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Tetrahedron Letters 45 (2004) 875–878
Tetrahedron Letters

24-Thiacycloartanol, a potent mechanism-based inactivator of plant sterol methyltransferase

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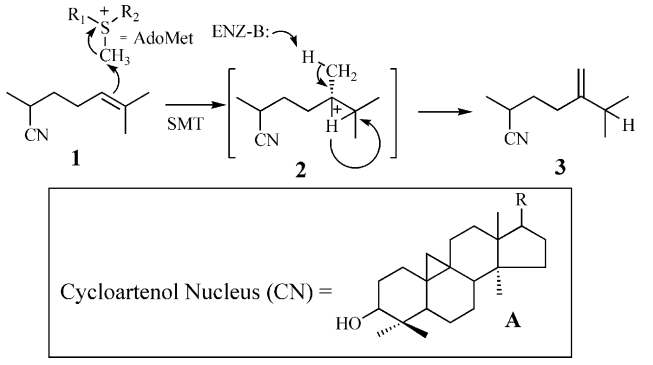
Received 30 August 2003; revised 28 October 2003; accepted 30 October 2003

Abstract—The rationally designed substrate mimic of cycloartenol, 24-thiacycloartanol (24-TC) **4A**, and its corresponding sulfonium salt **5A** were tested against the recombinant sterol methyltransferase (SMT) from *Glycine max* (soybean). Analog **4A** was found to irreversibly inactivate the enzyme generating competitive- and time-dependent inhibition of activity accompanied by a K_i value of 2 μM and k_{inact} of 0.3 min^{-1}. Analog **5A**, a presumptive high-energy intermediate of the SMT catalyzed reaction, was found to be a reversible, non-competitive-type inhibitor generating a K_i value of 55 nM.

Sterol methyltransferase (SMT: EC 2.1.1.41) from plants catalyzes the coupled *C*-methyl-addition–hydrogen-elimination reaction involving AdoMet and cycloartenol **1A** to produce 24(28)-methylene cycloartanol **3A** (Scheme 1). Sterol *C*-methylation represents the first committed step in the phytosterol pathway generating membrane inserts (sitosterol) and hormones (brassinosteroids) that control plant growth and development.[1]



Scheme 1. Non-covalent pathway for the first C_1-transfer reaction catalyzed by plant SMT1.[1]

Although some half-dozen SMTs catalyzing the first and second C_1-transfer reaction (SMT1 and SMT2, respectively) have now been cloned, sequenced, and functionally expressed,[2,3] little is known about the identity of key amino acid residues that are present in the active site to interact with sterol and AdoMet. Substrate studies using crude microsomes or solubilized material from different plant species suggested that an ionic process was involved with sterol *C*-methylation.[1] Studies with sterol *C*-methylation inhibitors fed to cell cultures and cell-free systems provided additional support for a high-energy intermediate in the *C*-methylation reaction.[1] The known SMT inhibitors include (i) transition state analogs (e.g., 25-azacycloartenol[4]), (ii) product mimics (e.g., stigmasterol[5]) or (iii) substrate mimics (e.g., 26,27-dehydrozymosterol[6]). The last group includes mechanism-based inactivators, which have been prepared in our laboratory for the first time based on the steric–electric features of the native substrate for fungal catalysis.[7] These inhibitors were designed to undergo rearrangement or delocalization during catalysis to place a positive charge in the region of the SMT active site that does not normally encounter electrophilic species. Elimination of the positive charge on the sterol side chain is expected to render the enzyme inactive thereby impairing phytosterol synthesis and the resulting affinity labeled SMT to be a source of enzyme characterization.

Sulfur-containing analogs of sterol acceptor molecules that bind to SMTs produced in fungi, protozoa, and plants have been prepared in which sulfur has replaced carbons at C-23, C-24, or C-25 and many are potent

Keywords: Sterol methyltransferase; Mechanism-based inhibitor; Enzymology; Phytosterol.
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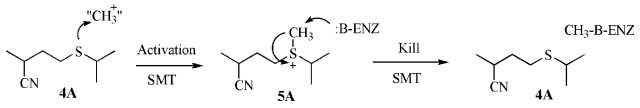
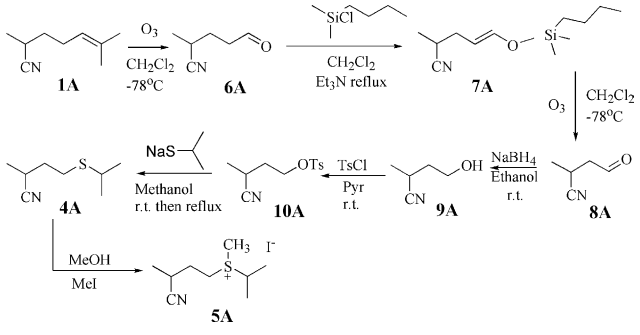
doi:10.1016/j.tetlet.2003.10.203

inhibitors of SMT action in vivo as well as in vitro.[4,8] Charged sulfonium analogs (e.g., **5A**) of predicted 24-methyl cycloartanyl C24–C25 cationic intermediates of sterol C-24 methylation reaction were designed as reversible inhibitors and shown to exhibit non-competitive-type kinetic patterns against sterol in the SMT assay.[4] In the case of 24-thiacholestanol tested with the fungal SMT, mechanism-based inactivation was considered (Scheme 2).[8a] As a logical extension of this line of research to the plant area and because we are searching for sterol-based inhibitors that can affinity label different amino acids in the active site of SMT enzymes, we now report the synthesis of two sulfur-based analogs **4A** and **5A** of the native substrate for SMT1 action, cycloartenol **1A**, and show their differential effects on SMT.

The requisite sulfonium compounds (24-thiacycloartanol **4A** and the diastereomeric mixture of (3β,24*R*,*S*)-24-methyl-24-thionacycloart-3-ol iodide **5A**) were prepared by straightforward synthetic methods involving ozonolysis of the cycloartenol side chain **1A** and its reconstruction introducing the sulfur atom at position 24 followed by methylation of the resulting sulfide as shown in Scheme 3.[4,8b,e] From 100 mg of starting material **1A** as the C3-acetate was recovered 19 mg of **4A** as the free alcohol. 24-TC was purified by HPLC using a Phenomenex, Selectosil C_{18} semi-preparative column eluted with methanol at 4 mL/min at ambient temperature. GLC RRT_C (Retention time relative to cholesterol on 3% SE-30 packed column operated isothermally at 245 °C), 2.64; Mass spectrum (m/z) (M^+ and other diagnostic ions in high mass region) 446, 431, 428, 413, 314 (retained 19β cyclopropyl ring); ^{1}H NMR (in ppm relative to TMS at 500 MHz, in $CDCl_3$): δ 0.330 (1H, d, $J = 4$, H-19); 0.550 (1H, d, $J = 4$, H-19); 0.809 (3H, s, H-18); 0.894 (1H, d, $J = 6.5$, H-21); 0.967 (6H, s, H-30/31); 1.261 (3H, d, $J = 6.5$, H-26); 1.274 (3H, d, $J = 6.5$, H-27); 2.45 (2H, m, H-23); 2.91 (1H, m, H-25); 3.29 (1H, m, H-3). ^{13}C NMR (75 MHz, in $CDCl_3$) δ terminal side chain signals) 34.73 (C-23); 35.51 (C-25); 23.41 (C-26); 23.42 (C-27).



Scheme 2. Proposed pathway for the mechanism-based inactivation of SMT. Activation implies SMT catalyzed *C*-methylation of substrate and kill implies covalent attachment of inhibitor to the enzyme.



Scheme 3. Preparative route for the synthesis of 24-thiacycloartanol **4A** and its sulfonium salt **5A**.

Conversion of the sulfide **4A** (10 mg) to the crystalline salt **5A** generated crystals (ca. 30% yield), which were collected by filtration, yielding **5A** as a mixture of diastereoisomers: ^{1}H NMR (in ppm relative to TMS at 500 MHz, in MeOH-d_4) δ 0.373 (1H, d, $J = 4$, H-19); 0.578 (1H, d, $J = 4$, H-19); 0.808 (3H, s, H-18); 0.949 (3H, s, H-30); 0.962 (3H, s, H-31); 3.780 (1H, m, H-25); 3.214 (1H, m, H-3); 2.863 (3H, s, Me–S). Mass spectrum (m/z) 461 (M^+). Anal. ($C_{30}H_{53}IOS$). No attempt was made to separate the diastereo-isomeric mixture of **5A** for activity assay.

On the basis of substrate activity of cycloartenol **1A** with soybean SMT,[3] 24-thiacycloartanol **4A** was designed to act as a mechanism-based inactivator via methylation of the thioether to yield the monoactivated sulfonium ion. This intermediate, rather than functioning as a charged sulfonium analog to impair SMT action, is postulated to act as a substrate that donates methyl to the enzyme during the reaction progress thereby forming a covalent adduct that inactivates the SMT activity and in turn generates 24-thiacycloartanol as a product (Scheme 2). In order for the intermediate generated during catalysis to function as a substrate for enzyme methylation rather than acting as transition state analog (cationic intermediate) of the *C*-methylation reaction, we postulate that a distinct enzyme conformation exists to recognize the charged intermediate generated during the reaction progress from the enzyme conformation that involves **5A** added exogenously. To test these postulates, we performed a series of kinetic experiments with soybean SMT described as follows.

Measurements of the inhibition of the SMT catalyzed conversion of cycloartenol to 24(28)methylene cycloartanol by **4A** and **5A** were carried using the purified soybean SMT as described.[3] The general proposal for SMT catalysis (Scheme 1) involves a high-energy intermediate in the construction of a C-24 methylated sterol side chain. The sulfonium analog **5A** of the intermediate **2A** was found to be an inhibitor of the enzymatic *C*-methylation of cycloartenol to 24(28)-methylene cycloartanol, exhibiting non-competitive-type kinetics against cycloartenol and generating a K_i value of 55 nM (Fig. 1, panel B). The β-epimer of the diastereomixture is thought to be the more potent inhibitor for mechanistic reasons.[8c] As expected for a cationic intermediate analog to act as a 'transition state analog',[9] **5A** was found to bind many orders of magnitude more tightly than the substrate. (K_m for **1A** = 30 μM vs K_i for **5A** = 55 nM yielding a ratio of 545). An overnight incubation with saturating concentrations of **5A** and preparative amounts of SMT (10 μM) failed to generate any 24-thiacycloartanol as determined by GC–MS and HPLC. Alternatively, administering **4A** to the SMT under identical conditions as with **5A** led to a competitive-type inhibition pattern (Fig. 1, panel A) and a K_i value for the inhibitor of 2.6 μM.

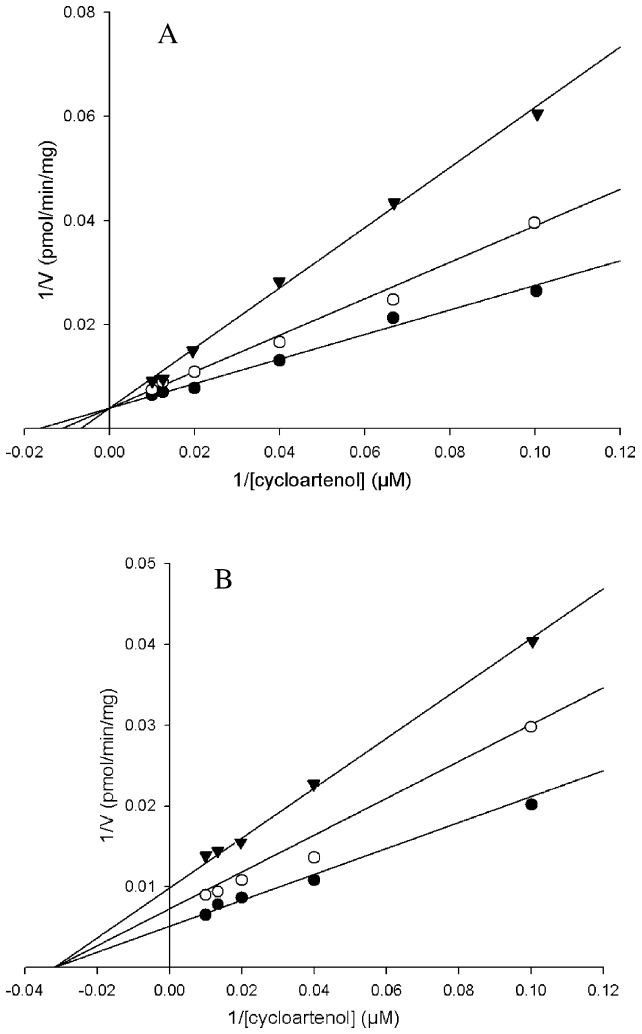


Figure 1. Lineweaver–Burk plot of reciprocal velocity versus concentration of SMT substrate **1A** using 24-thiacycloartanol **4A** (panel A) and (3β,24*R*,*S*)-24-methyl-24-thionacycloart-3-ol iodide **5A** (panel B)[2] as inhibitors. Incubations were performed with 0.10 μM SMT in a final volume of 600 μL of the standard assay.[2] The reactions were performed at 35 °C for 45 min.[2] The constant substrate-[$^3H_3$-*methyl*]AdoMet (20 μCi/μmol) in these experiments was held at a saturating concentration of 100 μM and the varied substrate–cycloartenol was added to the activity assays at several fixed concentrations ranging from 10 to 100 μM. The inhibitor **4A** was varied from 2.5 to 10 μM and **5A** was varied from 5 to 60 nM. Results are the average of samples from three separate experiments with variation amongst the trials at <10%.

These results suggested the two inhibitors were interacting in different environments of the active site and that **4A** may be acting as a mechanism-based inactivator. Indeed, a requirement for this class of inhibitor is to yield a competitive-type inhibition versus the native substrate.

The enzyme solution was preincubated with inhibitor at 0 μM (control), 2, 4, and 5 μM, using 0.1 μM SMT in 1 mL buffer A [50 mM Tris–HCl, 2 mM $MgCl_2$, 2 mM 2-mercaptoethanol and 20% (v/v) glycerol, pH 7.5] and incubated for up to 10 min at 35 °C with 100 μM of AdoMet. At the times indicated incubation mixtures were put into a pre-cooled (dry ice in ethanol) test tube to prevent catalysis. The samples were thawed on ice, diluted 20-fold in ice-cold buffer A and the remaining SMT activities were measured by catalytic capacity to convert **1A** to **3A**. The enzymatic reaction was initiated by addition of saturating concentrations of **1A** (100 μM) and [*methyl*-$^3H_3$]AdoMet (100 μM). The result of activity assay led to pseudo-first-order time and concentration-dependent loss of enzyme activity (Fig. 2).

From a replot of the half-lives for inactivation ($t_{1/2}$) versus the inverse of the inactivator concentrations (1/[*I*]) were obtained K_i and K_{inact} values of 2.10 ± 0.02 μM and 0.28 ± 0.02 min^{-1} (Fig. 2).

As a positive and a negative control for the irreversible inhibition, two sets of experiments were performed: (i) SMT was assayed with AdoMet and **4A** was assayed at 0 and 20 μM (10 times of its K_i value) and (ii) **5A** or 25-azacycloartenol (a high-energy intermediate of the *C*-methylation reaction of soybean $K_i = 40$ nM[3] considered to be protonated at physiological pH and therefore should behave as the charged sulfonium analog during catalysis[4]) were employed at concentrations of 550 and 400 nM (10 times of K_i values for the respective compounds). Using the standard assay with 0.1 μM SMT and the relevant inhibitor at the indicated concentration followed by temperature controlled dialysis against buffer A for 24 h with several buffer changes, we found that **4A** failed to restore enzyme activity, while the other three treatments gave quantitative recovery of SMT activity. The assay was repeated with only [*methyl*-$^3H_3$]AdoMet and SMT (no added **4A**) and there was no significant radioactivity remaining in the aliqout of enzyme solution, ruling out the possibility that the enzyme was methylated directly, even though, AdoMet can bind to the SMT with a K_d of 4 μM.[6]

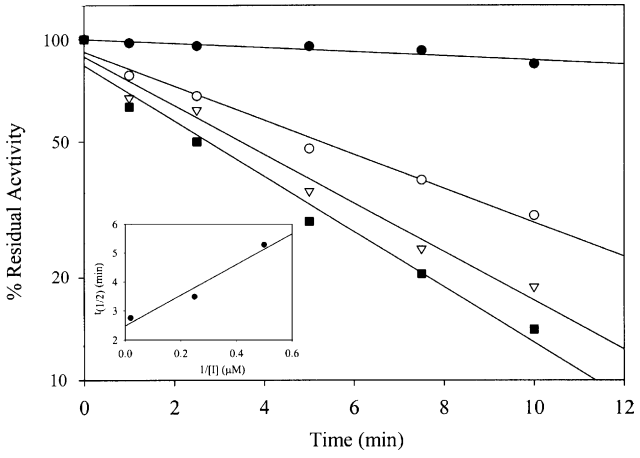


Figure 2. Time-dependent inactivation of soybean SMT with the 24-TC. Semilog plots of residual activity versus time at 0 (●), 2 (○), 4 (▽) and 5 (■) μM concentrations of inhibitor. The times indicated in the figure are 1, 2.5, 5, 7.5, and 10 min. (Inset) Replot of enzyme half life ($t_{1/2}$) for inactivation versus 1/[*I*].

Co-incubation of 10 μM of **4A** and 100 μM of native substrate cycloartenol and 100 μM of AdoMet, afforded protection against inactivation conserving ca. 30% of *C*-methylation activity after the incubation period.

To determine the stoichiometry of binding, 50 μM of **4A** was incubated with 0.25 μM pure SMT and 100 μM of [*methyl*-$^3H_3$]AdoMet in the standard assay for 45 min at 35 °C. Assay of an aliquot of the incubation mixture confirmed the loss of >98% of the original SMT activity. The remaining aliquot was dialyzed against buffer A until no detectable radioactivity remained in the dialysis buffer to give a final ratio of tritium activity to protein, which was calculated to correspond to 1.21 ± 0.1 equiv of inhibitor per mole of enzyme (native enzyme is a tetramer[3]).

The above results are readily explained by a process in which *C*-methylation of 24-thiacycloartanol **4A** by SMT catalysis generates intermediate **5A** (Scheme 2), which is charged and activated to serve as a methyl donor. The latter compound can, when the enzyme assumes an appropriate conformation, react with an active site base or a nearby nucleophilic amino acid side chain to covalently inactivate the enzyme. Experiments to further establish the mechanism of inactivation and to determine the site of covalent protein modification are in progress. It is anticipated the **4A** may also prove to be useful in specific and irreversible labeling of a region of the active site distinct from, for example, 26,27-dehydrocycloartenol,[1] thereby providing additional material for as yet an X-ray crystallographic determination of this class of AdoMet-dependent methyltransferase.

Acknowledgements

This work was supported by grants from the National Science Foundation (MCB 0115401 and IBN 0115401) and Welch Foundation (D-1276) to W.D.N.

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