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Ca^{2+}-ATPase INHIBITORY ACTIVITY OF A LOCKED ANALOGUE OF THAPSIGARGIN

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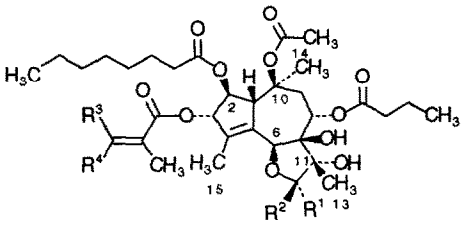
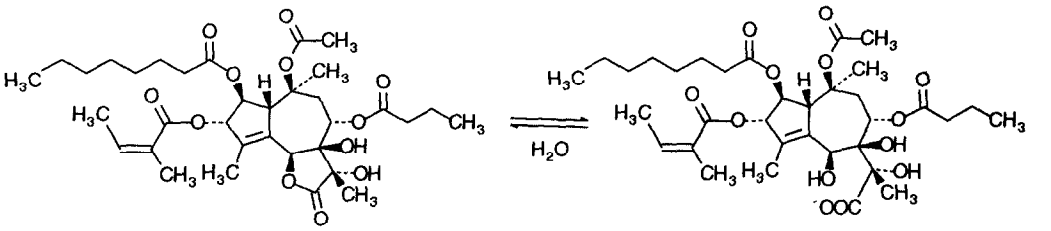
Abstract: The preparation of a nonionic desoxy-analogue of thapsigargin possessing a Ca^{2+}-ATPase inhibitory potency similar to that of thapsigargin is described.

Calcium ion is a secondary messenger of crucial importance for regulation of a number of cell activities such as secretion[1,2], contraction, metabolism, growth and development.[3,4] Maintenance of a low free cytosolic Ca^{2+} concentration (100 nM) is necessary for this second messenger function. Since Ca^{2+} cannot be metabolically converted, the only way of controling the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is by removing the ion to intra- or extracellular compartments.[5] Major enzymes for regulating $[Ca^{2+}]_i$ are Ca^{2+}-ATPases, classified into the SERCA family situated in the membranes of the sarco- or endoplasmic reticulum, and the PMCA family found in the plasma membrane. The distinction between these two families includes their different sensitivities to pharmacological inhibitors.[6,7] The sesquiterpene lactone, thapsigargin (**1**), selectively inhibits the enzymes of the



1	R^1, R^2 = =O		R^3 = CH_3	R^4 = H
2	R^1 = H	R^2 = OH	R^3 = CH_3	R^4 = H
3	R^1 = OH	R^2 = H	R^3 = CH_3	R^4 = H
4	R^1=R^2 = H		R^3 = H	R^4 = CH_3
5	R^1 = SCH_2CH_3	R^2 = H	R^3 = CH_3	R^4 = H
6	R^1 = H	R^2 = OCH_2CH_3	R^3 = CH_3	R^4 = H
7	R^1, R^2 = =O		R^3 = H	R^4 = CH_3

SERCA family with subnanomolar affinity[8] and has become a major tool for studying the Ca^{2+} homeostasis and the mechanism of action of the Ca^{2+}-pump on a molecular level. Thapsigargin (**1**) appears not to affect the activity of the PMCA-type Ca^{2+}-ATPases. Upon binding, **1** induces a state of muscle sarcoplasmic reticulum Ca^{2+}-pump in which several of the partial reactions involved in the Ca^{2+} transport (e.g. Ca^{2+} binding, Ca^{2+}-independent phosphorylation by Pi and nucleotide binding), spanning rather distant domains of the ATPase, are blocked.[6] Thus, understanding the binding mechanism between **1** and the enzyme, including the location of the binding site, would contribute significantly to the goal of elucidating the mechanism of the SERCA-ATPase action on a molecular basis.



1a 1b

Except for studies on the lactol **2**, previously performed structure-activity relationship studies[9] have all been done on **1** or analogues, which under physiological conditions (i.e. aqueous medium at pH 7.4) may exist either as uncharged molecules, that is as γ-lactone **1a**, or as negatively charged γ-hydroxycarboxylate **1b**.[10] To understand the mechanism of thapsigargin binding to the ATPase, it is important to know whether the presence of a charged carboxylate group influences the binding. The carboxylate group might contribute to binding by coulombic interaction. On the other hand if binding requires **1** to fit into a lipophilic cleft, it is conceivable that the uncharged lactone form **1a** is preferred. The affinity of the lactol **2**, which cannot exist as a charged form, shows that coulombic interaction is not essential for binding of the ligand to the enzyme. Since **2** might exist as an open γ-hydroxyaldehyde, as well as a closed lactol form, it is also relevant to ask how prevention of ring opening affects the affinity. In order to answer this question we have synthesized the desoxy-analogue **4**, which is locked as a tetrahydrofurane.

The starting material for the desoxy-analogue, the lactol **2** obtained as an anomeric mixture of **2** and **3**, had previously been prepared by reduction of **1** with sodium borohydride.[10] The use of 15 mol of lithium borohydride for each mol of thapsigargin and dry ether as solvent, however, afforded better yield (37%) and shorter reaction time (20 min at 0 °C). The lactols **2** and **3** were converted into the thioacetal **5**[11] by reaction with ethanethiol. Best yield (61%) was obtained by using ethanethiol as solvent and dry hydrogen chloride as a catalyst. Attempts to use methylene chloride, which is stabilized with ethanol, led to concurrent formation of the acetal **6**[12]. Desulfurization of **5** with Raney nickel or nickel boride[13] led to a small amount of the

desulfurated and debutanoylated derivative of **5** or isolation of unreacted **5**. respectively.[14] In contrast, the desoxy-analogue **4**[15] was formed in a yield of 39% by reacting one mol of **5** with 6 mol of triphenyltin hydride in the presence of a catalytic amount of α,α'-azo-isobutyronitrile using reaction conditions analoguous to those described by Nicolau.[16] Concurrent with the desulfurization the angelic ester isomerized to give the tiglic ester.

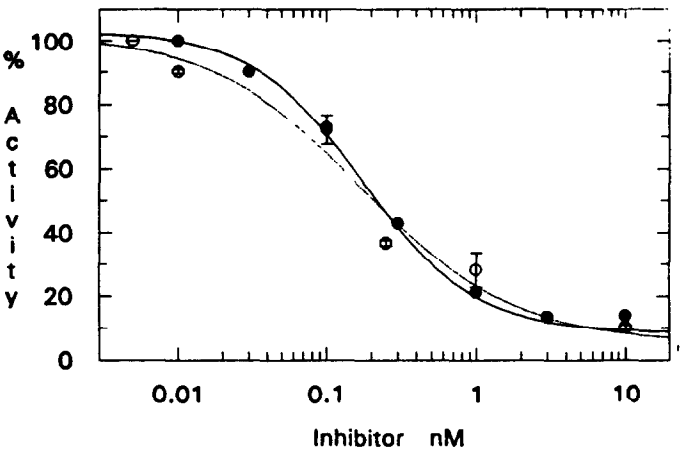


Figure 1: Inhibition of bovine cerebellum Ca^{2+} ATPase caused by **1** (o) or **4** (•).

The inhibitory effect of **4** was determined by measuring the ATP-dependent Ca^{2+} uptake in membranes isolated from bovine cerebellum.[17] In this preparation about 90% ot the ATP-dependent Ca^{2+}-uptake can be inhibited by **1** (Figure 1), and appears to be predominantly due to the SERCA 2b ATPase isozyme.[18] The assay conditions were as in Ref. 17. The IC_{50} value for **4** was determined to be 0.180±0.007 nM (n=3) similar to that of thapsigargin (**1**) [0.17±0.1 nM (n=3)][11] and the thapsigargin analogue **7** [0.264±0.04 nM (n=2)][15], in which the angelic residue has been isomerized to a tiglic residue under the condition used for desulfurization of **5**. This finding confirms the earlier conclusion based on the activity of **2**,[10] that a charged carboxylate group is not essential for enzyme inhibiting activity. In addition, the strong inhibition exerted by **4** demonstrates that locking the molecule into a closed ring form preserves the high affinity.

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11. Data of the sesquiterpene nucleus of **5**: ^{1}H NMR ($CDCl_3$) δ 5.70 (br.s., H-3), 5.50 (m, H-2 and H-8), 5.11 (s, H-12), 5.09 (br.s., H-6), 4.09 (br.s., H-1), 2.68 (overlapped by the methylene protons from the ethyl group, H-9), 2.50 (dd, *J* 14.6 and 4.3, H-9'), 1.81 (H-15), 1.44 (s, H-13 and H-14). The configuration at C-12 was established by NOESY. ^{13}C NMR δ 139.5 (C-4), 133.2 (C-5), 90.6 (C-12), 84.5 (C-10), 84.4 (C-11), 84.3 (C-3), 80.4 (C-7), 78.0 (C-2), 76.4 (C-6), 66.8 (C-8), 57.8 (C-1), 38.1 (C-9), 22.5 (C-14), 15.6 (C-13), 12.5 (C-15). FABMS (HEDS) 695 $[M-1]^-$.

12. Data of the sesquiterpene nucleus of **6** ^{1}H NMR ($CDCl_3$) δ 5.75 (br.s., H-3), 5.52 (dd *J* 3.4 and 4.8, H-2), 5.47 (t *J* 3.7 Hz, H-8), 5.18 (br.s., H-6), 4.69 (s, H-12), 4.14 (br.s., H-1), 2.75 (dd *J* 4.0 and 14.5, H-9), 2.51 (dd *J* 4.0 and 14.5, H-9'), 1.84 (br.s., H-15), 1.43 (s, H-14), 1.39 (s, H-13). The configuration at C-12 was established by NOESY. ^{13}C NMR δ 137.8 (C-4), 133.7 (C-5), 108.0 (C-12), 84.5 (C-10), 84.4 (C-11), 82.2 (C-3), 79.6 (C-7), 78.2 (C-2), 77.3 (C-6), 67.1 (C-8), 57.5 (C-1), 38.0 (C-9), 22.6 (C-14), 15.4 (C-13), 12.3 (C-15). FABMS (HEDS) 679 $[M-1]^-$.

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14. Anchimeric assistance from the 11-hydroxy groups makes the butanoate ester very sensitive to basic reagents. Probably base remaining on the surface of the Raney- nickel even after extensive wash is responsible for the cleavage of the ester.

15. Data of the sesquiterpene nucleus of **4**: ^{1}H NMR ($CDCl_3$) δ 5.62 (br.s., H-3), 5.57 (t *J* 3.4, H-8), 5.47 (br.s., H-2), 4.98 (br.s., H-6 and H-1), 3.92 (d *J* 10.8, H-12), 3.89 (d *J* 10.8, H-12'), 2.90 (dd *J* 4.3 and 15.1, H-9), 2.45 (dd *J* 4.3 and 15.1, H-9'), 1.80 (br.s., H-15), 1.45 (s, H-13 and H-14). ^{13}C NMR δ 140.7 (C-4), 132.8 (C-5), 84.3 (C-3), 84.0 (C-10), 82.0 (C-11), 80.1 (C-7), 78.3 (C-12), 77.3 (C-2), 76.0 (C-6), 67.1 (C-8), 58.0 (C-1), 38.1 (C-9), 22.2 (C-14), 13.7 (C-13), 11.8 (C-15). FABMS (HEDS) 635 $[M-1]^-$.

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