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22-Hydroxycholesterol Derivatives as HMG CoA Reductase Suppressors and Serum Cholesterol Lowering Agents

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A series of 22-hydroxycholesterol derivatives with a modified side chain terminus was prepared. These agents were evaluated in vitro and in vivo for their ability to suppress HMG CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis. In tissue culture assays, 22-hydroxycholesterol as well as the side chain modified analogues were potent inhibitors of HMG CoA reductase. However, only those sterols with a modified side chain terminus were effective suppressors of liver reductase when administered ig to rats. 22-Hydroxy-25-methylcholesterol (**4a**) and 25-fluoro-22-hydroxycholesterol (**15a**) significantly lowered serum cholesterol levels when administered ig to primates; 25-chloro-22-hydroxycholesterol (**15b**) and the analogue with a cyclopropyl terminus, **20b**, were ineffective. The cholesterol-lowering sterols did not significantly alter lipoprotein levels; however, the two compounds have been shown to inhibit acyl-coenzyme A:cholesterol acyl-transferase (ACAT) in tissue culture studies.

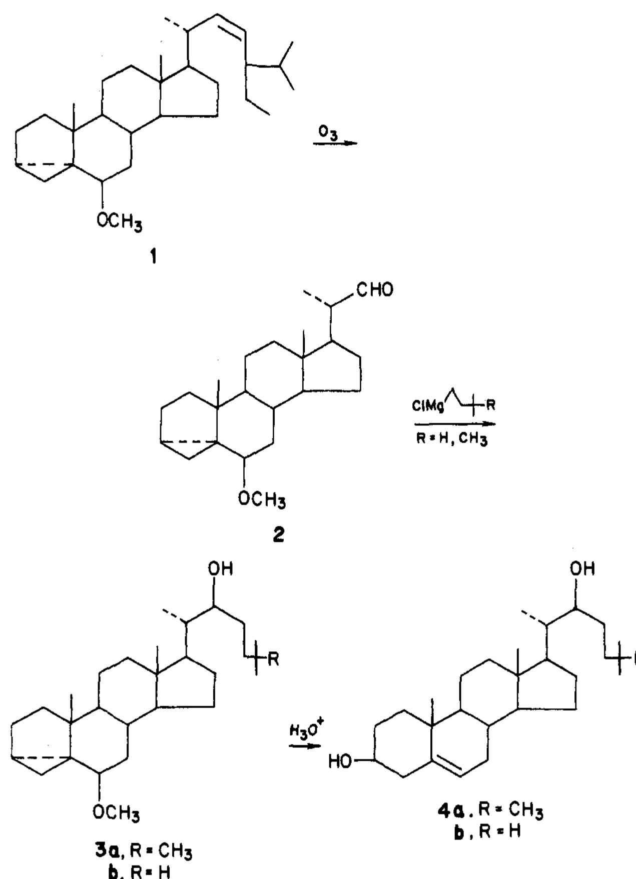
Cholesterol, a necessary component of all mammalian membranes, is able to control its own biosynthesis by regulating the activity of the rate-limiting enzyme in this sequence, HMG CoA reductase.[1] In this receptor-mediated process,[2] the feedback regulation of this enzyme should maintain plasma lipoprotein concentrations high enough to supply cells with cholesterol but not so high to potentiate cholesterol deposition in the arterial wall, thereby accelerating the development of atherosclerosis.[3] Consequently, when this regulatory process is deranged, certain diseased states are produced.[4]

Recent in vitro studies by a number of groups using both animal[5–7] and human[8,9] tissue cultures have demonstrated that various oxygenated sterols are potent suppressors of HMG CoA reductase activity. Included in this group are sterols oxygenated at both the 3- and the 6-, 7-, 15-, 20-, 22-, or 25-positions. However, with the exception of a 15-keto sterol and its derivatives which have shown protracted in vivo regulatory activity in both rodents[10a–c] and primates,[10d] oxygenated sterols have been devoid of biological activity in the whole animal.[11,12] The lack of in vivo activity by most of these compounds might be predicted, however, since oxygenated sterols are known to be rapidly metabolized and eliminated.[13] Thus, the inhibitory effect of oxygenated sterols on cholesterol synthesis would be transient and subsequent lowering of serum cholesterol levels would be minimal.

In an effort to determine whether the apparent increased in vitro potency of these oxygenated sterols might be exploited for the regulation of cholesterol biosynthesis, we undertook a program to prepare sterols containing substituents which would prevent, or at least retard, metabolism. By prolonging in vivo duration, these molecules would be better able to exert their influence on HMG CoA reductase and this modulation, in turn, would result in a lowering of serum cholesterol levels.

The work of Mosbach et al. and others on the metabolism of cholesterol to bile acids has shown that hydroxylations at the 25- or 26-positions are important biochemical transformations in the elimination of the side-chain terminus.[14] These studies suggested that sterols oxygenated at other sites in the molecule and also containing appropriate blocking groups at, or around, these two key positions should have a greater chance of exerting their enzyme regulating influence in vivo. Thus, these compounds would be resistant to liver hydroxylation and subsequent conversion to bile acids, acidic metabolites of cholesterol which are ineffective as feedback regulators of cholesterol biosynthesis.[8]

Scheme I



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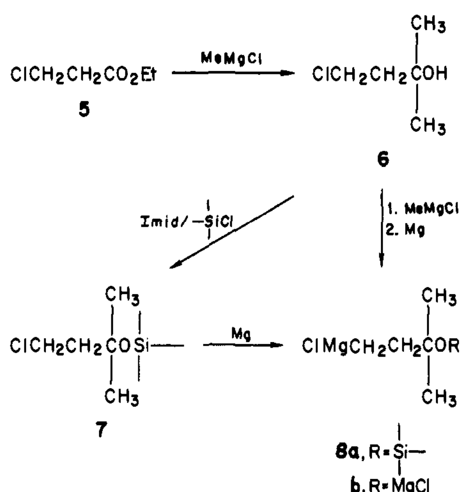
Scheme II



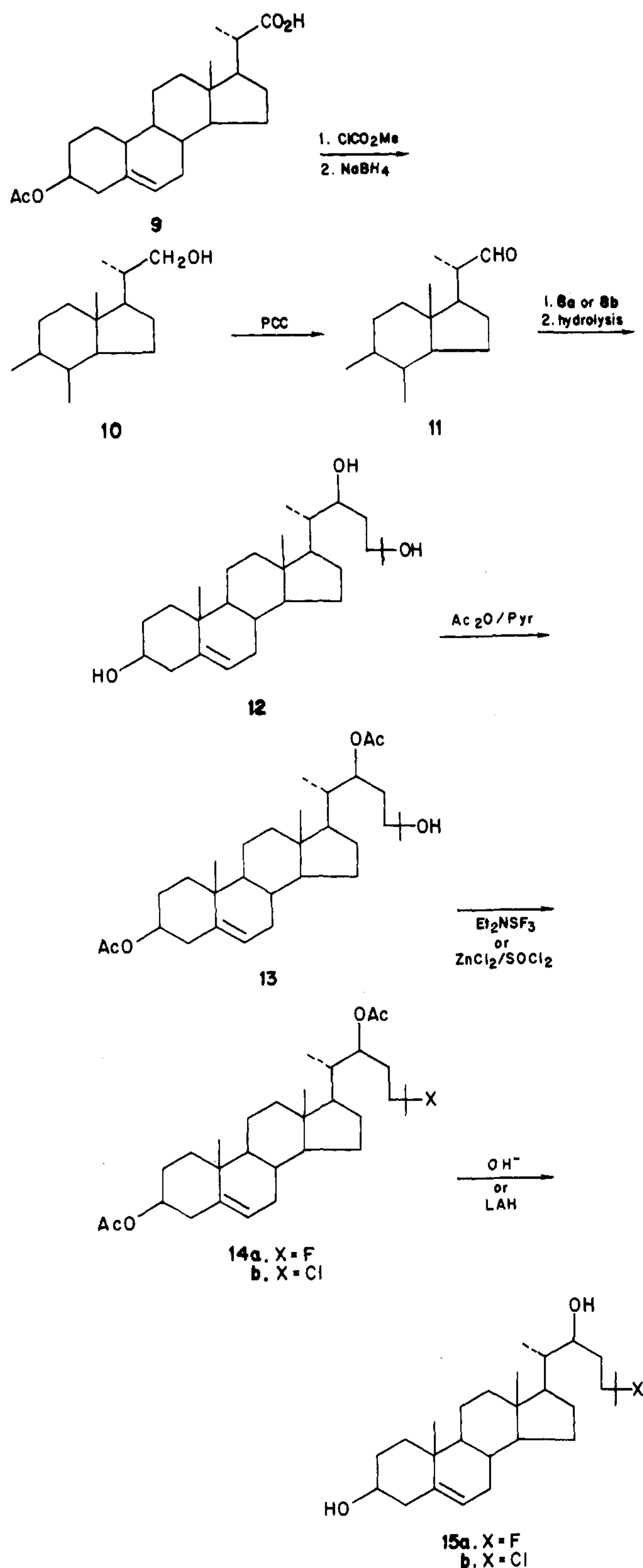
To test this hypothesis, we prepared analogues of 22-hydroxycholesterol with various metabolism-blocking groups at the 25-position or with 26- and 27-carbon atoms incorporated into a cyclopropyl ring. 22-Hydroxycholesterol has been shown to be a potent regulator of HMG CoA reductase in tissue culture assays,[5] and modifications of this oxygenated sterol were synthetically expedient.[15]

Chemistry. Initially, 22-hydroxy-25-methylcholesterol (**4a**) was prepared as shown in Scheme I. The i-steroidal methyl ether of stigmasterol **1**[16] was converted to the corresponding bisnorcholanaldehyde **2** upon ozonolysis and reductive workup.[17] Treatment of **2** with the appropriate Grignard reagent gave **3a**, which upon acid hydrolysis afforded the diol **4a**.[18]

Cram's rules of asymmetric induction predict that the predominant isomer from this Grignard reaction should possess the 22*S* configuration.[19] In previous work on the synthesis of 22-hydroxycholesterol (**4b**), the 22*S* isomer was that which predominated in a mixture comprised of a 6:1 ratio of isomers.[18] After purification of **4a**, we observed, from ^{13}C NMR spectroscopy, a single isomer for this compound (or its acetate). Moreover, the optical rotation of **4a** matched well with that of 22(*S*)-hydroxycholesterol either prepared by us using this route or reported by other groups.[20]

Sterols with a substituent other than methyl at the 25-position were synthesized from Grignard **8** whose preparation is shown in Scheme II. Ethyl 3-chloropropionate

Scheme III



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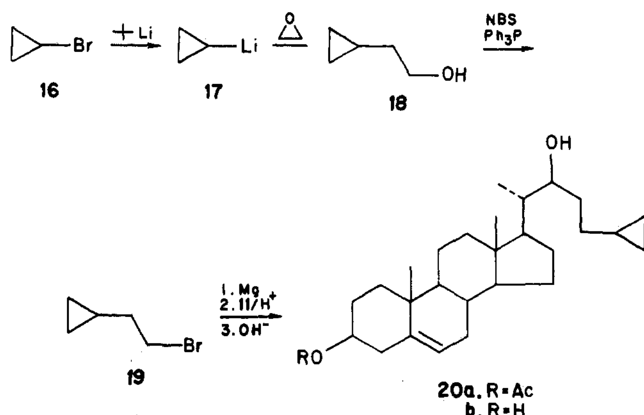
Scheme IV



(5) was treated with excess methylmagnesium chloride to yield the carbinol 6. Carbinol 6 was initially protected as the silyl ether 7[21] before conversion to the Grignard reagent 8a. Subsequently, we found that the unprotected carbinol 6 could be converted to the Grignard reagent 8b by treatment of 6 with about 1 equiv of methylmagnesium chloride prior to Grignard formation with magnesium turnings, thus making the alcohol protection step unnecessary.[22]

An alternate, more readily synthesized aldehyde precursor of the 22-hydroxysterols was then utilized for the preparation of the subsequent analogues (Scheme III). Commercially available 3-acetoxy-22,23-bisnorcholenic acid (9) was converted to the 3-acetoxy alcohol 10 via its mixed anhydride and subsequent reduction with sodium borohydride according to the method of Ishiguro.[23] Oxidation of the alcohol 10 with pyridinium chlorochromate[24] afforded the requisite aldehyde 11.[23] Treatment of 11 with either 8a or 8b gave, after hydrolysis, triol 12,[25] which readily underwent acetylation of the 3- and 22-hydroxyl groups to give 13. Conversion of the 25-alcohol 13 to the 25-fluoro or 25-chloro compounds 14a and 14b was carried out with use of Et_2NSF_3[26] or $ZnCl_2/SOCl_2$,[27] respectively. The fluoro diol 15a was formed by aqueous basic hydrolysis of 14a, whereas the chloro diol 15b was produced from 14b by LAH treatment since 14b underwent partial olefin formation during aqueous hydrolysis.

The desired cyclopropyl sterol 20 required the preparation of cyclopropylethyl bromide (19). Hart had reported that the precursor alcohol 18 was formed upon treatment of cyclopropyllithium (17) with ethylene oxide.[28] Rather than forming 17 by treatment of cyclopropyl chloride with finely dispersed lithium metal as previously reported,[29] we prepared 17 from cyclopropyl bromide (16) and *tert*-butyllithium; subsequent treatment with ethylene oxide gave 18 (Scheme IV).[28,30] Following a procedure by Hanessian,[31] 18 was converted to the bromide 19 in high yield with use of triphenylphosphine and *N*-bromosuccinimide in DMF.[30] The bromide 19 was converted to the Grignard reagent in the usual manner, and condensation with aldehyde 11 gave the steroidal cyclopropyl diol 20b after saponification of the 3-acetate 20a.

Table I. Effects of Oxygenated Sterols on HMG CoA Reductase Activity in Human Fibroblast Tissue

compd	concn, μg/mL	% inhibn[a]
4a	1	95[b]
4b	NT[c]	
15a	1	>95
20b	1	>95

[a] As determined by the method of Brown and Goldstein; see ref 32. [b] Results are expressed as a percentage of control activity determined on the same day as the test compound. Each value is the result of at least two sets of experiments. [c] Not tested in this assay. Other investigators had previously found this compound to be a potent suppressor of HMG CoA reductase levels in other tissue culture assays.[5]

Table II. Effects of Oxygenated Sterols on Elevated Liver Reductase Levels in the Diazasterol Treated Rat[12,33]

compd	MED,[a] mg/kg	% suppression[b]
4a	0.5	39 ± 6[c]
4b	>30	
15a	0.5	26 ± 2
15b	0.2	28 ± 5
20b	0.5	30 ± 3

[a] Minimum effective dose (ig) necessary to produce a ≥25% decrease of elevated liver HMG CoA reductase levels in diazasterol treated rats. [b] Percent suppression of elevated reductase levels from control at indicated dose as determined in groups of four to seven animals per dose, including SEM values. [c] Statistical analysis utilized the Student's *t* test.

Biological Results and Discussion

Selected compounds were initially evaluated in human fibroblast tissue culture assays where suppression of HMG CoA reductase was determined according to the method of Brown and Goldstein.[32] These data, shown in Table I, indicated that the 22-hydroxylated sterols with a modified side chain terminus were potent suppressors of reductase, decreasing enzyme activity by at least 95% at 1 μg/mL concentration. Thus, the modifications made to the side chain did not significantly diminish the ability of these oxygenated compounds to suppress the activity of HMG CoA reductase. These agents were then evaluated in an in vivo assay to determine their ability to suppress elevated HMG CoA reductase levels in rats.[33] In this test, animals were pretreated with 20,25-diazacholesterol,[34] a compound which had previously been reported to cause a twofold enhancement of normal hepatic reductase activity in the rat upon subcutaneous (sc) administration.[12] Rather than sc administration, the diazasterol was administered intragastrically (ig), a route which we found to cause a three- to fourfold enhancement of liver reductase levels in the rat.[33] Pretreated animals were then admin-

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Table III. Effects of Oxygenated Sterols on Serum Cholesterol and Lipoprotein Levels in the Primate

	cholesterol levels, mg/dL		lipoprotein levels, %					
			VLDL		LDL		HDL	
compd[a]	control[b]	14 day treated[c]	control	14 day treated	control	14 day treated	control	14 day treated
4a	205 ± 2	167 ± 16[d] (−18%)[e]	5 ± 2	6 ± 2 (+14%)	26 ± 2	25 ± 3 (−3%)	66 ± 0	66 ± 6 (−1%)
15a	178 ± 13	150 ± 8[d] (−15%)	10 ± 1	8 ± 0[d] (−20%)	26 ± 1	25 ± 5 (−4%)	57 ± 3	61 ± 4 (+7%)
15b	184 ± 11	168 ± 7 (−8.6%)	4 ± 1	4 ± 1 (−5%)	26 ± 5	22 ± 6 (−15%)	67 ± 6	70 ± 7 (+4%)
20b	182 ± 6	179 ± 19 (−1.6%)	6 ± 0	4 ± 0 (−34%)	24 ± 2	23 ± 2 (−2.9%)	69 ± 2	68 ± 2 (−3.5%)
control vehicle	176 ± 19	180 ± 22	8 ± 1	4 ± 1	28 ± 2	29 ± 3	62 ± 2	64 ± 4

[a] Administered at 10 mg/kg ig for 14 days in 0.1% Tween 80-saline (1mL/kg of animal weight). [b] Control values are average levels determined over a 2-week period prior to administration of test compound, including standard error of the mean of these measurements. [c] Statistical evaluation utilized a Student's t test. [d] Statistically significant lowering, $p \leq 0.05$. [e] Percent change from control.

istered test compounds (ig) to determine the ability of these substances to suppress elevated enzyme levels. The results, shown in Table II, indicated that in the 22-hydroxycholesterol series, only compounds bearing a modified side chain terminus manifested the enhanced potency that would be expected on the basis of the tissue culture results of Table I. 22-Hydroxycholesterol itself (**4b**) was an ineffective suppressor of reductase activity even at 30 mg/kg, whereas the other 22-hydroxysterols showed statistically significant lowering of reductase activity at a dose of 0.5 mg/kg or lower.

The active compounds in the latter assay were then evaluated for their ability to lower serum cholesterol. Experience in our own and other laboratories had indicated that the normal rat was a poor model for the evaluation of agents which might lower serum cholesterol levels through suppression or inhibition of HMG CoA reductase.[35] However, it has been shown by the Sankyo group that the primate was a suitable model for this determination.[36] Thus, male rhesus monkeys, previously screened for elevated serum cholesterol levels and maintained on a normal diet, were treated orally (10 mg/kg per day) with these sterols over a 2-week period. In addition to changes in serum cholesterol levels, effects on lipoproteins were recorded,[37] as shown in Table III.

Compounds **4a** and **15a** were most effective in lowering serum cholesterol levels, attaining statistically significant depression over this time period. Neither of these two compounds, however, significantly decreased low-density lipoprotein (LDL) levels or increased high-density lipoprotein (HDL) levels. Only **15b** indicated a tendency to decrease LDL levels, though this was not statistically significant. The lowering of LDL levels has been established as a goal of hypercholesterolemic therapy on the basis of epidemiologic and animal data.[38] The raising of HDL levels has also been associated with a decrease in mortality from cardiovascular disease.[39] The absence of a favorable effect on these lipoprotein fractions contrasts effects observed in similar primate studies with compactin, a competitive inhibitor of HMG CoA reductase,[36] as well as 3β-hydroxy-5α-cholest-8(14)-en-15-one, a suppressor of sterol biosynthesis.[10d]

Interestingly, compounds **4a**[40] and **15a**[41] have been shown to suppress the synthesis of cholesterol ester formation in intact fibroblasts by inhibiting the activity of of acyl-coenzyme A:cholesterol acyltransferase (ACAT) as well as down-regulating LDL surface receptors. This latter effect may also contribute to the decrease in the cholesterol esterification process[41] as well as accounting for the absence of a lowering of serum LDL which we observed in our monkey studies. ACAT plays an important role in the accumulation of cholesterol esters within smooth muscle cells of the arterial wall during the development of atherosclerosis.[4a,42] Inhibition of this enzyme suggests that the pathological accumulation of cholesterol esters may be reduced by these sterols which are acting as modulators of the enzyme activity. This modulation, along with the ability of these sterols to suppress HMG CoA reductase, lower serum cholesterol levels, and reduce LDL receptor populations, represents a unique biological profile[40] with potentially beneficial therapeutic action.

Experimental Section

Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. NMR spectra were obtained on Varian A60-A, T-60, or 100XL-15 spectrometers using Me_4Si as an internal standard. UV spectra were run on a Beckman DK-2A and rotations were determined on a Perkin-Elmer 141 instrument. The spectra were recorded by the group of A. J. Damascus and the microanalyses were performed by the group of E. Zielinski. (Diethylamino)sulfur trifluoride preparation was carried out by M. Scaros and associates. All new compounds were subjected to elemental analysis and results were within ±0.4% of theoretical values.

3α,5α-Cyclo-6β-methoxy-5α-23,24-bisnorcholan-22-al (2). The i-steroid of stigmasterol was prepared according to the procedure of Nes and Steele.[16] Ozonolysis was carried out as described by Hutchins et al. with dimethyl sulfide added (2 g/3 g of steroid) to the cold reaction mixture (−65 °C) rather than zinc dust and acetic acid during the reductive workup.[17] For optimal yields the i-steroidal aldehyde was used in the crude state as soon as possible after preparation.

3α,5α-Cyclo-22-hydroxy-6β-methoxy-5α-25-methylcholestane (3a). To 13.4 g (0.04 mol) of freshly prepared crude aldehyde **2** in 100 mL of THF in a nitrogen atmosphere cooled to ca. 0 °C was added 50 mL of 1.35 M (0.0675 mol) (3,3-dimethylbutyl)magnesium chloride in THF dropwise over a 25-min period. The cooling bath was then removed and the reaction was stirred at room temperature for 1 h before addition of 100 mL of saturated NH_4Cl solution. A portion of ether was added, and the two layers were separated. The aqueous phase was extracted with an additional portion of ether, and the combined extracts were washed with saturated NaCl solution and dried (Na_2SO_4). Solvent removal in vacuo gave 17.3 g of oil which was used for the subsequent hydrolysis reaction without further purification: NMR ($CDCl_3$) δ 0.73 (3 H, s, 13-CH_3), 0.90 (9 H, s, side-chain terminus), 1.03 (3 H, s, 10-CH_3), 3.32 (3 H, s, OCH_3).

22(*S*)-Hydroxy-25-methylcholesterol (4a). To 17.3 g (0.04 mol) of **3a** in 250 mL of dioxane and 75 mL of H_2O was added

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0.5 g of tosyl acid monohydrate and the reaction mixture was heated at steam bath temperature for 5 h. After cooling, H_2O was added and the aqueous solution was extracted with three portions of ether. The combined extracts were washed with saturated NaCl solution and dried (Na_2SO_4). Solvent removal in vacuo gave an oil which solidified upon trituration with EtOH. Recrystallization from aqueous EtOH gave 5.3 g (32%) of **4a**. Low-pressure chromatography of the mother liquors using a Waters LC/System 500 instrument and a Prep PAK-500 silica cartridge and eluting with 10% EtOAc/Skelly B gave an additional 1.5 g (40% total yield from **2**) of **4a**: mp 191–193 °C; NMR ($CDCl_3$) δ 0.70 (3 H, s, 13-CH_3), 0.88 (9 H, s, side-chain terminus), 1.01 (3 H, s, 10-CH_3), 5.35 (1 H, m, 6-H); ^{13}C NMR (C_5D_5N) δ 12.0 (18-CH_3), 12.3 (21-CH_3), 19.6 (19-CH_3), 71.2 (3-CH), 73.3 (22-CH), 121.2 (6-CH), 142.0 (5-C); $[\alpha]_{589}$ −51° (*c* 0.96, $CHCl_3$). Anal. ($C_{28}H_{48}O_2$), C, H.

The ^{13}C NMR (C_5D_5N) of the mother liquors indicated the presence of the 22*R* isomer: 12.0 (18-CH_3), 13.2 (21-CH_3), 19.6 (19-CH_3), 71.2 (3-CH), 73.7 (22-CH), 121.2 (6-CH), 142.0 (5-C).

Compound **4b** was prepared in a similar manner from **2**, utilizing the appropriate Grignard reagent: mp 179–181 °C (lit.[20] mp 180 °C); ^{13}C NMR (C_5D_5N) 12.0 (18-CH_3), 12.3 (21-CH_3), 19.6 (19-CH_3), 71.2 (3-CH), 72.7 (22-CH), 121.1 (6-CH), 142.0 (5-C); $[\alpha]_{589}$ −51° (*c* 0.99, $CHCl_3$) (lit.[20] $[\alpha]_{589}$ −54°).

4-Chloro-2-methyl-2-butanol (6). To 27.3 g (0.2 mol) of ethyl 3-chloropropionate (**5**) in 150 mL of THF at ca. −20 °C under a nitrogen atmosphere was added 135 mL of 2.95 M (0.40 mol) methylmagnesium bromide in ether solution over a 45-min period. After addition, the cooling bath was removed and the reaction mixture was stirred at room temperature for 4 h. Saturated ammonium chloride solution was then added followed by 1 N HCl solution to dissolve the inorganic salts present. After extraction of the aqueous solution with three portions of ether, the combined extracts were washed with saturated NaCl solution and dried (Na_2SO_4). Solvent removal gave 23.5 g of oil. This residue was distilled in vacuo to give 16 g (67%) of **6**: bp 58–63 °C (10–11 mm); NMR ($CDCl_3$) δ 1.23 (6 H, s, CH_3's), 1.97 (2 H, t, 3-CH_2), 3.63 (2 H, t, 4-CH_2). Anal. ($C_5H_{11}ClO$) C, H, Cl.

4-Chloro-2-methyl-2-[(trimethylsilyl)oxy]butane (7). To 7.3 g (0.0585 mol) of chloride **6** in 35 mL of DMF were added 7.35 g (0.068 mol) of trimethylsilyl chloride and 4.1 g (0.06 mol) of imidazole, and the solution was stirred at room temperature for 3 h. The reaction mixture was then added to ice water and extracted with four portions of pentane. The combined extracts were washed with saturated NaCl solution and dried (Na_2SO_4). Solvent removal gave 9.55 g (82%) of oil. Distillation in vacuo gave 6.26 g (54%) of **7**: bp 35–37 °C (1.2 mm); NMR ($CDCl_3$) δ 0.08 (9 H, s, $SiCH_3$'s), 1.22 (6 H, s, CH_3's), 1.90 (2 H, t, 3-CH_2), 3.58 (2 H, t, 4-CH_2). Anal. ($C_8H_{19}ClOSi$) C, H, Cl.

Cholest-5-ene-3β,22(*S*),25-triol (12) via 8a. To 0.364 mol of **8a** prepared from 70.8 g (0.364 mol) of **7** and 9.3 g (0.39 mol) of Mg turnings in 270 mL of THF under a nitrogen atmosphere was added at room temperature 30.0 g (0.08 mol) of aldehyde **11** in 100 mL of THF over a 30-min period. After addition, the reaction was stirred for ca. 1 h, cooled (ice bath), and then hydrolyzed with 140 mL of 10% HCl solution. After the solution was stirred at room temperature for 1.5 h, the layers were separated, and the aqueous phase was extracted with EtOAc (twice); the combined extracts were washed with saturated NaCl solution and dried (Na_2SO_4). Solvent removal gave a white solid shown by TLC (40% EtOAc/Skelly B-silica) to be a mixture of the 3-acetoxy and 3-hydroxy compounds. Saponification of this solid using 230 mL of MeOH, 23 mL of H_2O, and 9.3 mL of 45% KOH solution for 1 h at reflux was followed by neutralization with HOAC. The volume of the solution was reduced to ca. 50 mL in vacuo before addition of water. The precipitate which formed was collected, washed with H_2O, and dried. Trituration with 200 mL of boiling cyclohexane afforded, after cooling, 31.4 g (93%) of triol **12**; mp 183.5–186 °C (lit.[25] mp 186–187 °C). Anal. ($C_{27}H_{46}O_3$) C, H.

Cholest-5-ene-3β,22(*S*),25-triol (12) via 8b. To 5.6 g (0.046 mol) of 4-chloro-2-methyl-2-butanol in 40 mL of THF cooled to ca. −20 °C under a nitrogen atmosphere was added dropwise 16.2 mL of 2.9 M (0.047 mol) methylmagnesium chloride. The reaction mixture was allowed to warm to room temperature over a 30-min period before 1.16 g (0.048 mol) of magnesium turnings was added. Upon heating of the solution to reflux, a small amount of 1,2-dibromoethane was added to initiate the Grignard formation and refluxing was continued for 2 h. After the solution cooled to room temperature, 4.5 g (0.012 mol) of aldehyde **11** in 25 mL of THF was added dropwise, and the reaction mixture was stirred for 1 h. Hydrolysis with 10% hydrochloric acid solution was followed by extraction with ethyl acetate (3×). The combined extracts were washed with saturated sodium bicarbonate solution and dried (Na_2SO_4), and the solvent was removed in vacuo. The residue was triturated with 50% aqueous methanol to afford after filtration 3.92 g (78%) of triol **12**, which was used without further purification for the subsequent reaction. Recrystallization from aqueous MeOH gave **12**: mp 181–184 °C (lit.[25] mp 186–187 °C). Anal. ($C_{27}H_{46}O_3$) C, H.

Cholest-5-ene-3,22(*S*),25-triol 3,22-Diacetate (13). To 31.3 g (0.074 mol) of triol **12** in 125 mL of pyridine was added 50 mL of acetic anhydride, and the reaction mixture was stirred at room temperature overnight. Water was added and the precipitate which formed was collected and dried. Trituration with ca. 100 mL of refluxing cyclohexane gave, after cooling, 28.4 g (76%) of **13**: mp 172–173 °C;[43] NMR ($CDCl_3$) δ 0.69 (3 H, s, 13-CH_3) 1.03 (3 H, s, 10-CH_3), 1.20 (6 H, s, 25-CH_3's), 2.02 (3 H, s, OAc), 2.03 (3 H, s, OAc), 5.40 (1 H, br m, 6-H); ^{13}C NMR ($CDCl_3$) δ 11.7 (18-CH_3), 12.9 (21-CH_3), 19.3 (19-CH_3), 27.1, 27.9 (26,27-CH_3's), 70.4 (25-C), 74.0 (3-CH), 76.8 (22-CH). Anal. ($C_{31}H_{50}O_5$) C, H.

22(*S*)-Acetoxy-25-fluorocholesterol 3-Acetate (14a). To 2.85 g (0.0057 mol) of **13** cooled to −70 °C in 50 mL of CH_2Cl_2 under a nitrogen atmosphere was added dropwise 1.5 g (0.0093 mol) of (diethylamino)sulfur trifluoride[44] in 10 mL of CH_2Cl_2 over a 5-min period. The reaction was stirred at the above temperature for 20 min and then allowed to warm to 0 °C whereupon 5% $NaHCO_3$ solution was added. The two layers were separated, and the aqueous phase was extracted with two additional portions of CH_2Cl_2. The combined extracts were washed with saturated NaCl solution and dried. Solvent removal in vacuo gave ca. 2.9 g (quantitative recovery) of solid. This material was generally used without purification in the subsequent reaction. Recrystallization of a portion of this residue from aqueous methanol afforded **14a**: mp 161–162 °C; NMR ($CDCl_3$) δ 0.68 (3 H, s, 13-CH_3), 1.02 (3 H, s, 10-CH_3), 1.35 (6 H, d, J_{FCCH} = 21 Hz, 25-CH_3's), 2.02 (6 H, s, OAc's), 5.40 (1 H, br m, 6-H). Anal. ($C_{31}H_{49}FO_4$) C, H.

22(*S*)-Acetoxy-25-chlorocholesterol 3-Acetate (14b). To 4.8 g (9.5 mmol) of **13** in 50 mL of toluene were added 0.2 g (1.46 mmol) of $ZnCl_2$ and 1.77 g (14.9 mmol) of $SOCl_2$, and the reaction mixture was stirred at room temperature under a nitrogen atmosphere for 1 h. The solution was then poured into a saturated $NaHCO_3$ solution, and the layers were separated. The organic phase was washed with water, dried over Na_2SO_4, and filtered through a cake of Celite. Solvent removal in vacuo gave a solid residue, which was used without purification in the subsequent reaction. Recrystallization of a small portion from aqueous MeOH gave 14b: mp 174–176 °C; NMR ($CDCl_3$) δ 0.70 (3 H, s, 13-CH_3), 1.03 (3 H, s, 10-CH_3), 1.57 (6 H, s, 25-CH_3's), 2.03 (3 H, s, OAc), 2.05 (3 H, s, OAc), 5.42 (1 H, br d m, 6-H). Anal. ($C_{31}H_{49}ClO_4$) C, H.

25-Fluoro-22(*S*)-hydroxycholesterol (15a). To 2.5 g (0.0050 mol) of **14a** in 80 mL of MeOH was added 20 mL of 5% NaOH solution, and the reaction mixture was heated at reflux for 4 h. Upon cooling, 1.89 g (90%) of crystalline **15a** separated from the solution and was collected. Recrystallization from aqueous MeOH gave 1.3 g (63%) of **15a**: mp 162–163 °C; NMR ($CDCl_3$) δ 0.68 (3 H, s, 13-CH_3), 1.00 (3 H, s, 10-CH_3), 1.33 (6 H, d, J_{FCCH} = 21 Hz, 25-CH_3's), 5.38 (1 H, br m, 6-H). Anal. ($C_{27}H_{45}FO_2$) C, H.

25-Chloro-22(*S*)-hydroxycholesterol (15b). To 90 mL of dry THF was added 0.5 g (13.1 mmol) of lithium aluminum

(43) The melting point of the 22(*R*)-diacetate has been reported to be 150 °C.[25]

(44) While (diethylamino)sulfur trifluoride has been purified via distillation on several occasions without incident, explosions have been reported with this reagent during this process.[45] We have since found that the crude reagent works equally as well as the distilled compound in this reaction.

(45) Cochran, *J. Chem. Eng. News* **1979**, *57*(12), 4. Middleton, W. J. *Ibid.* **1979**, *57*(21), 43.

hydride followed by ca. 5.0 g (ca. 9.5 mmol) of crude diacetate **14b** in 30 mL of THF over a 10-min period at room temperature under a nitrogen atmosphere. After 15 min the reaction mixture was quenched with a few milliliters of EtOAc followed by 2 mL of H_2O. After the solution was filtered through a pad of $MgSO_4$, the solvent was removed from the filtrate to give ca. 4.5 g of solid residue. Recrystallization from toluene afforded 3.4 g (82%) of diol **15b**: mp 157–159 °C; NMR ($CDCl_3$) δ 0.70 (3 H, S, 13-CH_3), 1.02 (3 H, s, 10-CH_3), 1.57 (6 H, s, 25-CH_3's), 5.38 (1 H, br m, 6-H). Anal. ($C_{27}H_{45}ClO_2$) C, H.

2-Cyclopropylethanol (18). To 115 g (0.95 mol) of cyclopropyl bromide (**16**) under nitrogen atmosphere in 1300 mL of anhydrous ether cooled to ca. −60 °C was added dropwise 500 mL of 1.9 M *tert*-butyllithium/hexane solution (0.95 mol) over a 45-min period. After stirring an additional 30 min at the above temperature, the solution was allowed to warm to ca. −25 °C, whereupon 42 g (0.95 mol) of ethylene oxide in 75 mL of ether was added. The reaction mixture was stirred at −20 °C for 1 h and at 0 °C for 30 min. Upon warming of the mixture to room temperature, the reaction became exothermic and cooling was again applied to maintain a temperature of ca. 20 °C where it was kept for $^1/_2$ h. Further cooling to 5 °C was followed by hydrolysis with 10% sulfuric acid solution. The layers were then separated, and the aqueous phase was extracted with additional ether. The combined extracts were washed with 5% sodium bicarbonate solution, dried over $MgSO_4$, and filtered through a cake of diatomaceous earth. Solvent removal in vacuo gave 53.4 g (65%) of crude carbinol. This crude material was suitable for conversion to the bromide. Distillation at atmospheric pressure afforded the pure carbinol: bp 135–136 °C (lit.[29] bp 136 °C) (39% distilled yield); NMR ($CDCl_3$) δ −0.02–1.05 (cyclopropyl H's), 1.47 (2 H, m, β-CH_2), 3.73 (2 H, t, J = 7 Hz, α-CH_2).

2-Cyclopropylethyl Bromide (19). To 53.2 g (0.62 mol) of crude alcohol **18** in 375 mL of DMF under a nitrogen atmosphere was added 165 g (0.63 mol) of triphenylphosphine. The reaction mixture was cooled to ca. 0 °C, whereupon 112 g (0.63 mol) of *N*-bromosuccinimide was added portionwise over a 30-min period at a rate so as to maintain a pot temperature of ca. 20 °C. The reaction mixture was stirred at room temperature for 45 min, quenched with 10 mL of methanol, and diluted with 375 mL of H_2O. The aqueous solution was extracted with three 200-mL portions of pentane, and the extracts were washed with 5% $NaHCO_3$ solution, dried over $MgSO_4$, and filtered through a cake of diatomaceous earth. Solvent removal gave an oil which upon distillation at atmospheric pressure afforded 42 g (47%) of bromide **19**: bp 132–134 °C (lit.[30] mp 129–131 °C); NMR ($CDCl_3$) δ −0.05–1.05 (cyclopropyl H's), 1.77 (2 H, m, β-CH_2), 3.41 (2 H, t, J = 7 Hz, α-CH_2).

24-Cyclopropylchol-5-ene-3β,22(S)-diol 3-Acetate (20a). To 6.5 g (0.28 mol) of magnesium turnings in 30 mL of THF in a nitrogen atmosphere was added 37.8 g (0.2 mol) of 2-cyclopropylethyl bromide (**19**) in 70 mL of THF. The reaction mixture was then stirred at room temperature, whereupon Grignard formation commenced. A cooling bath (MeOH–ice) was used to maintain a reaction temperature of ca. 20 °C. To the freshly formed Grignard reagent cooled to ca. 0 °C was then added dropwise 28 g (0.075 mol) of aldehyde **11** in 300 mL of THF at a rate to maintain the temperature below 10 °C. The reaction mixture was then stirred at 0 °C for 30 min before addition of saturated NH_4Cl solution. Some water was also added before extraction of the aqueous solution with two 400-mL portions of ether. The combined extracts were washed with saturated NaCl solution, dried over $MgSO_4$, and filtered through a cake of diatomaceous earth. Solvent removal gave 29 g of solid residue. Recrystallization from MeOH afforded 18.4 g (55%) of **20a**: mp 179–182 °C; NMR ($CDCl_3$) δ 0.70 (3 H, s, 13-CH_3), 1.03 (3 H, s, 10-CH_3), 2.02 (3 H, s, 3-OAc), 5.37 (1 H, br m, 6-H). Anal. ($C_{29}H_{46}O_3$) C, H.

24-Cyclopropylchol-5-ene-3β,22(S)-diol (20b). To 18.2 g (0.041 mol) of **20a** in 350 mL of MeOH was added 70 mL of 5% NaOH solution, and the reaction mixture was heated on a steam bath for 15 min. During this time a crystalline precipitate separated from the hot solution and was collected, affording 3.6 g of analytically pure **20b**. Further cooling of the filtrate afforded an additional 8.6 g of product which was recrystallized to afford another 8.0 g (11.6 g total, 71%) of 20b: mp 175–175.5 °C; NMR ($CDCl_3$) δ 0.70 (3 H, s, 13-CH_3), 1.02 (3 H, s, 10-CH_3), 5.37 (1 H, br m, 6-H); ^{13}C NMR (C_5D_5N) δ 4.8, 5.2 (26,27-CH_2's), 11.4 (25-CH), 12.0 (18-CH_3), 12.4 (21-CH_3), 19.6 (19-CH_3), 71.2 (3-CH), 72.2 (22-CH), 121.1 (6-CH), 141.2 (5-C); $[\alpha]_{589}$ −51° (c 1.01, $CHCl_3$). Anal. ($C_{27}H_{44}O_2$) C, H.

Biological Testing Procedures. Tissue Culture Assay. HMG CoA reductase activity was determined in extracts of detergent solubilized cells (Detroit, 551 human fetal skin fibroblasts) according to the method described by Brown and Goldstein.[32] The rate of conversion of [^{14}C]HMG CoA to [^{14}C]mevalonate was determined in cells incubated at 37 °C for 24 h in fetal calf lipoprotein deficient serum (lds) followed by an additional 24 h of incubation with test compound administered at the designated concentration in 10 μL of ethanol. Enzymatic activity was determined as pmol of [^{14}C]mevalonate min^{-1} (mg of soluble protein)$^{-1}$, and the results of two sets of experiments are expressed as a percentage of the control activity as determined on the same day.

20,25-Diazacholesterol-Treated Rat Assay. The procedure used to study the effect of oxygenated sterols on HMG CoA reductase in rats was adapted from a method previously described.[12] Male CD rats (180–250 g) were fed Purina Certified Rodent Chow (5002) and water ad lib. and housed in a reversed light cycle environment. 20,25-Diazacholesterol[34] was given intragastrically as a suspension (0.1% Tween 80/saline, 0.8 mL/kg of animal weight) at a daily dose of 5 mg/kg for 7 days to rats during the dark cycle of their day. Test compounds were administered as a suspension in the same Tween 80/saline vehicle and dose volume over the last 4 days of the test, 2 h after administration of the diazasterol. On the final day of the assay, rats were killed 2 h after administration of the test compounds, livers were removed, the microsomal fraction was prepared (6 × 10^6 G·min), and HMG CoA reductase activity was determined for control and treated groups of rats on the basis of [^{14}C]HMG CoA to [^{14}C]mevalonate.[32] Control values of elevated reductase levels were determined for groups of four to seven animals which were administered the diazasterol exclusively for the 7 days of the assay. Test compounds were administered to groups of four to seven animals at doses from 0.1 to 30 mg/kg and the lowest doses which reduced elevated reductase levels ≥ 25% are reported in Table II. Control groups possessed reductase levels 300–400% over that of vehicle-treated animals. Data was analyzed by using the Student's t test and standard error of the mean (SEM) is shown for the ED25 dose in their assay.

Primate Study. Blood cholesterol and lipoprotein levels (three determinations/animal) were determined over a 2-week period on adult male rhesus monkeys, maintained on a Purina Monkey Chow Jumbo (5039) diet, to determine primates which possessed elevated, stable serum cholesterol levels. Each test compound was administered as a suspension intragastrically in 0.1% Tween 80/saline solution (1 mL/kg of animal weight) at a daily dose of 10 mg/kg for 14 days to a group of four primates for which control values were previously established. All blood samples were then drawn via the saphenous vein and the serum was collected. Cholesterol levels were determined by using the Liebermann-Burchard method on a Technicon autoanalyzer.[46] Lipoprotein levels were determined by densiometric analysis of lipoprotein fractions separated by disc gel electrophoresis using a Quick-Disc Lipoprotein Gel Tube Kit (Ames Co.) and a Clifford Model 750 Densitometer (Medfield, Mass.).[37] The values listed in Table III were analyzed by using a Student's t test and are accompanied by the standard error of the mean (SEM). In a separate experiment, the Tween 80/saline vehicle was administered over a 2-week period and its effects on cholesterol and lipoprotein levels are also shown in the table.

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(*S* isomer), 83945-75-9; **4a** (*R* isomer), 93600-86-3; **4b**, 17711-16-9; **5**, 623-71-2; **6**, 1985-88-2; **7**, 76752-38-0; **11**, 10211-88-8; **12**, 63163-38-2; **13**, 79435-62-4; **14a**, 93527-43-6; **14b**, 93527-44-7; **15a**, 83945-76-0; **15b**, 93404-42-3; **16**, 4333-56-6; **17**, 3002-94-6; **18**, 2566-44-1; **19**, 36982-56-6; **20a**, 82937-67-5; **20b**, 82937-68-6; HMG CoA reductase, 9028-35-7; 3,3-dimethylpropyl chloride, 107-84-6; 3,3-dimethylbutyl chloride, 2855-08-5; *tert*-butyllithium, 594-19-4; ethylene oxide, 75-21-8; methyl bromide, 74-83-9.

Analogues of Aminoglutethimide: Selective Inhibition of Aromatase

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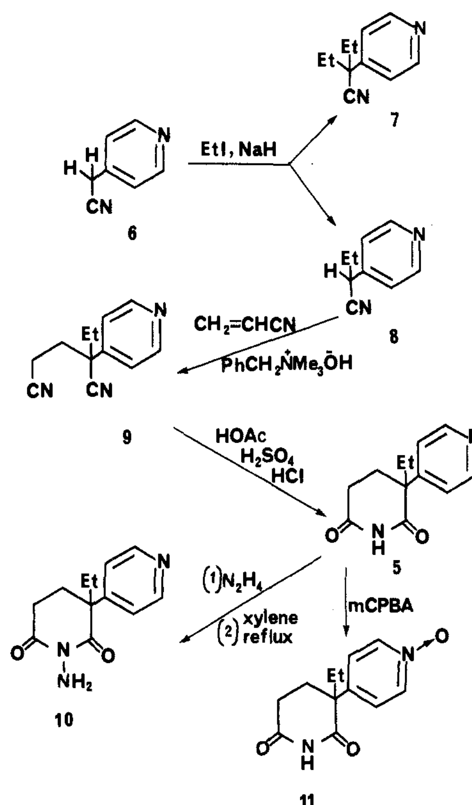
In exploring further the structural features that influence the relative efficacy of analogues of aminoglutethimide [1, 3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione] as inhibitors of the cholesterol side-chain cleavage enzyme system desmolase and the estrogen forming system aromatase, analogues have been synthesized in which the aminophenyl substituent is replaced by pyridyl or substituted pyridyl. The 4-pyridyl analogue **5** [3-ethyl-3-(4-pyridyl)-piperidine-2,6-dione] is a strong competitive inhibitor of aromatase (K_i = 1.1 μM; value for **1**, 0.60 μM), which exhibits a type II difference spectrum (K_s = 0.28 μM; value for **1**, 0.13 μM) but is noninhibitory toward desmolase. The 2- and 3-pyridyl analogues (**3** and **4**) inhibit neither enzyme system. 1-Amino-3-ethyl-3-phenylpiperidine-2,6-dione (**2**) is a strong and selective inhibitor of desmolase but the 4-pyridyl analogue **10** [1-amino-3-ethyl-3-(4-pyridyl)-piperidine-2,6-dione] is a weak inhibitor of desmolase and aromatase. Analogues of **5** having a less basic aromatic substituent, namely, the *N*-oxide **11** and the 2,3,5,6-tetrafluoro derivative **13**, were also prepared. The latter is a weak inhibitor of aromatase and the former inhibits neither enzyme system.

Aminoglutethimide [**1**, 3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione] is becoming increasingly used for the treatment of hormone-dependent metastatic breast carcinoma.[2,3] The drug inhibits the growth of such tumors by interfering with estrogen biosynthesis, the strongest action being against the enzyme complex, aromatase, which converts the androgens androstene-3,17-dione and testosterone into estrone and estradiol.[4] Its other major action is against the initial step in estrogen biosynthesis, namely, the conversion of cholesterol into pregnenolone by the enzyme complex desmolase.[5] Inhibition of desmolase depletes corticosteroid production, and consequently, patients receiving aminoglutethimide require hydrocortisone as replacement therapy to prevent the reflex rise in adrenocorticotropic hormone (ACTH) which might counteract the initial blockade of desmolase. Hence, it might be advantageous therapeutically to use a drug that inhibits aromatase but not desmolase. The steroid analogue 4-hydroxy-4-androstene-3,17-dione is a strong inhibitor of aromatase, and there is indirect evidence, based on its effects on testosterone levels when it is administered in vivo, that it is not inhibitory toward desmolase.[6] In contrast to aminoglutethimide, the binding of 4-hydroxy-4-androstene-3,17-dione to aromatase is irreversible.[7,8] Furthermore, steroidal inhibitors exhibit type I difference spectra in binding to cytochrome P_{450} type enzymes whereas aminoglutethimide gives a type II difference spectrum.[9] Because of these mechanistic differences between the inhibitory actions of the two types of agent, a comparative evaluation as inhibitors of aromatase is desirable and the present study concerns the development of an analogue of aminoglutethimide that selectively inhibits aromatase.

Our previous investigations into the structural features favorable for inhibition of aromatase or desmolase[10] were concerned with the effect of relocating the amino function and of introducing an additional amino group into the molecule. Although 1-amino-3-ethyl-3-phenylpiperidine-2,6-dione (**2**) was identified as a strong inhibitor of desmolase without activity against aromatase, no leads to a pure inhibitor of aromatase emerged. However, these and

Scheme I



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