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A Structural Analog of the Protosterol Cation Is Not a Strong Inhibitor of Sterol Biosynthesis

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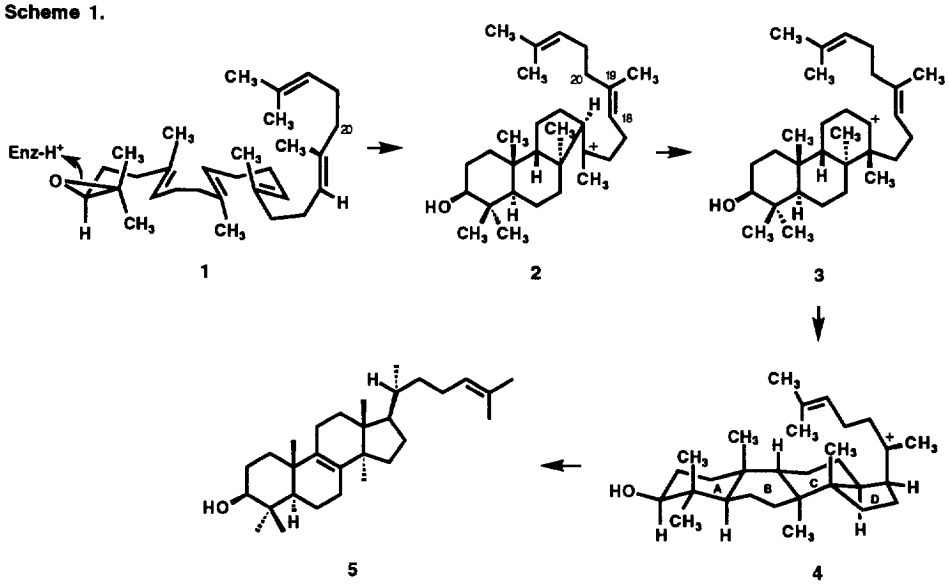
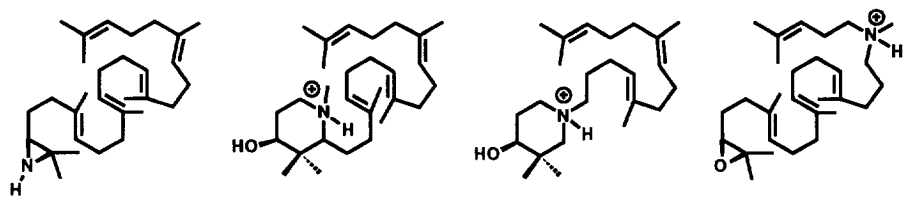
Summary: *The relatively modest potency of inhibition of lanosterol synthase by the tetracyclic ammonium ion **10**, a structural analog of the protosterol cation **4**, IC_{50} = 22 μM, indicates that the protosterol cation is not strongly bound by the enzyme.* Copyright © 1996 Elsevier Science Ltd

On the basis of several lines of evidence it has recently been proposed that the conversion of (*S*)-2,3-oxidosqualene (**1**) to lanosterol (**5**) by lanosterol synthase proceeds with the intermediacy of the discrete cations shown in Scheme 1.[1,2] In this pathway the six-membered C-ring of the sterol nucleus is formed by a ring expansion, **2** → **3**, which results in part from a favorable three-dimensional geometry of the prefolded, enzyme-bound substrate **1**. The substrate shape/envelope for this geometry differs considerably from the overall shape of the protosterol cation (**4**), which suggests that structural analogs of the protosterol cation might not be highly effective inhibitors of lanosterol synthase. This paper reports an experimental test of this premise and also of the binding of **4** by the enzyme using an ammonium analog (**10**) of the protosterol cation.

Almost three decades ago it was discovered that 2,3-iminosqualene (**6**) is a potent inhibitor of lanosterol synthase (IC_{50} = 0.4 μM).[3] Subsequently, a number of other aza compounds which are analogs of

Scheme 1.





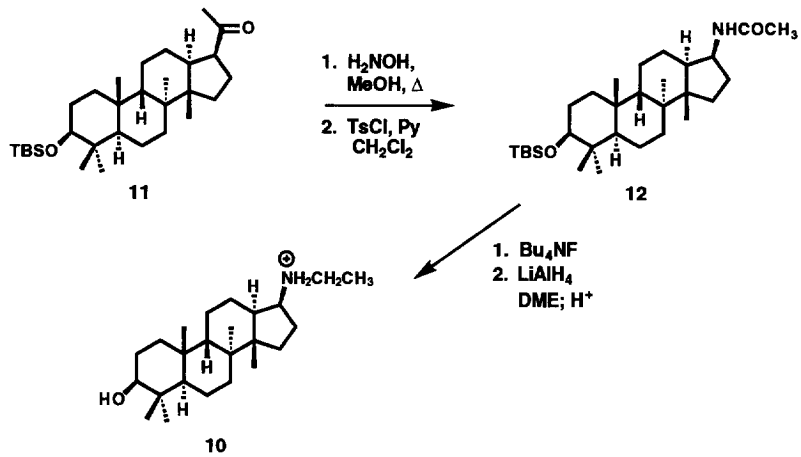
6 7 8 9

oxidosqualene-derived cations have been shown to inhibit lanosterol synthase, for example **7** (IC_{50} = 0.2 μM),[4] **8** (IC_{50} = 1 μM),[5] and **9** (IC_{50} = 1.5 μM).[6,7] The ammonium analog of the protosterol cation which was studied as an inhibitor of lanosterol synthase in the present work represents a logical extension of this line of research to the tetracyclic protosterol series. The starting material for the synthesis of **10** was the methyl ketone **11**, a protosterol derivative which is available either by total synthesis[8] or by the action of lanosterol cyclase on 20-oxa-2,3-oxidosqualene.[1,9] The preparative sequence is outlined in Scheme 2. Ketone **11** was converted to the corresponding oxime (excess 1 : 1 NH_2OH•HCl–$NaHCO_3$ in MeOH at reflux for 4 h; 78% yield after isolation and silica gel (sg) chromatography using 20 : 1 hexane–EtOAc[10]). A solution of this oxime in CH_2Cl_2 was treated with 5 equiv of tosyl chloride–pyridine at 23 °C for 21 h to give the 17β-acetylamino derivative **12** in quantitative yield after sg chromatography using 2.5% MeOH in CH_2Cl_2 for elution.[11] Desilylation of **12** with $Bu_4N^+F^-$ (dried by two azeotropic concentrations under vacuum at 23 °C using toluene containing a little THF) in THF at reflux for 20 h and reduction of the resulting hydroxy amide with excess $LiAlH_4$ in dimethoxyethane at reflux for 24 h provided the amine corresponding to **10** in 55% yield (overall from **12**) as a colorless solid after sg chromatography with 7 : 0.9 : 0.1 CH_2Cl_2–MeOH–NH_4OH for elution.[12]

Measurements of the inhibition of the lanosterol synthase-catalyzed conversion of 2,3-oxidosqualene to lanosterol by the amino protosterol derivative **10** were carried out using the purified yeast enzyme[13] at pH 6.4 in 200 mM aqueous sodium phosphate buffer containing 20% glycerol, 1% Triton X-100, and 3 mM dithiothreitol at an enzyme concentration of 0.04 μM. The enzyme solution was preincubated with inhibitor at 0 μM (control), 20 μM, 30 μM and 40 μM for 1 h at 23 °C, then tritiated 2,3-oxidosqualene was added, and the conversion to lanosterol was determined at 10 min intervals (assay by tlc separation and radiometric determination of lanosterol). The IC_{50} value measured in this way for inhibitor **10** was 22 μM.

Given that the IC_{50} for the acyclic inhibitor **9** (1.5 μM) is considerably lower than that measured for the amino protosterol **10** (22 μM), it is clear that the potential entropic advantage of rigidity of **10** vs. flexibility of **9** does not translate into superior inhibitory potency. The difference between IC_{50} values of **9** and **10** appears to indicate that the binding sites available to lanosterol synthase do not accommodate the protosterol structure very well. This result is consistent with the cyclization pathway shown in Scheme 1, since the spatial envelope of 2,3-oxidosqualene just prior to cyclization to **2** is expected to be quite different from that for inhibitor **10**, or the protosterol cation. On the other hand, it is necessary to consider the possibility that conformational changes occur in the enzyme as cyclization of **1** proceeds through the various intermediates such as **2**, **3** and **4** in Scheme 1. Such dynamic conformational change of the enzyme in response to structural modification of the substrate during

Scheme 2.



11 12 10

reaction seems not unreasonable given that charge-stabilizing interactions probably occur between the various intermediate carbocations and the enzyme (akin to solvation of cations in solution, but probably operating at longer than contact range). Even if these dynamic conformational changes accompany cyclization and play a key role in channeling and controlling the reaction pathway, it still follows that inhibitor **10** is not bound strongly by the enzyme in whatever conformation it adopts when the cyclization reaches the protosterol cation (**4**) stage.

It seems reasonable to suppose that the exothermicity of each ring closure step (roughly 20 kcal/mole assuming the same degree of electrostatic cation stabilization) makes for fast reaction once cyclization is initiated, with the enzyme providing suitable conformational control of the substrate. Thus, no further catalytic acceleration of C–C bond formation by the enzyme should be required, but only conformational control (i.e. proper folding) which precludes any other reaction pathway. Tight conformational control by the enzyme also implies proximity of the cationic center with the nearest double bond of the substrate during cyclization, another charge-stabilizing mechanism. It is also possible that weaker binding of the protosterol cation **4** assists the subsequent 1,2-methyl or H migration steps. If **4** is more poorly stabilized by the enzyme than the subsequent cations which intervene in the rearrangement of **4** to lanosterol, then the 1,2-rearrangement steps which are required for the transformation of the protosterol cation to lanosterol are driven not only by intrinsic stability of the cations but also improvement in "solvation-like" stabilization by the enzyme. The modest inhibition of lanosterol synthesis exhibited by **10** underscores this possibility.

The definitive clarification of all these mechanistic issues will require much further study, including not only the determination of the three dimensional structure of lanosterol synthase bound to 2,3-oxidosqualene (**1**) or a stable analog such as 2,3-iminosqualene (**6**), but also detailed analysis of how this structure changes as the complex, multi-step reaction progresses. For these and other reasons, it is clear that the study of lanosterol biosynthesis from 2,3-oxidosqualene is of extraordinary interest and complexity. The full understanding of lanosterol synthase will add greatly to our knowledge of enzyme function, and explain how the versatile substrate 2,3-oxidosqualene can be channeled by enzymes with great fidelity to so many different natural products.[14]

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10. Data on the oxime of **11** were found to be as follows: $R_f = 0.25$ (tlc on sg plate using 9 : 1 hexane–EtOAc; R_f of **11** under these conditions = 0.34). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.15 (s, 1 H), 3.18 (dd, J = 5.6, 12.2 Hz, 1 H), 2.75 (m, 1 H), 2.22 (m, 1 H), 2.15 (m, 1 H), 1.92 (m, 1 H), 1.82 (s, 3 H), 1.80-1.05 (m, 16 H), 1.09 (s, 3 H), 0.89 (s, 3 H), 0.88 (s, 3 H), 0.87 (s, 9 H), 0.86 (s, 3 H), 0.73 (s, 3 H), 0.02 (s, 6 H). IR (cm^{-1}, thin film): 3260, 2948, 2936, 2892, 2858, 1249. HRMS (CI): found for $(C_{30}H_{55}NO_2Si + NH_4)^+$ 507.43460; calculated 507.43426.
11. Physical data for **12**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.32 (d, J = 8.2 Hz, 1 H), 4.36 (qd, J = 3.6, 8.9 Hz, 1 H), 3.18 (dd, J = 6.0, 10.4 Hz, 1 H), 2.26 (m, 1 H), 1.85 (m, 1 H), 1.70-1.20 (m, 17 H), 1.94 (s, 3 H), 1.09 (s, 3 H), 0.91 (s, 3 H), 0.89 (s, 3 H), 0.88 (s, 12 H), 0.74 (s, 3 H), 0.03 (s, 6 H). IR (cm^{-1}, thin film): 3344, 2952, 2933, 2859, 1651. HRMS (EI): found for $(C_{30}H_{55}NO_2Si)^+$ 489.40021; calculated 489.39991.
12. Physical data for **10** free base: ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.25 (dd, J = 5.6, 11.1 Hz, 1 H), 3.10 (m, 1 H), 2.65 (m, 1 H), 2.55 (m, 1 H), 2.00 (m, 2 H), 1.80-1.20 (m, 19 H), 1.26 (s, 3 H), 1.09 (m, 3 H), 1.03 (s, 3 H), 1.00 (s, 3 H), 0.94 (s, 3 H), 0.80 (s, 3 H). IR (cm^{-1}, thin film): 3300, 2961, 2930, 2850. HRMS (EI): found for $(C_{24}H_{43}NO)^+$ 361.33450; calculated 361.33424.
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