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Steroidal Inhibitors of Microbial Degradation of Sterol Side Chains

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Methyl esters of acetylene carboxylic acids (**2** and **3**), an α-bromo acid (**4**), and α,α-difluoro acids (**5** and **6**) were synthesized as analogues of the C-24 carboxylic acid which is an intermediate in the microbial degradation of sterol side chains, and these compounds were shown to be inhibitors of the degradation reaction.

Keywords——inhibitor; microbial degradation; sterol side chain; acetylene carboxylic acid methyl ester; bromo acid methyl ester; difluoro acid methyl ester; cholesterol; sitosterol; cholenic acid; *Mycobacterium* sp.

The microbial degradation of the side chains of cholesterol, sitosterol, and campesterol has been reported to proceed through the C-26, C-24, and C-22 carboxylic acids, as shown in Chart 1.[1] Almost 20 years ago, a number of substances were tested by Arima and other researchers for ability to block the complete degradation of these sterols, and this work resulted in the successful microbial production of 17-keto steroids.[2] In an attempt to regulate this multi-step enzymatic reaction at earlier stages, in particular the stage involving the C-24

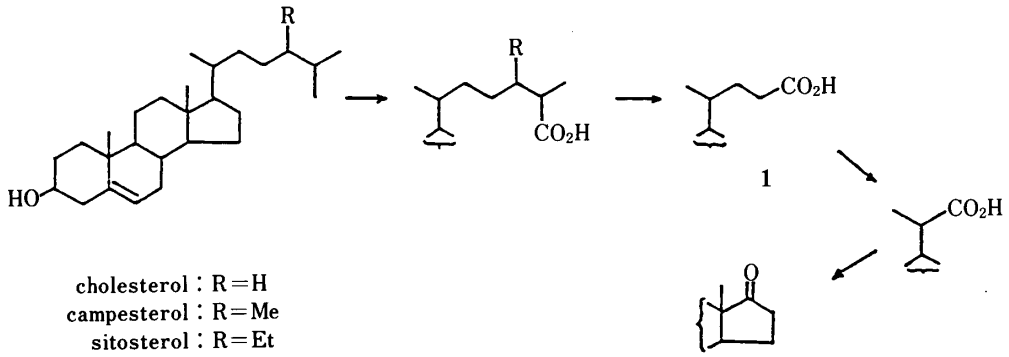

Chart 1

carboxylic acid (**1**), we have prepared several steroidal compounds (**2**—**6**) as analogues of the C-24 acid, and investigated their inhibitory effects on the microbial degradation of sterol side chains. The C-24 acid, which we hoped would be accumulated, is a useful compound, since a C-24 carboxylic acid (cholenic acid) has been utilized as a starting material for the synthesis of vitamin D_3 metabolites and their analogues.[3]

Treatment of the known dibromide **7**[4] with *n*-BuLi (2.5 eq) and then with dry ice afforded the C-24 acetylene acid **8** in 64% yield. Acidic treatment (*p*-TsOH in refluxing aq. dioxane) of **8** afforded the 3β-alcohol **9** in 77% yield. This was esterified with diazomethane to give the methyl ester **2**. The enone **10** was also prepared by a modified Oppenauer oxidation[5] of **2**. The synthesis of the C-25 acetylene acid **3** was carried out by a slight modification of the

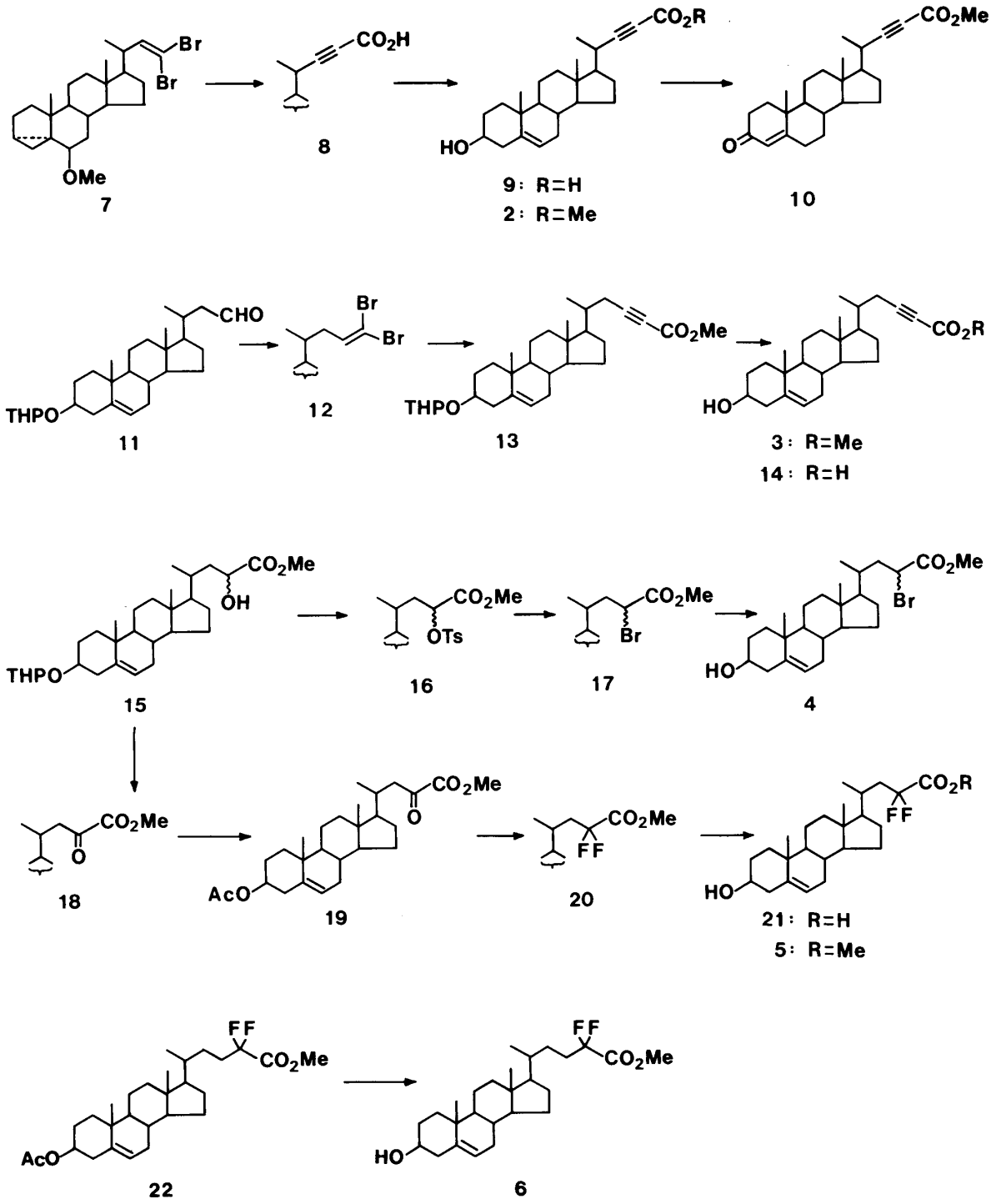

Chart 2

above method. The starting dibromide **12**, obtained from the C-23 aldehyde **11**[6] according to the known method (CBr_4–Ph_3P, 79% yield),[7] was converted into the methyl ester **13** using chloromethyl formate instead of dry ice, in 77% yield. Acidic treatment of the tetrahydropyranyl (THP) ether **13** afforded the 3β-alcohol **3** in 88% yield. Hydrolysis of **3** with base gave the acetylene acid **14** in 84% yield.

At this stage, a preliminary biological test showed that the methyl esters **2** and **3**, but not the acids **9** and **14**, inhibited sterol side chain degradation. Thus, the following compounds were prepared in the form of methyl esters.

TABLE I. Inhibitory Effect of Compounds **2**—**6** on Cholesterol Side Chain Degradation

Compound	Added weight (mg)	Conversion into AD[a] (%)	Compound	Added weight (mg)	Conversion into AD[a] (%)
Not added	—	51	**4**	0.8	36
2	0.8	45		2.0	20
	2.0	15		5.0	16
	5.0	7	**5**	0.8	41
10	0.8	50		2.0	11
	2.0	13		5.0	1
	5.0	6	**6**	0.8	44
3	0.8	57		2.0	20
	2.0	33		5.0	6
	5.0	35			

a) Androst-4-ene-3,17-dione.

The synthesis of the bromo ester **4** was started with the hydroxy ester **15** (epimeric mixture at the C-23 position), which was obtained by hydroxylation[8] (lithium diisopropylamide, O_2) of 3β-tetrahydropyranyloxycholenic acid methyl ester. Treatment of the corresponding tosylate **16** with LiBr afforded the bromide **17** in 89% yield. Deprotection of the THP ether of **17** gave the bromo ester **4** in 89% yield.

Swern oxidation of **15** gave in 95% yield the keto ester **18**, which was converted into the acetate **19** in 59% yield upon treatment with HCl in methanol followed by acetylation (acetyl chloride–pyridine). Treatment of **19** with diethylaminosulfur trifluoride (DAST) in CH_2Cl_2 gave the α,α-difluoro ester **20** in 67% yield. Hydrolysis (KOH/methanol) of **20** smoothly afforded the hydroxy acid **21** in 95% yield. Diazomethane treatment of **21** furnished the methyl ester **5** quantitatively. The homologous difluoro ester **6** was obtained from the acetate **22**[9] in the same manner.

With the possible inhibitors in hand, their effect on the sterol side chain degradation was examined. The microorganism used was *Mycobacterium* sp. NRRL B-3805,[10] which is known to produce androst-4-ene-3,17-dione (AD), and cholesterol was incubated with this organism in the presence of compounds **2**—**6** and the enone **10**. The results are listed in Table I. Among the compounds tested, the difluoro ester **5** exhibited the strongest inhibitory action; cholesterol degradation was completely blocked and cholesterol was recovered unchanged at the dose level of 5 mg. The carbon length of the side chain appeared to be of importance for activity since the C-24 acetylene ester **2** and the C-24 difluoro ester **5** were more potent than the respective C-25 homologues **3** and **6**. Both 3-hydroxy-5-ene and 4-en-3-one functionalities in the steroid nucleus were found to be equally effective, since the acetylene ester **2** and the corresponding enone **10** exhibited comparable levels of inhibition. When commercial sitosterol was incubated in the presence of these inhibitors, the difluoro ester **5** also showed the strongest inhibition activity. In order to confirm that the observed inhibitory effect is related to the structural features of the test compounds, some steroids such as cholestane and 3β-hydroxychol-5-ene were also investigated.[11] These compounds proved to be practically inactive.

Although we have not succeeded in accumulating any intermediates as yet, further studies including *in vitro* experiments are under way to define the enzymatic step(s) blocked by these inhibitors.

Experimental

General——Melting points were determined on a Yazawa hot stage microscope and are uncorrected. The proton

nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Hitachi R-24A (60 MHz) (unless otherwise noted), JEOL JNH-PS-100 (100 MHz), or JEOL FX-200 (200 MHz) spectrometer in $CDCl_3$ solution with tetramethylsilane (TMS) as an internal reference. Ultraviolet (UV) spectra were measured on a Shimadzu UV-200 spectrometer in a solution of ethanol. Mass spectra (MS) (70 eV) were obtained with a Shimadzu GC-MS 9020 DF spectrometer operating in a GC-MS mode except for compounds **8**, **9**, **13**, **14** and **21**, which were recorded in a direct inlet system. Column chromatography was performed with Kieselgel 60 (70—230 mesh, E. Merck). Gas liquide chromatography (GLC) was performed on a Shimadzu GC-7A instrument equipped with a 1 m × 3 mm i.d. glass column containing 1% OV-17 on Shimalite W, operating at 260 °C. The usual work-up refers to dilution with sat. aq. NH_4Cl, extraction with the organic solvent indicated in parenthesis, washing to neutrality, drying, filtration and evaporation under a vacuum. THF refers to tetrahydrofuran.

6β-Methoxy-3α,5-cyclo-5α-chol-22-yn-24-oic Acid (8)——*n*-Butyllithium (3.31 ml of 1.6 M solution in hexane, 5.3 mmol) was added to a solution of the dibromide[4] **7** (1.09 g, 2.21 mmol) in THF (15 ml) at −78 °C, and the mixture was stirred for 30 min. Then, dry ice (1 g) was added at −78 °C and the reaction mixture was warmed to room temperature over 1 h. After the usual work-up (ethyl acetate), the crude product was purified by silica gel column chromatography. Elution with hexane–ethyl acetate (3 : 1) gave 543 mg (1.41 mmol, 64%) of the carboxylic acid **8** as an oil. ^{1}H-NMR δ: 0.73 (3H, s, 18-CH_3), 1.01 (3H, s, 19-CH_3), 1.25 (3H, d, J=7.2 Hz, 21-CH_3), 2.79 (1H, m, 6-H), 3.32 (3H, s, 6-OCH_3), 8.40 (1H, br s, COOH). MS *m/z*: 384 (M^+), 340 ($M-CO_2$).

3β-Hydroxychol-5-en-22-yn-24-oic Acid (9)——A mixture of the *i*-ether **8** (543 mg, 1.41 mmol), dioxane (6 ml), H_2O (2 ml) and a catalytic amount of *p*-toluenesulfonic acid was refluxed for 2 h. The usual work-up (ethyl acetate) afforded a crude product, which was crystallized from methanol to give the carboxylic acid **9** (403 mg, 77%), mp 213.5—215 °C. UV λ_{max}^{EtOH} nm ($\log\varepsilon$): 218.5 (3.3). MS *m/z*: 326 ($M-CO_2$).

Methyl 3β-Hydroxychol-5-en-22-yn-24-oate (2)——An excess of diazomethane (ether solution) was added to a suspension of the carboxylic acid **9** (205 mg) in ether at 0 °C, and the mixture was stirred for 10 min. The solvent was evaporated off and the residue was chromatographed on silica gel. Elution with hexane–ethyl acetate (4 : 1) gave the methyl ester **2** (202 mg, 0.53 mmol, 95%), mp 167—169 °C (from ethyl acetate). ^{1}H-NMR (100 MHz) δ: 0.76 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), 1.31 (3H, d, J=6 Hz, 21-CH_3), 3.60 (1H, m, 3-H), 3.76 (3H, s, CO_2CH_3), 5.36 (1H, m, 6-H). MS *m/z*: 384 (M^+).

Methyl 3-Oxochol-4-en-22-yn-24-oate (10)——A solution of the alcohol **2** (100 mg, 0.26 mmol) and 1-methyl-4-piperidone[5] (0.32 ml, 2.6 mmol) in toluene (10 ml) was refluxed under a Dean-Stark trap until *ca.* 2 ml of distillate had been collected. Aluminum isopropoxide (80 mg, 0.39 mmol) was added to the solution, and the mixture was refluxed for 6 h. The usual work-up (ethyl acetate) gave a crude product, which was chromatographed on silica gel. Elution with hexane–ethyl acetate (4 : 1) gave the enone **10** (72 mg, 0.19 mmol, 73%) as an amorphous solid. ^{1}H-NMR δ: 0.75 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), 1.30 (3H, d, J=6 Hz, 21-CH_3), 3.75 (3H, s, CO_2CH_3), 5.63 (1H, s, 4-H). MS *m/z*: 382 (M^+).

24,24-Dibromo-3β-tetrahydropyranyloxychola-5,23-diene (12)——A mixture of triphenylphosphine (4.24 g, 16.2 mmol) and carbon tetrabromide (2.68 g, 8.09 mmol) in CH_2Cl_2 (10 ml) was stirred at 0 °C for 40 min. A solution of the aldehyde **11**[6] (1.15 g, 2.70 mmol) in CH_2Cl_2 (8 ml) and pyridine (1.5 ml) was added dropwise, and the reaction mixture was stirred for 30 min at room temperature. Hexane was added, and insoluble material was removed by decantation. The insoluble material was dissolved in CH_2Cl_2 and hexane was added to the solution. The insoluble material was removed similarly, and the combined hexane–CH_2Cl_2 solution was concentrated under a vacuum. The residue was applied to a silica gel column. Elution with hexane–ethyl acetate (15 : 1) gave the dibromide **12** (1.24 g, 2.13 mmol, 79%), mp 139—140 °C (from acetone). ^{1}H-NMR δ: 0.69 (3H, s, 18-CH_3), 0.95 (3H, d, J=6 Hz, 21-CH_3), 1.02 (3H, s, 19-CH_3), 3.2—4.2 (3H, m, 3-H, 6′-H of THP), 4.67 (1H, m, 2′-H of THP), 5.32 (1H, m, 6-H), 6.39 (1H, t, J=8 Hz, 23-H). *Anal.* Calcd for $C_{29}H_{44}Br_2O_2$: C, 59.57; H, 7.59. Found: C, 59.48; H, 7.46.

Methyl 3β-Tetrahydropyranyloxychol-5-en-23-yne-24-carboxylate (13)——*n*-Butyllithium (3.33 ml of 1.6 M hexane solution, 5.33 mmol) was added to a solution of the dibromide **12** (1.24 g, 2.13 mmol) in THF (12 ml) at −78 °C, and the mixture was stirred for 30 min. Then, methyl chloroformate (0.49 ml, 6.39 mmol) was added dropwise to the mixture and the reaction mixture was stirred for 30 min at −78 °C. The usual work-up (ether) afforded a crude product, which was chromatographed on silica gel. Elution with hexane–ethyl acetate (10 : 1) gave the methyl ester **13** (791 mg, 1.64 mmol, 77%) as an oil. ^{1}H-NMR δ: 0.68 (3H, s, 18-CH_3), 1.01 (3H, s, 19-CH_3), 1.08 (3H, d, J=6 Hz, 21-CH_3), 3.2—4.2 (3H, m, 3-H, 6′-H of THP), 3.77 (3H, s, CO_2CH_3), 4.70 (1H, m, 2′-H of THP), 5.33 (1H, m, 6-H). MS *m/z*: 482 (M^+), ($M-CH_3O$).

Methyl 3β-Hydroxychol-5-en-23-yne-24-carboxylate (3)——A mixture of the THP-ether **13** (791 mg, 1.64 mmol), THF (5 ml), methanol (5 ml) and several drops of 2 N HCl was stirred at room temperature for 2 h. The usual work-up (ether) gave the alcohol **3** (601 mg, 1.51 mmol, 92%), mp 151—153 °C (from hexane). ^{1}H-NMR (100 MHz) δ: 0.72 (3H, s, 18-CH_3), 1.01 (3H, s, 19-CH_3), 1.10 (3H, d, J=6 Hz, 21-CH_3), 3.60 (1H, m, 3-H), 3.78 (3H, s, CO_2CH_3), 5.35 (1H, m, 6-H). *Anal.* Calcd for $C_{26}H_{38}O_3$: C, 78.35; H, 9.61. Found: C, 77.77; H, 9.47.

3β-Hydroxychol-5-en-23-yne-24-carboxylic Acid (14)——A mixture of the methyl ester **3** (600 mg, 1.51 mmol) in 1,2-dimethoxyethane (10 ml) and $LiOH\cdot H_2O$ (1.2 g) in H_2O (12 ml) was stirred at room temperature for 2 h. The mixture was acidified by addition of 2 N HCl and extracted twice with ethyl acetate. The combined organic layer was

washed with brine, dried over $MgSO_4$ and concentrated. The residue was crystallized from methanol to give the hydroxy acid **14** (546 mg, 1.42 mmol, 94%), mp 221—223 °C. UV λ_{max}^{EtOH} nm (log ε): 218 (2.8). MS *m/z*: 340 ($M-CO_2$). The ^{1}H-NMR spectrum was recorded for the methyl ester of **14**, obtained by treatment with diazomethane, and was identical with that of **3**.

Methyl 23-Hydroxy-3β-tetrahydropyranyloxychol-5-en-24-oate (15)——*n*-Butyllithium (17.7 ml of 1.6 M hexane solution, 28.3 mmol) was added dropwise to a solution of diisopropylamine (3.96 ml, 28.3 mmol) in THF (50 ml) at −78 °C. The solution was stirred at −78 °C for 10 min, at room temperature for 10 min and finally at −78 °C for 20 min. A solution of 3β-tetrahydropyranyloxycholenic acid methyl ester (3.56 g, 7.55 mmol) in THF (13 ml) was added dropwise to the solution of lithium diisopropylamide at −78 °C, and the mixture was stirred for 1 h. Then, oxygen gas was bubbled into the mixture for 30 min at −78 °C. Triethyl phosphite (2.6 ml, 15 mmol) was added, and the whole was stirred for 15 min. Next, aq. NH_4Cl was added, and the usual work-up (ether) gave a crude product which was chromatographed on silica gel. Elution with hexane–ethyl acetate (10 : 1) afforded **15** (2.03 g, 4.15 mmol, 55%) as an amorphous solid. ^{1}H-NMR (200 MHz) δ: 0.68 and 0.71 (each 1.5H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 1.03 (3H,d, J=5.4 Hz, 21-CH_3), 2.68 (1H, dd, J=6, 18 Hz, 23-H), 3.4—4.0 (3H, m, 3-H, 6′-H of THP), 3.78 (3H, s, CO_2CH_3), 4.71 (1H, m, 2′-H of THP), 5.35 (1H, m, 6-H). MS *m/z*: 404 (M − THP + H).

Methyl 3β-Tetrahydropyranyloxy-23-*p*-toluenesulfonyloxychol-5-en-24-oate (16)——*p*-Toluenesulfonyl chloride (1.11 g, 5.85 mmol) was added to a solution of the alcohol **15** (948 mg, 1.95 mmol) in pyridine (10 ml) at 0 °C, and the mixture was stirred overnight. After addition of ice, the mixture was stirred for 20 min, then extracted twice with ethyl acetate. The combined organic layer was washed with 4 N HCl, $NaHCO_3$ and brine, and dried over $MgSO_4$. Evaporation of the solvent gave a crude product, which was chromatographed on silica gel. Elution with hexane–ethyl acetate (7 : 1) afforded the tosylate **16** (774 mg, 1.21 mmol, 62%) as an amorphous solid. ^{1}H-NMR (200 MHz) δ: 0.57 and 0.58 (each 1.5H, s, 18-CH_3), 0.96 (3H, d, J=7 Hz, 21-CH_3), 1.00 (3H, s, 19-CH_3), 2.44 (3H, s, CH_3-Ph), 3.4—4.0 (3H, m, 3-H, 6′-H of THP), 3.65 and 3.66 (each 1.5H, s, CO_2CH_3), 4.72 (1H, m, 2′-H of THP), 4.85 (1H, m, 23-H), 5.35 (1H, m, 6-H), 7.36 (2H, d, J=7 Hz, Ph), 7.82 (2H, d, J=7 Hz, Ph). MS *m/z*: 386 (M − THP − TsOH + H).

Methyl 23-Bromo-3β-tetrahydropyranyloxychol-5-en-24-oate (17)——LiBr (315 mg, 3.62 mmol) was added to a solution of the tosylate **16** (774 mg, 1.21 mmol) in acetone (15 ml), and the mixture was refluxed for 6 h, then concentrated under a vacuum and diluted with ether. The solution was washed with 2 N HCl, $NaHCO_3$ and brine, and dried over $MgSO_4$. The solvent was evaporated off and the residue was chromatographed on silica gel. Elution with hexane–ethyl acetate (20 : 1) gave the bromide **17** (595 mg, 1.08 mmol, 89%), mp 118—140 °C (from MeOH). ^{1}H-NMR (200 MHz) δ: 0.65 and 0.72 (each 1.5H, s, 18-CH_3), 0.94 and 0.96 (each 1.5H, d, J=6 Hz, 21-CH_3), 1.01 (3H, s, 19-CH_3), 3.4—4.0 (3H, m, 3-H, 6′-H of THP), 3.79 (3H, s, CO_2CH_3), 4.30 and 4.33 (each 0.5H, dd, J=3.5, 11.4 Hz, and 4.4, 11.2 Hz, respectively, 23-H), 4.73 (1H, m, 2′-H of THP), 5.37 (1H, m, 6-H). *Anal.* Calcd for $C_{30}H_{47}BrO_4$: C, 65.32; H, 8.59. Found: C, 65.30; H, 8.60.

Methyl 23-Bromo-3β-hydroxychol-5-en-24-oate (4)——Several drops of 2 N HCl were added to a solution of the THP-ether **17** (595 mg, 1.08 mmol) in THF (10 ml)–MeOH (10 ml), and the mixture was stirred at room temperature for 2 h. Then, ethyl acetate was added and the usual work-up gave a crude product, which was chromatographed on silica gel. Elution with hexane–ethyl acetate (3 : 1) gave the bromide **4** (449 mg, 0.96 mmol, 89%), mp 119—132 °C[12] (from MeOH). ^{1}H-NMR (200 MHz) δ: 0.65 and 0.72 (each 1.5H, s, 18-CH_3), 0.94 and 0.96 (each 1.5H, d, J=6 Hz, 21-CH_3), 1.01 (3H, s, 19-CH_3), 3.50 (1H, m, 3-H), 3.77 (3H, s, CO_2CH_3), 4.31 and 4.34 (each 0.5H, dd, J=11.4, 3.5 Hz, and 12.0, 4.0 Hz, respectively, 23-H), 5.33 (1H, m, 6-H). MS *m/z*: 466/468 (M^+). *Anal.* Calcd for $C_{25}H_{39}BrO_3$: C, 64.23; H, 8.41. Found: C, 64.22; H, 8.44.

Methyl 23-Oxo-3β-tetrahydropyranyloxychol-5-en-24-oate (18)——Dimethyl sulfoxide (1.77 ml, 24.9 mmol) was added dropwise to a solution of oxalyl chloride (1.09 ml, 12.5 ml) in CH_2Cl_2 at −78 °C, and the mixture was stirred for 5 min. A solution of the alcohol **15** (2.03 g, 4.15 mmol) in CH_2Cl_2 (8 ml) was then added dropwise at −78 °C. The whole was stirred for 20 min, then triethylamine (3.50 ml, 24.9 mmol) was added, and the reaction mixture was warmed gradually to room temperature. The usual work-up (ether) gave a crude product which was chromatographed on silica gel. Elution with hexane–ethyl acetate (15 : 1) gave **18** (1.92 g, 3.94 mmol, 95%) as an amorphous solid. ^{1}H-NMR δ: 0.71 (3H, s, 18-CH_3), 0.95 (3H, d, J=6 Hz, 21-CH_3), 0.99 (3H, s, 19-CH_3), 2.71 (2H, m, 22-H_2), 3.2—4.1 (3H, m, 3-H, 6′-H of THP), 3.85 (3H, s, CO_2CH_3), 4.70 (1H, m, 2′-H of THP), 5.33 (1H, m, 6-H). MS *m/z*: 402 (M − THP + H).

Methyl 3β-Acetoxy-23-oxochol-5-en-24-oate (19)——A mixture of the THP-ether **18** (1.92 g, 3.94 mmol), THF (7 ml), methanol (7 ml) and several drops of 2 N HCl was stirred at room temperature for 2 h. The usual work-up (ethyl acetate) and purification by silica gel column chromatography (elution with hexane–ethyl acetate (5 : 2)) gave the 3β-alcohol (1.22 g, 3.04 mmol, 77%), mp 163.5—165 °C (from methanol). ^{1}H-NMR δ: 0.75 (3H, s, 18-CH_3), 1.00 (3H, d, J=7 Hz, 21-CH_3), 1.07 (3H, s, 19-CH_3), 2.69 (2H, m, 22-H_2), 3.60 (1H, m, 3-H), 3.86 (3H, s, CO_2CH_3), 5.33 (1H, m, 6-H). *Anal.* Calcd for $C_{25}H_{38}O_4$: C, 74.59; H, 9.52. Found: C, 74.34; H, 9.45. Acetyl chloride (0.21 ml, 3.04 mmol) was added to a solution of the 3β-alcohol (1.22 g, 3.04 mmol) in pyridine (15 ml) at 0 °C, and the mixture was stirred for 30 min. The usual work-up gave a crude product, which was chromatographed on silica gel. Elution with hexane–ethyl acetate (5 : 1) gave **19** (933 mg, 2.10 mmol, 76%), mp 143—145 °C (from hexane). ^{1}H-NMR δ: 0.73

(3H, s, 18-CH_3), 0.97 (3H, d, J=6 Hz, 21-CH_3), 1.02 (3H, s, 19-CH_3), 2.04 (3H, s, 3-OAc), 2.72 (2H, m, 22-H_2), 3.86 (3H, s, CO_2CH_3), 4.58 (1H, m, 3-H), 5.38 (1H, m, 6-H). *Anal.* Calcd for $C_{27}H_{40}O_5$: C, 72.94; H, 9.07. Found: C, 73.09; H, 9.04.

Methyl 3β-Acetoxy-23,23-difluorochol-5-en-24-oate (20)——Diethylaminosulfur trifluoride (1.79 ml, 14.7 ml) wad added dropwise to a solution of **19** (933 mg, 2.10 mmol) in CH_2Cl_2 (10 ml) at 0 °C, and the mixture was stirred at room temperature for 16 h. After addition of 5% $NaHCO_3$, the usual work-up (ether) gave a crude product which was chromatographed on silica gel. Elution with hexane–ethyl acetate (50 : 1) gave **20** (651 mg, 1.40 mmol, 67%), mp 135—136.5 °C (from hexane). ^{1}H-NMR δ: 0.70 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 1.03 (3H, d, J=7 Hz, 21-CH_3), 2.02 (3H, s, 3-OAc), 3.86 (3H, s, CO_2CH_3), 4.58 (1H, m, 3-H), 5.34 (1H, m, 6-H). *Anal.* Calcd for $C_{27}H_{40}F_2O_4$: C, 69.50; H, 8.64. Found: C, 69.67; H, 8.54. MS *m/z*: 406 (M − AcOH).

23,23-Difluoro-3β-hydroxychol-5-en-24-oic Acid (21)——A saturated methanol solution of K_2CO_3 (4 ml) was added to a solution of the acetate **20** (651 mg, 1.40 mmol) in THF (8 ml) and methanol (5 ml), and the mixture was stirred at room temperature for 1 h, acidified by addition of 2 N HCl, and extracted twice with ethyl acetate. The combined organic layer was washed with brine, dried over $MgSO_4$ and concentrated. The residue was crystallized from ethyl acetate to afford **21** (561 mg, 1.37 mmol, 98%), mp *ca.* 210 °C (dec.). MS *m/z*: 410 (M^+).

Methyl 23,23-Difluoro-3β-hydroxychol-5-en-24-oate (5)——The acid **21** was esterified with diazomethane as described for **9**. Purification of the crude product on a silica gel column (eluted with hexane–ethyl acetate (2 : 1)) afforded the methyl ester **5** (19 mg, 91%), mp 154—156.5 °C (from ethyl acetate). ^{1}H-NMR (100 MHz) δ: 0.72 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 1.04 (3H, d, J=6 Hz, 21-CH_3), 3.60 (1H, m, 3-H), 3.88 (3H, s, CO_2CH_3), 5.36 (1H, m, 6-H). MS *m/z*: 424 (M^+).

Methyl 24,24-Difluoro-3β-hydroxychol-5-ene-24-carboxylate (6)——A saturated methanol solution of K_2CO_3 (1 ml) was added to a solution of the acetate[9] **22** (100 mg, 0.21 mmol) in THF (2 ml) and methanol (1 ml), and the mixture was stirred at room temperature for 1 h, acidified by addition of 2 N HCl, and extracted twice with ethyl acetate. The combined organic layer was washed with brine, dried over $MgSO_4$ and evaporated to dryness. The residue was treated with a large excess of diazomethane at 0 °C to give the hydroxy methyl ester **6** (81 mg, 0.19 mmol, 90%), mp 128—132 °C (from methanol). ^{1}H-NMR (200 MHz) δ: 0.68 (3H, s, 18-CH_3), 0.94 (3H, d, J=6 Hz, 21-CH_3), 1.00 (3H, s, 19-CH_3), 3.54 (1H, m, 3-H), 3.87 (3H, s, CO_2CH_3), 5.35 (1H, m, 6-H). *Anal.* Calcd for $C_{26}H_{40}F_2O_3$: C, 71.20; H, 9.19. Found: C, 71.34; H, 9.15.

Incubation——*Mycobacterium* sp. NRRL B-3805 was grown for 1 d in liquid medium[10] (20 ml) containing nutrient broth (160 mg), yeast extract (20 mg) and cholesterol (1.0 mg), and then a solution of cholesterol (10 mg) and the test inhibitor (a variable amount) in dimethylformamide (0.7 ml) was added. After incubation for another 4 d, the mixture was extracted with ethyl acetate and the conversion yield into AD was analyzed, after CH_2N_2 treatment, by GLC.

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11) 3β-Hydroxycholenic acid methyl ester showed some inhibitory activity, but was less active than **3**.
12) This is due to the presence of a mixture of C-23 epimers. Stereochemically homogeneous isomer (23*S*)- or (23*R*)-**4** could be obtained since the epimers of the tosylate **16** are separable on thin-layer chromatography.