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SYNTHESIS OF RATIONALLY DESIGNED MECHANISM-BASED INACTIVATORS OF THE (*S*)-ADENOSYL-L-METHIONINE: $\Delta^{24(25)}$-STEROL METHYL TRANSFERASE

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Abstract: A series of 26,27-cyclopropylidine side chain modified sterols were prepared for the first time from the known aldehydes by Wittig olefination with the ylide from cyclopropyltriphenylphosphonium bromide in butyllithium. Two novel by-products were detected; sterols with nine carbon side chains which lack the terminal isopropyl group and with double bonds in positions 23,24 or 24,25. The structures of the compounds were confirmed by a combination of chromatographic (GLC and HPLC) and spectral (IR, ^{1}H,^{13}C-NMR) methods.

For some time this laboratory has been interested in the biorational design of sterol biosynthesis inhibitors (SBIs) which interfere with the biomethylation process of the sterol side chain[1]. The enzyme which catalyzes methyl transfer from AdoMet to a sterol acceptor molecule is (S)-adenosyl-L-methionine:$\Delta^{24(25)}$-sterol methyl transferase (SMT). The SMT from plants and fungi promotes the same *si*-face biomethylation mechanism (Scheme 1)[1-3]. However, the SMT from plants evolved different sterol substrate specificities from that in fungi; for instance, sterols with C-4 methyl groups were bound and catalyzed by the plant SMT, but they were not bound by the fungal SMT[1,4,5]. The difference at C-4 was considered in the design of transition state analogs (TSA), which were prepared to act

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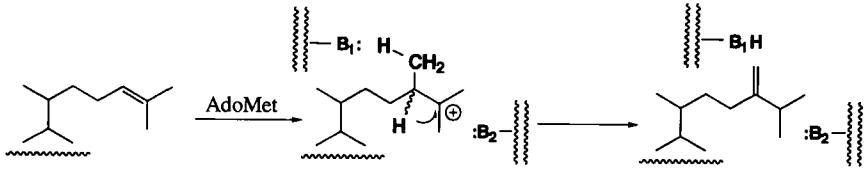
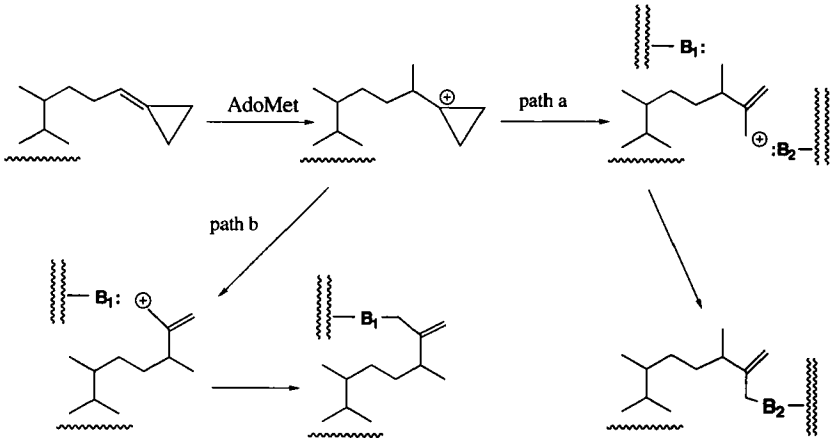
reversibly on biomethylation[6,7]. TSAs with intact C-4 groups were efficient inhibitors, indicating the geminal methyls were not significantly harmful to the activity of the inhibitor, particulary in the fungal enzyme assay. The purpose of this study was to prepare a series of SBIs which were designed to act *irreversibly* on the biomethylation reaction by a mechanism-based type inhibition process. In our design of a mechanism-based probe, we considered first the structural relationship of the inhibitor with that of the sterol side chain bound in the substrate-SMT complex. As shown in scheme 1, biomethylation of the 24,25-double bond involves the participation of a base (negative point charge) from the active site which is used to extract the hydrogen from C-28. Whereas a C-25 carbenium ion is formed transiently, there is no requirement for the enzyme to eliminate the positively charged high energy intermediate, since the resulting 1,2-hydride of H-24 to C-25 shift may be concerted. Hence the interaction of the mechanism-based inactivator may not operate according to route A, shown in scheme 2, which requires that a C-25 carbocation complexes with a complimentary nucleophilic site on the enzyme. TSAs which interfere with biomethylation are thought to operate mechanistically by mimicking the C-25 carbenium ion which is produced during the reaction progress[6,7]. An alternative possibility for interaction of the mechanism-based inhibitor with an appropriate residue from the active site involves route B, shown in scheme 2. Here, the inhibitor may replace the native substrate in the active site and covalently attach itself to the negative point charge involved in removing the C-28 hydrogen from the methylated acceptor molecule. For this to occur, following methylenation of the 24,25-double bond a positive charge must be generated away from C-24 or C-25, e.g., at C-26. During the course of the movement of the intermediate carbocation, the side chain is expected to rotate to a position where the "activated C-26" orients into spatial relationship with the negative point charge, followed by the formation of a chemical bond which establishes irreversible inhibition of catalysis.

We report here the synthesis of a series of mechanism-based inactivators that differ structurally in the nature of the nucleus: 26,27-cyclopropyl 4,4-dimethyl 14α-methyl cholesta-8,24-dien-3β-ol (**3**, Scheme 3), 26,27-cyclopropyl-9β,19-4,4-dimethyl 14α-methyl cholest-24-en-3β-ol (**6**), which should mimic structurally the preferred substrate of the plant SMT and 26,27-cyclopropyl cholesta-8,24-dien-3β-ol (**7**), which should mimic structurally the preferred substrate of the fungal SMT. In our efforts to develop a facile method to prepare these compounds, we discovered that the reaction temperature affected the product distribution, with minimal influence on the 8,9-double bond to isomerize (**7**) or the 9β,19-cyclopropane ring (**6**) to cleave open. In order to characterize the sterols and to make NMR assignments, we performed the usual spectroscopic examinations of the compounds and additional DEPT, COSY and HETCOR experiments, as described in the Experimental.



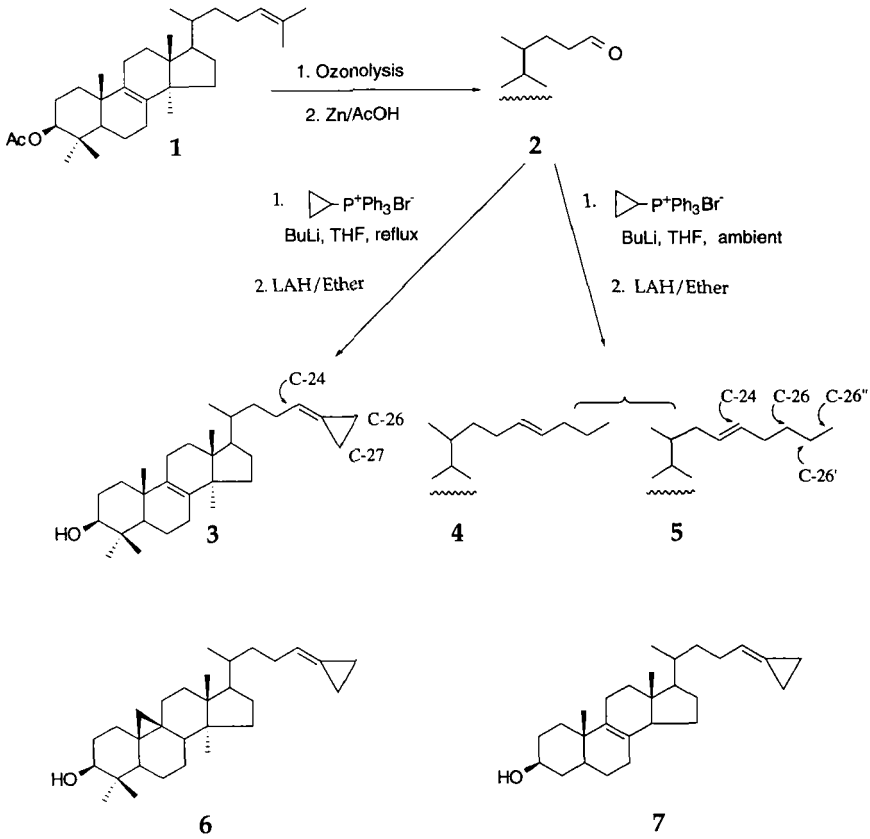
Scheme 1



Scheme 2

EXPERIMENTAL SECTION

Melting points were determined in open ended capillary tubes and are uncorrected. IR spectra were recorded as KBr pellets on a Perkin-Elmer 1600 series FT-IR Spectrophotometer. 1D and 2D NMR (^{1}H- and ^{13}C-NMR) spectra were recorded on a Bruker AC-300 or a AF-200 Spectrometer, with samples referenced to tetramethyl silane. Mass spectra were obtained on a Table-top model 5870 detector and HP 5989 GC-MS mass detector using the DIP/EI method[8]. Thin layer chromatography was performed on Analtech glass plates precoated with silica gel (250μ thickness). Plates were developed with benzene-ether (85/15) or pure toluene. Developed plates were visualized by spraying the plates with a solution of 50% sulfuric acid in methanol, then charring. Column chromatography was performed with silica gel (60-200 mesh, Fischer) eluted isocratically



Scheme 3

with either benzene or benzene-ether. The yield from each reaction was calculated on the basis of the final products purified. HPLC was performed on a semi-preparative Zorbax C_{18} - column (1 x 25 cm) which was eluted with methanol at 4 ml/min, as described[8]. GLC was performed on a HP 5890 gas-chromatograph, using a 3 ft. spiral column packed with 3% SE-30 and operated isothermally at 245 °C. Lanosterol, cycloartenol and zymosterol were purified from commercial sources or plant material as described earlier[8,9].

Ozonolysis: A solution of sterol acetate (100 mg) in dry dichloromethane (10 mL) containing 100 µl of pyridine was ozonized at -70 °C in the presence of ozone generated by O_3 generator (Sandpoint). The reaction progress was monitored by GLC and visually by

color changes in an aqueous KI AcOH trap connected to the reaction vessel. After the reaction was complete (about 30 minutes), the mixture was warmed to room temperature. Zn (25 mg) and acetic acid (1 ml) were added, the solution was stirred for 1 hour and the resulting residual dust filtered off. The filtrate was washed with water and dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue purified by flash chromatography to afford pure 24-aldehyde sterol acetate. The products of zymosterol ozonolysis were more complex than either the lanosterol or cycloartenol products, particularly when an excess of ozone was introduced during the reaction.

Preparation of 26,27-cyclopropyl 4,4-dimethyl,14α-methyl choleta-8,24-dien-3β-ol (**3**). To a solution of cyclopropyltriphenylphosphonium bromide (Aldrich) (383 mg, 1mM) in anhydrous THF (10 ml) was added n-butyllithium in hexane (1.6 M, 0.64 ml). The reaction was allowed to proceed at room temperature for 1 hour, and a solution of 24-aldehydelanosterol acetate (80 mg, 0.2 mM) in 5 ml anhydrous THF was introduced. The mixture was refluxed for 40 minutes and moist ethyl ether was added to decompose any excess butyl lithium. The material was chromatographed on silica gel eluted with benzene/ether (85/15) and the fraction containing acetylated sterol was converted to the free alcohol with LAH dissolved in ether. HPLC purification afforded the desired product (56 mg, 70%): m.p. 125.0-127 °C (from MeOH). GLC (RRT_c, relative retention time to cholesterol) 2.0. IR ν_{max}: 3424, 2942.7, 1572.3, 1454.8, 1372.0, 1196, 1020.0, 720.3, 608.7 cm^{-1}. EI-MS (*m/z*, relative intensity) 424 (M^+, 12%), 409 (M^+-CH_3, 39%), 391(M^+-CH_3-H_2O, 18%), 259 (14 %), 241 (16 %). ^1H and ^{13}C-NMR data of the side chain are reported in Tables 1 and 2. The diagnostic cyclopropylidine side chain signals were characterized partly in the ^1H-NMR from the olefinic proton resonating at the δ 5.74 ppm (H-24) and by DEPT where the isopropyl carbons could be assigned resonating down field from those in lanosterol at δ 2.19 (C-26) and 1.80 (C-27) ppm respectively (Table 1). We recently prepared ^{13}C-isotopically labeled C-26 and C-27 in the lanosterol series (Zhou and Nes unpublished) to confirm the assignments for C-26 and C-27 (see also, 10,11). We found that when the reaction is allowed to proceed under reflux for minimally 30 min. the ratio of side chain **3** to **4/5** was ca. 99:1. When the reaction was allowed to continue at room temperature for another 10 hours the ratio of side chain **3** to **4/5** was 1:2 (optimal conditions, data not shown). The sterols **3** and **4/5** were separated by reversed-phase HPLC (α_C, relative to cholesterol, **3**=0.88 and **4/5**=1.09). Note, that while we follow the recommended revised 1989 IUPAC rules for sterol nomenclature in the paper, sterol biochemists often use the modified Fieser/Nes system, cf. ref. 8 and references cited.

Using the same approach as described above, 26,27-cyclopropyl-9β,19-cyclopropyl cholest-24-en-3β-ol (**6**) was synthesized from its corresponding 24-aldehyde product (80 mg) in similar yield (54.4 mg, 68)%. M.p. 78.0-79.0 °C (from MeOH). GLC RRT_c:

3	0.686 s	0.980 s	0.920 d	ND	5.740 m	1.101 bs	1.101 bs	NP	NP	0.999 s	0.809 s
4 & 5	0.689 s	0.982 s	0.921 d	5.371 m	5.371 m	ND	NP	ND	0.891 t	0.811 s	0.877 s
6	0.965 s	0.551 d 0.333 d	0.905 d	ND	5.742 m	1.010 bs	1.010 bs	NP	NP	0.964 s	0.891 s
7	0.690 s	0.950 s	0.953 d	ND	5.734 m	1.010 bs	1.010 bs	NP	NP	NP	NP

*Chemical shifts given in ppm values from TMS. s, singlet; d, doublet; bs, broad singlet; t, triplet.
ND-not determined; NP-not present.
†Sterol structures are given in scheme 3.

Table 2 ^{13}C-NMR Spectral Values for the Side Chain of Compounds 1-7*

sterol	C-20	C-21	C-22	C-23	C-24	C-25	C-26	C-27	C-26'	C
1	36.25	18.61	36.32	24.90	125.23	130.92	25.73	17.63	NP	
2	35.94	18.32	41.05	28.07	203.12	NP	NP	NP	NP	
3	36.14	18.54	35.86	28.77	118.84	120.59	2.19	1.80	NP	
4	36.17	18.58	36.25	29.25	129.42	130.43	24.10	NP	22.87	1
5	36.01	18.58	34.69	129.86	130.96	29.49	29.69	NP	22.72	1
6	35.76	18.12	35.86	28.79	118.86	120.59	2.19	1.80	NP	
7	35.93	18.55	35.69	28.63	118.80	120.59	2.17	1.78	NP	

*Chemical shifts given in ppm values from TMS. NP-not present.

2.26. IRν_{max}:3436.3, 2931.0, 2860.5, 1648.7, 1625.2, 1460.7, 1372.0, 1096.4, 1049.4, 685.0, 497.0. EI-MS (*m/z*): 424 (M^+, 5%), 409 (M^+-CH_3, 15%), 391 (M^+-CH_3-H_2O, 32%), 363 (12%), 337 (8%), 315 (7%), 284 (24%), 259 (10%). ^1H and ^{13}C-NMR data of the side chain are reported in Tables 1 and 2. Similarly prepared was 26,27-cyclocholesta-8,24-dien-3β-ol (**7**, 24.0 mg, 30%): m.p. 100-101 °C. GLC RRT_c: 1.57. IRν_{max}: 3448.1, 3424.6, 2931.0, 2860.5, 1654.6, 1637.0, 1460.7, 1372.5, 1102.2, 1049.4, 849.6, 685.0, 579.3, 479.4. ^1H and ^{13}C NMR data are given in Tables 1 and 2.

Production of by-products under optimal reaction conditions (10 hr., ambient): 4,4-dimethyl 14αmethyl-20(*R*)-n-nonyl cholesta-8,E24,25-dien-3β-ol **4**; 4,4-dimethyl 14αmethyl-20(*R*)-n-nonyl cholesta-8,E23-dien-3β-ol **5** (unresolved mixture of **4** and **5**, 30.4 mg, yielding 38%): m.p.119.5-120.5 °C (from MeOH). GLC RRTc: 1.92. IRν_{max}: 3448.1, 2942.7, 2860.5, 1648.7, 1631.1, 1454.8, 1366.7, 1025.9, 767.3, 679.2, 491.1. EI-MS (*m/z*): 440 (M^+, 10%), 424 (M^+-CH_3, 25%), 407 (M^+-CH_3-H_2O, 30%), 391 (M^+-CH_3-H_2O, 22%), 259 (8%). ^1H and ^{13}C-NMR data of the side chain are reported in Tables 1 and 2 (assignments based in part on COSY and HETCOR).

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