Pergamon

Tetrahedron Letters 39 (1998) 8575–8578

TETRAHEDRON LETTERS

Mechanism-Based Active Site Modification of Sterol Methyl Transferase by Tritium-Labeled 26-Homocholesta-8,14,24-trien-26-yn-3β-ol

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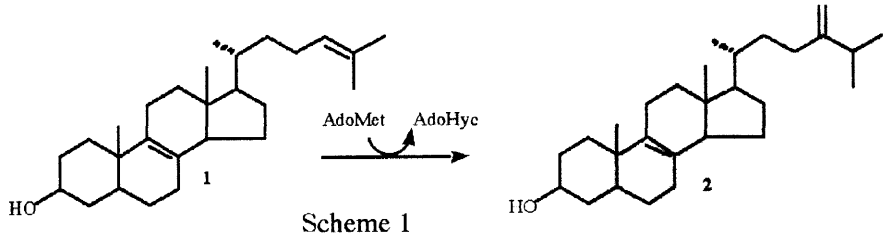
Received 12 August 1998; revised 3 September 1998; accepted 9 September 1998

Abstract: The title compound (26-HC, **5**) was synthesized and tested as a mechanism-based inhibitor of the sterol methyl transferase (SMT) enzyme from *Saccharomyces cerevisiae*. Enzyme assays were performed with SMT enzyme using zymosterol as substrate and AdoMet as coenzyme. The inhibition of SMT enzyme by 26-HC showed an apparent k_i of 72.5 μM and k_{inact} of 2.4 min^{-1}. Covalent modification of the active site of a SMT enzyme was demonstrated for the first time using [3-^{3}H]26-HC.

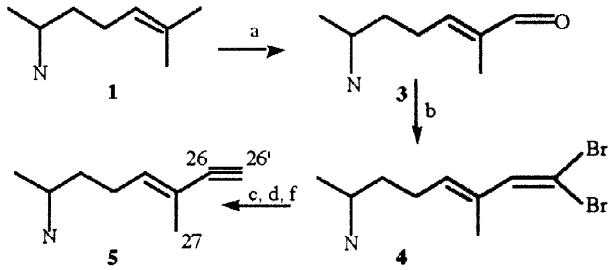
Sterol methyl transferase catalyzes the transformation of the universal Δ^{24}-sterol acceptor molecule in plants and fungi, to any of 200 distinct C24-methyl sterols,[1] which in turn can serve as membrane inserts and as metabolic precursors of phytosteroid hormones.[2] The crucial role of SMT enzymes in regulation of phytosterol synthesis has stimulated considerable interest in the synthesis and kinetic evaluation of substrate analogs (e.g., 24-thiasterols, and 24-cyclopropylidene sterols), product analogs (e.g., 24-methyl desmosterol) or transition state analogs (e.g., 24(*R,S*),25-epiminosterols).[3] The first group includes mimics that function as mechanism-based inactivators designed to test distinctly different modes of enzyme inactivation, *viz.*, covalent modification *versus* tight binding via ionic interactions. However, in the absence of a pure protein to establish the nature of the inactivation, the molecular details of how these inhibitors interact mechanistically with the SMT enzyme remain enigmatic. Several SMT enzymes from fungi and plants have been cloned and expressed.[4] However, only the SMT enzyme from *Saccharomyces cerevisiae* has been purified to homogeneity,[5] a tetramer of 4 identical subunits of M_D 43,000 that exhibits cooperativity and down-regulation of activity from the native membrane insert, ergosterol (24β-methyl cholesta-5,7,22*E*-trien-3β-ol).[3b,4a] Using the recombinant (S)-adenosyl-L-methionine: $\Delta^{24(25)}$-sterol methyl transferase (SMT) enzyme from *S. cerevisiae* (EC 2.1.1.41) cloned into *Escherichia coli*, we now show unambigously that 26-homocholesta-8,14,24-trien-26-yn-3β-ol (26-HC, **5**) is an effective mechanism-based inactivator of the SMT enzyme which catalyzes C-methyl transfer from AdoMet to the 24,25-double bond of the sterol side chain stereoselectively (Scheme 1).



Scheme 1

PII: S0040-4039(98)01961-3

The synthetic route leading to 26-HC is outlined in Scheme 2. The C26*trans* methyl group of zymosteryl acetate was selectively oxidized by selenium dioxide to afford aldeyde **3**.[6] Using the Corey-Fuchs procedure, vinyl aldehyde **3** was converted to the corresponding enyne **5** *via* the dibromide intermediate **4** in 31% overall yield.[7,8] [3-^{3}H]26-HC (126.4 mCi/mmol) was prepared by oxidation of 26-HC with PCC, followed by reduction with [^{3}H]$NaBH_4$, as previously described.[9]



Reagents and conditions: a) SeO_2,95% EtOH, reflux; b) CBr_4, PPh_3, Zn/CH_2Cl_2; c) n-BuLi, THF, -78°C; d) LAH, ether; f) C_{18}-HPLC, elute with MeOH; nucleus with Δ^8-structure in **1** and **3** and nucleus with $\Delta^{8,14}$-structure in **4** and **5**.

Scheme 2

Incubation of increasing concentrations of 26-HC with pure recombinant SMT enzyme (0.42 μM) and [$^3H_3$-*methyl*]AdoMet (100 μM; 20.0 mCi/mmol) at 30°C in Buffer A (50 mM Tris-HCl, pH 7.5, 2mM $MgCl_2$, 2mM β-mercaptoethanol, 20% glycerol) failed to generate a C-methylated sterol product in the absence of zymosterol (**1**), but in the presence of **1** (50 μM) gave rise to pseudo-first-order time-dependent inactivation of the SMT enzyme activity, as evidenced by the linear dependence of the log of residual activity against time (Figure 1). The rate of inactivation by 26-HC was saturable, with a maximum rate of inactivation , k_{inact} of 2.4 ± 0.01 min^{-1} at 0.833 μM protein and a K_i for 26-HC of 72 ± 0.01 μM. These values compare favorably with the steady state kinetic parameters for the normal substrate, zymosterol **1** (k_{cat} = 0.6 min^{-1}; two k_m values due to cooperating subunits of 22 μM and 45 μM).[5] Co-incubation of 5.0 μM 26-HC with 50 μM and 100 μM **1**, afforded protection against inactivation generating 53 % and 78% C-methylation activity, respectively, relative to the C-methylation activity of a control incubation that contained saturating amounts of substrate and coenzyme only.

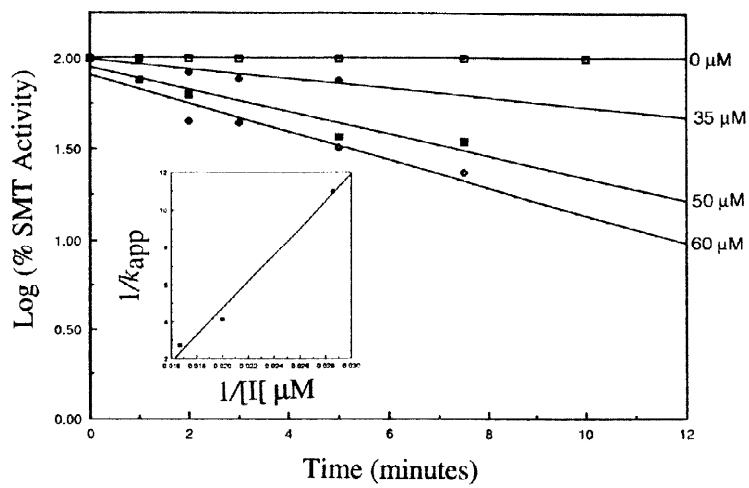


Figure 1. Kinetics of inactivation of SMT enzyme by 26-HC. Inset: Plot of $1/k_{app}$ (range from 2 to 12) versus 1/[26HC] (Inhibitor (I) range from 0.016 to 0.030 μM).

The covalent nature of binding was established by incubation of 100 μM [3-^{3}H]26-HC and 100 μM nonisotopically labeled AdoMet with 0.833 μM SMT enzyme in Buffer A for 45 min. After dialysis and Centricon (Y30) filtration to concentrate the protein and remove unbound inhibitor, the protein sample was boiled in SDS (sodium dodecyl sulfate) buffer for 10 min prior to analysis by 12% SDS-PAGE (polyacrylamide gel electrophoresis). Staining with Coomassie blue confirmed the presence of a single protein of the expected M_r, while treatment of the gel with DMSO-2,5-diphenyloxazole and radiofluorography revealed the presence of a single radioactive band with mobility identical to that of authentic SMT enzyme (Figure 1). In control experiments, [3-^{3}H[26-HC was separately incubated with boiled SMT enzyme. Analysis of the corresponding SDS-PAGE gels, either by radiofluorography or by excision of the relevant protein bands and direct liquid scintillation counting, failed to indicate any comigration of tritium with protein, thereby confirming the specificity of the covalent modification of SMT enzyme by 26-HC. To determine the stoichiometry of binding, 100 μM of [3-^{3}H]26-HC was incubated with 100 μM AdoMet and 0.833 μM SMT in Buffer A for 45 min at 30 ^{0}C. Assay of an aliquot of the incubation mixture pre-equilibrated with inhibitor confirmed the loss of 99% of the original SMT activity. The incubation mixture was dialyzed for 48 h against Buffer A, and then subjected to repeated ultrafiltration using an Amicon concentrator exclusion membrane to give a final ratio of tritium activity to protein calculated to correspond to 1.00 ± 0.10 equiv. of inhibitor per mole of enzyme (native enzyme as tetramer). The ability of 25% of the enzyme monomers to inactivate the enzyme is consistent with the previously observed strong cooperativity among the subunits[5]. Thus, disruption of the quaternary structure of the SMT protein by covalent modification of at least one subunit with a suicide substrate can lead to complete loss of C-methylation activity.

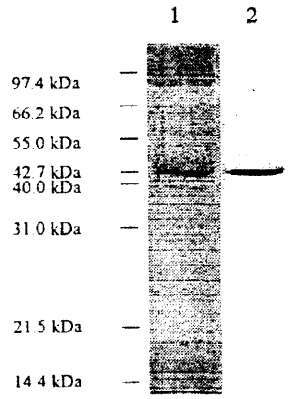
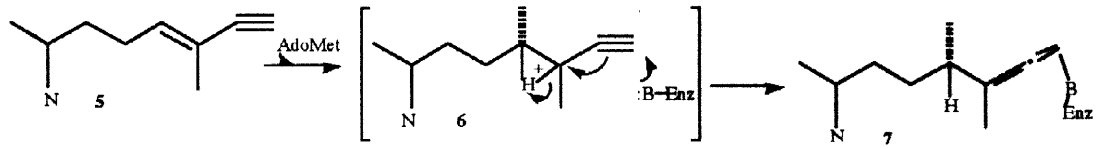


Figure 2. Labeling of SMT enzyme by [3-^{3}H]26-HC (**5**). Lane 1, Coomasssie blue-stained 12% SDS-PAGE gel of SMT enzyme after treatment with [3-^{3}H]26-HC and boiling with SDS bufer, as described in text. Lane 2, Radiofluorogram of SDS-PAGE gel. Migration of standards is indicated. SMT migrates with a Mr of ca. 43,000.

The above results from incubation with a novel 26-homo sterol bearing a triple bond in conjugation with a double bond in the side chain are readily explained by a mechanism-based inactivation process in which **5** can undergo rearrangement or delocalization to place a positive charge in a region of the active site that does not normally encounter electrophilic species. Allylic cation species **6** generated during C-methylation of **5** serves as substrate to react with an active site base or nearby nucleophilic amino acid side chain generating adduct **7**, Scheme 3. Experiments to establish the residues involved with covalent protein modification are in progress.[10]



Scheme 3

References and Notes.

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8. Physical and spectral constants of 26-HC: MP, 92-94 ^{0}C; GLC (Retention time relative to the retention time of cholesterol on 3% % SE-30 packed column operated isothermally at 245 ^{0}C), 1.61; MS (M^+ and other diagnostic ions in high mass region) 392, 377, 359, 297, 270, 255, 237, 207 amu; ^{1}H NMR (in ppm relative to TMS at 300 MHz) 0.82 (s, 18H), 0.96 (d, J = 6.38 Hz, 21H), 0.99 (s, 19H), 1.80 (s, 27H), 2.76 (s, 26'H), 3.63 (m, 3H), 5.36 (s, 15H), 5.95 (m, 24H); ^{13}C NMR (75 MHz) Relevant signals are as follows. C8 (123), C9 (140.9), C14 (151.0), C15 (117.3), C23 (25.2), C24 (140.4), C25 (116.4), C26 (87.0), C26'(73.3), C27 (16.9).
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10. The financial support of the Welch Foundation (D-1276) and Texas THECB-Advanced Technology Program is gratefully acknowledged.