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4,4,14α-TRIMETHYL 9β,19-CYCLO-5α-26-HOMOCHOLESTA-24,26-DIEN-3β-OL: A POTENT MECHANISM-BASED INACTIVATOR OF $\Delta^{24(25)}$- TO $\Delta^{25(27)}$-STEROL METHYL TRANSFERASE

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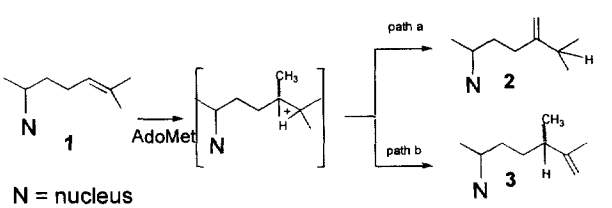
Abstract: The title compound (**4A**) was synthesized and tested as a mechanism-based inactivator of the sterol methyl transferase (SMT) enzyme from *Prototheca wickerhamii*. Using cycloartenol as substrate, **4A** was found to exhibit time-dependent inactivation kinetics, generating a K_i value of 30 μM and K_{inact} value of 0.30 min^{-1}.

Sterol methyl transferase (SMT) enzyme catalyzes the conversion of a Δ^{24}-sterol acceptor molecule to C-methylated product(s) (Scheme 1, **1** to **2** and **3**: bold numbers refer to side chain construction and bold letters refer to the sterol nucleus) and is thought to function as a committed step in phytosterol synthesis (viz., cycloartenol **1A** to Δ^5-sterol end products).[1] The SMT enzyme from the alga *Prototheca wickerhamii* catalyzes the coupled methylation-deprotonation reaction of the sterol side chain to generate a $\Delta^{25(27)}$-olefin (path b, Scheme 1)[2] and the SMT enzyme from the fungus, *Saccharomyces cerevisiae,* generates the $\Delta^{24(28)}$-olefin (path a, Scheme 1).[3,4] Whereas both microorganisms are nonphotosynthetic and yeast-like in appearance, *P. wickerhamii* operates a cycloartenol-ergosterol based pathway[2a] and *S. cerevisiae* operates a lanosterol-ergosterol based pathway.[5] As a result of the morphological similarities between the alga and fungus, algal infections of the skin are often treated with antifungal compounds, such as nystatin, which complexes with ergosterol in the membrane,[5,6] prompting us to design and test for the first time an algal-specific mechanism-based sterol biosynthesis inhibitor that incorporates the cycloartenol nucleus.



Scheme 1

Kinetic, mechanistic and sterol specificities studies on the $\Delta^{24(25)}$- to $\Delta^{24(28)}$-SMT enzyme from *S. cerevisiae* catalyzed reaction indicate an S_N2 mechanism in which methyl addition occurs from the *Si*-face (β-face attack) and H24 migrates to C25 in a concerted manner.[3,7] The stereochemistry at C24 of cyclolaudenol (side chain **3**) isolated from *P. wickerhamii* has been determined to be β-oriented by ^1H and ^{13}C NMR,

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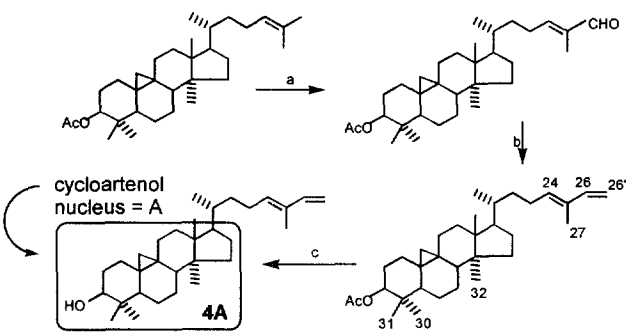
consistent with *Si*-face C-methylation of cycloartenol.[2] Although much is known about the SMT enzyme from *S. cerevisiae*, little is known about enzyme mediated C-methylation of related SMT enzymes, particularly as it relates to the identity of key amino acid residues that are present at the active site. It was anticipated, on the basis of the pathway shown in Scheme 1, that substrate analog **4A** could undergo rearrangement or delocalization to place a positive charge in a region of the active site that does not normally encounter electrophilic centers and under the influence of the SMT enzyme would be susceptible to alkylation. To test our hypothesis, the active site and mechanism of action of the enzyme SMT from *P. wickerhamii* have been probed using **4A** as an inhibitor of the SMT-catalyzed reaction. In our studies described herein, we have taken advantage of the fact that the preferred sterol substrate for plant SMT enzymes,[8] including *P. wickerhamii*,[2b] is cycloartenol (**1A**).

The vinyl analog of cycloartenol **4A** was readily prepared from the known C3 acetate **1A**[9] (50 mg) by selective oxidation using selenium dioxide followed by Wittig olefination and reaction with excess $LiAlH_4$ in anhydrous THF to deprotect the C3 hydroxyl group. The preparative sequence is outlined in Scheme 2.[10] Compound **4A** was purified by semi-preparative HPLC using a C_{18}-Zorbax column eluted with methanol.[11,12] The overall yield (following HPLC) was 25%.



Reagents and conditions: (a) SeO_2, 95% EtOH, reflux; (b) $CH_3PPh_3Br^-$, *n*-BuLi, NaH, THF reflux 12 h; (c) $LiAlH_4$, rt.

Scheme 2

Incubations were conducted at 29 °C with acetone-precipitated SMT enzyme from *P. wickerhamii* microsomes using cycloartenol (50 μM) as substrate, AdoMet (50 μM; 20.0 mCi/mmol) as coenzyme,[2b,7a] and increasing concentrations of **4A** as inhibitor. Incubation of **4A** in the presence of AdoMet with SMT enzyme resulted in pseudo-first-order time-dependent inactivation of the SMT enzyme, as evidenced by the linear dependence of the log of residual activity against time (Figure 1). When the enyzme is preincuabted with **4A** in the absence of AdoMet, no enzyme activity is observed. The rate of inactivation by **4A** was saturable, with a maximum rate of inactivation, K_{inact} of 0.30 min^{-1} ± 0.01 min^{-1} and a K_i for **4A** of 30 ± 0.01 μM. These values compare very favorably with the steady-state kinetic parameters for the normal substrate cycloartenol (K_{mapp} = 30 μM and V_{maxapp} = 21 pmol/min/mg protein). Co-incubation with the normal substrate, cycloartenol, afforded protection against inactivation. Compound **4A** was found not to be a substrate for C-methylation by the *P. wickerhamii* SMT enzyme.

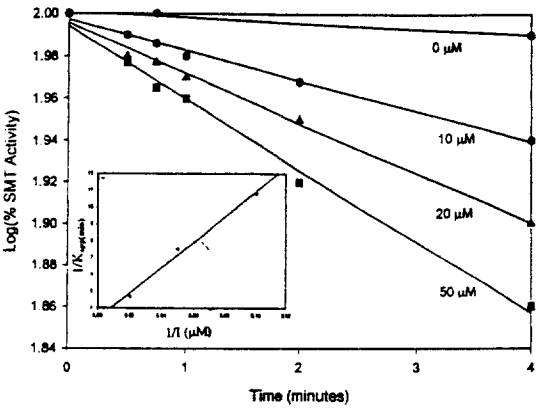
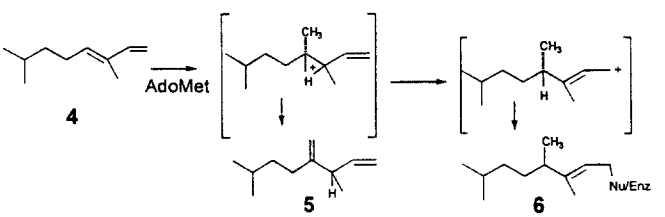


Figure 1. Time dependency of inactivation of *P. wickerhamii* SMT enzyme by **4A**. Six experiments were performed and the variation among the trials did not exceed 5%.

In separate incubations, using a soluble enzyme assay with cycloartenol (range tested; 5 to 150 μM) and AdoMet (fixed at saturation concentration; 50 μM) as substrates, 24(28)-methylene cycloartanol (**2A**) and cyclolaudenol (**3A**) were tested as inhibitors (range tested; 5 μM to 150 μM). Both C24-methylated sterols, **2A** and **3A**, exhibited reversible and competitive-type patterns of inhibition of SMT enzyme activity with K_i values of 85 μM (**2A**) and 23 μM (**3A**), respectively. The K_i value of **4A** is close to the K_i value of **3A** which is consistent with the C-methylation mechanism of the algal SMT enzyme proceeding from the *Si*-face of the Δ^{24}-bond to generate the 24β-methyl group, as proposed by Arigoni and Mihailovic for *Trebouxia* species.[7b,13]

The above results from incubation with **4A** are readily explained by a mechanism-based inactivation process in which C-methylation of **4A** by the SMT enzyme gives rise to an allylic cation species that reacts with the active site base or a nearby nucleophilic amino acid side chain generating adduct **6**. We postulate that inhibition and C-methylation both occur through a common intermediate, as illustrated in Schemes 1 and 3. Normal C-methylation of **4A** can occur to give a tertiary C-26 cation, which may undergo the usual hydride migration and proton loss to a cycloartenol analog (**4** to **5**), or it can be trapped by an incipient carbocationic species during C-methylation of the Δ^{24}-bond (**4** to **6**). Interestingly, the related substrate analog with the 24-cyclopropylidene containing side chain (26,27-dehydocycloartenol) failed to act as a mechanism-based inactivator, and was metabolized to a C-methylated product by the *P. wickerhamii* SMT enzyme.[2b]



Scheme 3

Alternatively, the zymosterol substrate analog (26,27-dehydrozymosterol) serves as a mechanism-based inactivator toward the SMT enzyme from *S. cerevisiae* and demonstrably is alkylated by the SMT enzyme.[14] These results suggest that the nature and position of amino acid residues in the active site of SMT enzymes from algae and fungi are different and these differences may affect binding specificity, and regio- and stereospecificity involved in the $\Delta^{24(28)}$- and $\Delta^{25(27)}$-C-methyl pathways leading to ergosterol. Studies in progress are designed to establish the nature of covalent modification of the SMT enzyme by **4A**.

References and Notes

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