolysis of the secondary hexanoic ester and the tertiary acetyl ester in **9** might again involve an anchimeric assistance. It is assumed that the hydroxy group formed by cleavage of the hexanoic ester assists the cleavage of the acetate ester. Selective acylation of the secondary hydroxy group in **13** affords

Table 2. Additive Inhibition of the Ca^{2+}–ATPase by Coincubation of Thapsigargicin Analogues, Modified at the γ-Lactone Ring, with Thapsigargin[a]

		activity of the Ca^{2+}–ATPase compared to control (%)		
analogue	concntration (nM)	1	analogue	1 + analogue
7	1		101.4	52.5
	20	53.3	98.4	41.6
	100		78.6	36.8
9	1		100.3	55.7
	20	53.9	81.2	49.3
	100		66.6	34.7
12	100		97.0	52.3
	500	56.5	87.1	49.0
	5000		53.7	24.0
13	1000		98.6	53.8
	10 000	56.7	82.6	43.5
	50 000		50.9	25.1
14	1		98.4	51.1
	30	49.4	68.9	34.6
	200		21.9	16.2

[a] Sarcoplasmic vesicles isolated from white rabbit muscles are incubated with either different concentrations of the inhibitor, a concentration of thapsigargin inducing a 50% inhibition, or different concentrations of the inhibitor together with thapsigargin. The decrease in rate of formation of inorganic phosphate by hydrolysis of ATP in the presence of Ca^{2+} and Mg^{2+} is measured determining phosphate as a phosphate–malachite green complex.

12. The dioctanoate lactol **14** was prepared by reduction of the corresponding lactone **15**.[20]



14

Biological Results

The Ca^{2+}–ATPase inhibitory potencies of the analogues were determined by incubation of the analogues with membrane vesicles of rabbit white muscles containing Ca^{2+}–ATPases. The rate of ATP hydrolysis was estimated from the rate of formed phosphate, which was measured spectrophotometrically as a phosphate–malachite green complex. The IC_{50} values of the analogues were estimated by fitting the data to eq 1:

$$E = a/(1 + b \times S^c) + d \quad (1)$$

in which S is the concentration of the analogues, E is the rate of hydrolysis of ATP compared to the rate of the noninhibited ATPase, and a, b, c, and d are constants. The estimated IC_{50} values are given in Table 1.

In order to test the hypothesis that some of the compounds might abolish the inhibition of Ca^{2+}–ATPases, the ATPase was incubated with thapsigargin as well as one of the analogues, **7, 9, 12, 13,** or **14**. As illustrated in Table 2, the two inhibitors had an additive effect on the ATPase.

Discussion

Replacement of the octanoyl residue in thapsigargin (**1**) with a hexanoyl residue (thapsigargicin, **3**) does not significantly reduce the IC_{50} value. In contrast, **3** was found to have almost only one-half the potency of **1** as a histamine secretagogue on peritoneal rat mast cells.[17] These conflicting observations might be explained by different absorption, metabolism, or distribution of the two analogues. Reduction of the carbonyl group of **3** to give a mixture of the two anomeric lactols **4** and **5** causes a small decrease in the IC_{50} value. This loss, however, is almost regained by locking the lactol into the β-anomer by acetalization with a small alkyl group like ethyl (**8**). If the lactol is locked into the α-anomer as the acetyl ester **10**, an additional loss of activity is observed, although the compound still is potent.

Comparison of the activity of the three compounds **6**, **7**, and **9**, all of which contain an additional five-membered ring, might be informative for understanding the binding of thapsigargin to the binding site. The orthoesters **6** and **7** both possess greater IC_{50} values than the lactols; by far the greatest loss of activity, however, is observed for the α-isomer **7**. The importance of the α-face of the lactone ring is further emphasized by the poor activity of the acetonide **9**. The decreased activity of the compounds **6, 7,** and **9** might be explained by the masking of the 11-hydroxy group. Acetylation of this group, however, previously has been found only to affect the potency to a limited extent. The strong decrease in the affinity by introduction of an α-substituent at the dioxolane carbon in the additional ring thus indicates that the α-face of the lactone ring is in close contact with the binding site.

Removal of the acetyl group leaving a free hydroxy group in the 10-position (**14**) strongly decreased the activity. The previously described trend that an increase of the volume of the α-face of the molecule, e.g., by acetonide formation, leads to decreased activity was confirmed. Additional cleavage of the hexanoic ester to give the very polar derivative **13** afforded a very poor inhibitor.

As illustrated in Table 2 an additive inhibition of the Ca^{2+}–ATPase was obtained by coincubation of the enzyme with thapsigargin (**1**) and one of the poor inhibitors, **7**, **9**, **12**, **13**, or **14**. Thus, none of these compounds prevents thapsigargin from binding to the enzyme.

Experimental Section

^1H NMR, ^{13}C NMR, NOESY, and DEPT (135°) were recorded on a Bruker AC-200F spectrometer. Only the signals originating in the sesquiterpene skeleton are given, since the signals due to the acyl groups were found as previously reported.[21] Accurate mass determinations (MS) were carried out on a Jeol AX505W mass spectrometer run in negative mode FAB. NBA was used as a matrix and PEG-600 as a reference with dual target probe. HPLC was performed using a Waters 6000 A pump, a prepacked Knauer column (8 × 250 mm, LiChrosorb RP18, 7 μm, flow 2.5 mL/min), and a Shimadzu SPD 6A UV detector at 230 nm. The homogeneities of all compounds were ensured by final purification by preparative HPLC (Knauer prepacked column, 16 × 250 mm, LiChrosorp RP18, eluent: methanol–water–acetic acid). Column chromatography (CC) was performed over silica gel-A (Merck, 0.063–0.200 mm) or silica gel-B (Merck, 0.040–0.063 mm).

Thapsigargicin Lactol (4 + 5). To a solution of thapsigargicin (**3**; 40 mg, 64 μmol) in dry toluene (4 mL) was added sodium bis(2-methoxyethoxy)ethoxyaluminum hydride (340 μmol) over 20 min at −70 °C under a nitrogen atmosphere. After stirring for 1 h water (10 mL) was added and the mixture acidified with 4 M hydrochloric acid. The aqueous phase was

extracted three times with ethyl acetate (15 mL), and the combined organic phases were dried ($MgSO_4$) and concentrated under vacuum. The residue was purified by CC [silica gel-A, eluent: toluene–ethyl acetate–acetic acid (85:15:1)] to give a mixture of **4** and **5** (17.2 mg, 43.0%) as a yellow oil and unreacted **3** (20.4 mg, 50%). MS: calcd ($C_{32}H_{48}O_{12}$), 623.3067; found, 623.3069. ^1H NMR and ^{13}C NMR data were in accordance with those given for the lactol of thapsigargin.[17]

11-*O*-12α-*O*-Thapsigargicin Lactol Ethyl Orthoformate (6 and 7) and 12β-*O*-Ethylthapsigargicin Lactol (8). To a solution of **4** and **5** (65.3 mg, 105 μmol) in dry tetrahydrofuran were added *p*-toluenesulfonic acid (10 mg) and triethyl orthoformate (3.0 mL, 18.0 mmol), and the solution was stirred for 25 min at room temperature. The solution was washed with a saturated aqueous solution of sodium hydrogen carbonate (10 mL) and a saturated aqueous solution of sodium chloride. The organic phase was dried ($MgSO_4$) and concentrated in vacuum, and the residue was purified by CC [siliga gel-B, 40 g, eluent: toluene–ethyl acetate (9:1)] to give **8** (18.3 mg, 26.8%), **6** (13.7 mg, 19.2%), and **7** (6.3 mg, 8.8%) as colorless amorphous powders.

Compound 6: MS calcd ($C_{35}H_{52}O_{13}$) 679.3329, found 679.3293; ^1H NMR (CD_3CN) δ 4.14 (bs, 1H, H-1), 5.37 (dd, *J* = 3.4, 2.5 Hz, 1H, H-2), 5.59 (bs, 1H, H-3), 4.94 (bs, 1H, H-6), 5.50 (t, *J* = 3.6 Hz, 1H, H-8), 2.91 (dd, *J* = 14.7, 3.3 Hz, 1H, H-9a), 2.25 (overlapped, localized by NOESY, 1H, H-9b), 5.51 (s, 1H, H-12), 1.43 (s, 3H, H-13), 1.27 (s, 3H, H-14), 1.76 (s, 3H, H-15), 5.99 (s, 1H, H-21), 3.52 (q, *J* = 7.1 Hz, 2H, H-22), 1.13 (t, *J* = 7.1 Hz, 3H, H-23); ^{13}C NMR (CD_3CN) δ 58.6 C-1, 79.0 C-2, 81.3 C-3, 134.6 C-4, 140.0 C-5, 76.6 C-6, 81.1 C-7, 66.6 C-8, 38.7 C-9, 85.4 C-10, 91.9 C-11, 107.1 C-12, 16.7 C-13, 23.4 C-14, 13.0 C-15, 117.5 C-21, 60.4 C-22, 16.1 C-23.

Compound 7: MS calcd ($C_{35}H_{52}O_{13}$) 679.3329, found 679.3359; ^1H NMR (CD_3CN) δ 4.14 (bs, 1H, H-1), 5.37 (dd, *J* = 3.1, 2.3 Hz, 1H, H-2), 5.58 (bs, 1H, H-3), 5.48 (bs, 1H, H-6), 5.55 (t, *J* = 3.7 Hz, 1H, H-8), 2.95 (dd, *J* = 14.5, 3.4 Hz, 1H, H-9a), 2.21 (overlapped, localized by NOESY, 1H, H-9b), 5.48 (s, 1H, H-12), 1.30 (s, 6H, H-13 and H-14), 1.74 (s, 3H, H-15), 5.89 (s, 1H, H-21), 3.60 (q, *J* = 7.1 Hz, 2H, H-22), 1.18 (t, *J* = 7.1 Hz, 3H, H-23); ^{13}C NMR (CD_3CN) δ 58.9 C-1, 79.3 C-2, 85.6 C-3, 135.5 C-4, 140.1 C-5, 76.4 C-6, 81.5 C-7, 66.5 C-8, 39.1 C-9, 85.7 C-10, 93.8 C-11, 108.2 C-12, 16.3 C-13, 24.0 C-14, 13.1 C-15, 119.4 C-21, 61.8 C-22, 16.0 C-23.

Compound 8: MS calcd ($C_{34}H_{52}O_{12}$) 651.3380, found 651.3380; ^1H NMR ($CDCl_3$) δ 4.14 (bs, 1H, H-1), 5.51 (dd, *J* = 4.5, 3.4 Hz, 1H, H-2), 5.75 (bs, 1H, H-3), 5.17 (bs, 1H, H-6), 5.49 (t, *J* = 3.7 Hz, 1H, H-8), 2.75 (dd, *J* = 14.7, 3.4 Hz, 1H, H-9a), 2.50 (dd, *J* = 14.7, 4.2 Hz, 1H, H-9b), 4.68 (s, 1H, H-12), 1.39 (s, 3H, H-13), 1.43 (s, 3H, H-14), 1.84 (s, 3H, H-15), 3.49 (dq, *J* = 9.8, 7.1 Hz, 1H, H-21a), 3.85 (dq, *J* = 9.8, 7.1 Hz, 1H, H-21b), 1.21 (t, *J* = 7.1 Hz, 3H, H-22); ^{13}C NMR ($CDCl_3$) δ 57.6 C-1, 78.2 C-2, 84.4 C-3, 133.8 C-4, 137.9 C-5, 77.6 C-6, 79.6 C-7, 67.2 C-8, 38.1 C-9, 84.8 C-10, 82.3 C-11, 108.1 C-12, 15.0 C-13, 22.8 C-14, 12.7 C-15, 64.1 C-21, 16.5 C-22.

11,12-*O*-Isopropylidenethapsigargicin Lactol (9). A solution of **4** and **5** (10 mg, 16 μmol) in dry acetone (12 mL) was added to a suspension of Dowex 50 H^+ (20 mg) in 2,2-dimethoxypropane (100 μL). After stirring for 3 h at room temperature, the reaction mixture was filtered. The filtrate was concentrated in vacuum and the residue purified by CC [silica gel-B, 4 g, eluent: toluene–ethyl acetate (6:1)] to give **9** (7.2 mg, 67.8%) as an amorphous powder. MS: calcd ($C_{33}H_{50}O_{11}$), 663.3380; found, 663.3362. ^1H NMR (CD_3CN): δ 4.15 (bs, 1H, H-1), 5.36 (dd, *J* = 3.2, 2.6 Hz, 1H, H-2), 5.59 (bs, 1H, H-3), 5.16 (bs, 1H, H-6), 5.50 (t, *J* = 3.4 Hz, 1H, H-8), 2.91 (dd, *J* = 15.0, 3.4 Hz, 1H, H-9a), 2.22 (overlapped, localized by NOESY, H-9b), 5.49 (s, 1H, H-12), 1.35 (s, 3H, H-13), 1.31 (s, 3H, H-14), 1.75 (bs, 3H, H-15), 1.47 (s, 3H, H-22), 1.35 (s, 3H, H-23). ^{13}C NMR (CD_3CN): δ 58.9 C-1, 79.3 C-2, 85.7 C-3, 135.6 C-4, 139.7 C-5, 76.6 C-6, 81.7 C-7, 66.8 C-8, 39.0 C-9, 85.6 C-10, 93.5 C-11, 109.5 C-12, 16.7 C-13, 23.6 C-14, 13.3 C-15, 114.9 C-21, 29.8 C-22, 29.1 C-23.

12α-*O*-Acetylthapsigargicin Lactol (10) and 12β-*O*-Ethylthapsigargicin Lactol (8). To a solution of **4** and **5** (35 mg, 56 μmol) in dry tetrahydrofuran (3.5 mL) were added *p*-toluenesulfonic acid (10 mg) and triethyl orthoacetate (1.4 mL, 7.7 mmol), and the solution was stirred for 2 h at room temperature. The solution was washed with a saturated aqueous sodium hydrogen carbonate solution (10 mL) and a saturated aqueous sodium chloride solution (10 mL). The organic phase was dried ($MgSO_4$) and concentrated in vacuum, and the residue was purified by CC [siliga gel-B, 20 g, eluent: toluene–ethyl acetate (9:1)] to give **10** (22.7 mg, 60.9%) and **8** (6.5 mg, 17.8%) as colorless oils. Compound **10**: MS calcd ($C_{34}H_{50}O_{13}$) 665.3173, found 665.3190; ^1H NMR (CD_3CN) δ 4.17 (bs, 1H, H-1), 5.35 (dd, *J* = 3.4, 2.4 Hz, 1H, H-2), 5.60 (bs, 1H, H-3), 5.30 (bs, 1H, H-6), 5.44 (dd, *J* = 3.4, 3.3 Hz, 1H, H-8), 2.89 (dd, *J* = 14.2, 3.3 Hz, 1H, H-9a), 2.20 (dd, *J* = 14.2, 3.3 Hz, 1H, H-9b), 5.95 (s, 1H, H-12), 1.21 (s, 3H, H-13), 1.31 (s, 3H, H-14), 1.73 (bs, 3H, H-15), 2.10 (s, 3H, H-22); ^{13}C NMR (CD_3CN) δ 58.8 C-1, 79.2 C-2, 85.6 C-3, 135.4 C-4, 140.0 C-5, 77.3 C-6, 81.8 C-7, 67.1 C-8, 39.0 C-9, 85.7 C-10, 82.8 C-11, 101.0 C-12, 18.5 C-13, 23.5 C-14, 13.0 C-15, 171.0 C-21, 21.6 C-22.

11,12-Anhydrothapsigargicin Lactol (11). To a solution of **4** and **5** (102.3 mg, 163.8 μmol) in toluene (5 mL) were added *N,N*-dimethylformamide dimethyl acetal (1 mL, 7.5 mmol) and 20 mg of Dowex 50 H^+. The mixture was left for 4 h at 70 °C in a sealed vessel. After filtration the filtrate was concentrated in vacuum and the residue was purified by CC [silica gel-A, 80 g, eluent: toluene–ethyl acetate (7:1)] to give 11 (50.1 mg, 50.5%) as an amorphous powder and unreacted **4** and **5** (36.7 mg, 35.8%). MS: calcd ($C_{32}H_{46}O_{11}$), 605.2961; found, 605.2939; ^1H NMR (CD_3CN): δ 3.78 (bs, 1H, H-1), 5.37 (bs, 1H, H-2), 5.51 (bs, 1H, H-3), 4.90 (bs, 1H, H-6), 4.98 (dd, *J* = 3.8, 3.5 Hz, 1H, H-8), 2.72 (dd, *J* = 14.8, 3.5 Hz, 1H, H-9a), 2.44 (dd, *J* = 14.8, 3.8 Hz, 1H, H-9b), 5.14 (s, 1H, H-12), 1.43 (s, 3H, H-13), 1.35 (s, 3H, H-14), 1.74 (bs, 3H, H-15). ^{13}C NMR (CD_3CN): δ 59.8 C-1, 78.5 C-2, 85.6 C-3, 136.4 C-4, 140.2 C-5, 70.5 C-6, 70.1 C-7, 66.6 C-8, 40.3 C-9, 85.1 C-10, 67.4 C-11, 98.3 C-12, 14.3 C-13, 23.8 C-14, 10.7 C-15.

11,12-*O*-Isopropylidene-10-*O*-deacylthapsigargicin Lactol (12). To a solution of **13** (115 mg, 219 μmol) in dry pyridine (10 mL) were added 4-(dimethylamino)pyridine (100 mg, 0.84 mmol) and hexanoic anhydride (400 μL, 1.73 mmol), and the mixture was stirred for 2 h at room temperature. The mixture was acidified with 4 M hydrochloric acid and extracted three times with ethyl acetate (20 mL). The combined organic phases were dried ($MgSO_4$) and concentrated in vacuum, and the residue was purified by CC [silica gel-A, 70 g, eluent: toluene–ethyl acetate–acetic acid (75:25:1)] to give **12** (128 mg, 94.1%) as a colorless amorphous powder. MS: calcd ($C_{33}H_{50}O_{11}$), 621.3274; found, 621.3282. ^1H NMR (CD_3CN): δ 3.44 (bs, 1H, H-1), 5.27 (dd, *J* = 3.1, 2.3 Hz, 1H, H-2), 5.61 (bs, 1H, H-3), 5.17 (bs, 1H, H-6), 5.44 (t, *J* = 3.6 Hz, 1H, H-8), 2.25 (localized by NOESY, *J* not measured due to overlap, 1H, H-9a), 1.80 (localized by NOESY, *J* not measured due to overlap, 1H, H-9b), 5.47 (s, 1H, H-12), 1.34 (s, 3H, H-13), 1.04 (s, 3H, H-14), 1.72 (bs, 3H, H-15), 1.46 (s, 1H, H-22), 1.34 (s, 1H, H-23). ^{13}C NMR (CD_3CN): δ 61.6 C-1, 80.0 C-2, 85.1 C-3, 138.3 C-4, 136.6 C-5, 76.6 C-6, 81.6 C-7, 66.6 C-8, 45.3 C-9, 73.5 C-10, 93.3 C-11, 109.3 C-12, 25.2 C-13, 16.5 C-14, 13.4 C-15, 114.6 C-21, 29.6 C-22, 28.9 C-23.

11,12-*O*-Isopropylidene-2,10-*O*-dideacylthapsigargicin Lactol (13). A solution of **9** (60 mg, 90.3 μmol) in 10% methanolic potassium hydroxide (5 mL) was stirred for 5 min at room temperature and neutralized with Dowex 50 H^+. The mixture was filtered and the filtrate concentrated in vacuum. The residue was purified by CC [silica gel-A, 6 g, eluent: toluene–ethyl acetate–methanol (65:33:1)] to give **13** (22.2 mg, 47.0%) as a colorless amorphous powder and unreacted **9** (26.1 mg, 43.5%). MS: calcd ($C_{27}H_{40}O_{10}$), 523.2543; found, 523.2579. ^1H NMR (CD_3CN): δ 3.16 (bs, 1H, H-1), 4.21 (dd, *J* = 4.6, 4.0 Hz, 1H, H-2), 5.45 (bs, 1H, H-3), 5.17 (bs, 1H, H-6), 5.45 (bt, 1H, H-8), 2.30 (localized by NOESY, *J* not measured due to overlap, H-9a), 1.80 (localized by NOESY *J* not measured due to overlap, H-9b), 5.45 (s, 1H, H-12), 1.34 (s, 3H, H-13), 1.06 (s, 3H, H-14), 1.72 (bs, 3H, H-15), 1.45 (s, 1H, H-22), 1.34 (s, 1H, H-23). ^{13}C NMR (CD_3CN): δ 62.7 C-1, 79.1 C-2, 87.8 C-3, 135.1 C-4, 137.7 C-5, 77.6 C-6, 81.8 C-7, 66.7 C-8, 45.5 C-9, 74.2 C-10, 93.3 C-11, 109.2 C-12, 24.9 C-13, 16.4 C-14, 13.2 C-15, 114.5 C-21, 29.6 C-22, 28.9 C-23.

2,8-*O*-Dioctanoyl-2,8,10-*O*-trideacylthapsigargin Lactol (14). To a solution of 2,8,10-*O*-trideacylthapsigargin[20] (**15**; 103 mg, 0.155 mmol) in dry toluene (10 mL) was added lithium borohydride (300 mg, 13.8 mmol). After the solution was stirred for 6 min at 0 °C, 4 M hydrochloric acid was added. The aqueous phase was extracted four times with ethyl acetate (10 mL), and the combined organic phases were dried ($MgSO_4$) and concentrated under vacuum. CC of the residue [silica gel-A, 100 g, eluent: toluene–ethyl acetate–acetic acid (87:12:1)] afforded **14** as a colorless amorphous powder (18.3 mg, 17.7%) and unreacted **3** (8.1 mg, 8%). MS: calcd ($C_{36}H_{58}O_{11}$), 665.3900; found, 665.3895. ^1H NMR (CD_3CN): δ 3.54/3.46 (bs, 1H, H-1), 5.36 (t, J = 3.0 Hz, 1H, H-2), 5.62/5.59 (bs, 1H, H-3), 5.12 (bs, 1H, H-6), 5.28/5.25 (t, J = 3.0 Hz, 1H, H-8), 2.30 (localized by NOESY, J not measured due to overlap, 2H, H-9), 4.83/5.02 (s, 1H, H-12), 1.17/1.25 (s, 3H, H-13), 1.01/1.02 (s, 3H, H-14), 1.75 (bs, 3H, H-15). ^{13}C NMR (CD_3CN): δ 61.7 C-1, 79.8/79.7 C-2, 85.2/85.3 C-3, 137.9 C-4, 138.0 C-5, 77.4 C-6, 80.0/80.1 C-7, 68.3/67.8 C-8, 45.1 C-9, 75.4 C-10, 73.3 C-11, 101.2/104.8 C-12, 14.2 C-13, 20.8 C-14, 12.8 C-15.

Biological Methods. Preparation of Sarcoplasmic Vesicles Containing Ca^{2+}–ATPases. Vesicular fragments of longitudinal sarcoplasmic reticulum were prepared from the hind leg muscle of New Zealand white rabbits as previously described.[3] The protein concentration was determined according to Bradford using bovine γ-globulin as a standard.

Ca^{2+}–ATPase Assay. Sarcoplasmic reticulum vesicles corresponding to a concentration of 5 μg/mL protein was dissolved in an aqueous buffer (pH 7.0) containing potassium chloride (120 mM), MOPS (25 mM), EGTA (0.5 mM), calcium chloride (0.44 mM), and magnesium chloride (2 mM). To the buffer was added the ionophore A23187 (1 μM) to render the vesicles leaky and avoid uptake inhibition by a rise of Ca^{2+} in the lumen of the vesicles. A solution of the analogue in dimethyl sulfoxide was added to the buffer keeping the concentration of dimethyl sulfoxide below 1%. After incubation for 5 min at 30 °C, an aqueous solution of ATP was added to give a final concentration in the buffer of 1 mM, and the incubation at 30 °C was continued. The amounts of inorganic phosphate at different times were determined according to the method of Lanzetta et al.,[22] and the best straight line obtained through at least eight points was estimated using the PC program Grafit 2.02 (Erithacus Software Ltd.). The rate of phosphate formation was calculated from the slope of the line. The IC_{50} values of the analogues were estimated by fitting the determined velocities as a function of the concentrations into eq 1, using the PC program Grafit 2.02 (Erithacus Software Ltd.).

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