# Inhibition of Cholesterol Side-Chain Cleavage. 2.[1] Synthesis of Epimeric Azacholesterols

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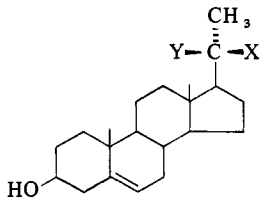
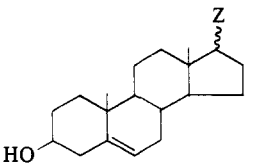
The potent inhibitory activity of 20α-22-azacholesterol (*N*-isoamyl-20α-aminopregn-5-en-3β-ol) and 17β-20-azacholesterol (*N*-methyl-*N*-isohexyl-17β-aminoandrost-5-en-3β-ol) on the conversion of cholesterol to pregnenolone prompted the synthesis and enzymic studies of several epimeric aza- and diazacholesterols in an attempt to ascertain the stereochemical requirements for inhibition of the cleavage reaction. In the 22-azacholesterols, the epimer having the same configuration as cholesterol at C-20 was clearly the most active inhibitor. Little or no difference was noted for the epimers in the 20-azacholesterol series. Minor changes in the length and type of side chain in 22-azacholesterol had little effect on inhibitory activity.

Inhibitors of cholesterol side-chain cleavage are of current interest because of their possible diagnostic and therapeutic utility in diseases associated with hyperfunctioning adrenal glands.[2] One such inhibitor, aminoglutethimide (α-ethyl-α-*p*-aminophenylglutarimide), has been shown to cause a reduction in the formation of all hormonal steroids including cortisol and aldosterone.[3] Several groups[4] have postulated that aminoglutethimide inhibits the conversion of cholesterol to 20α-hydroxycholesterol, but the evidence is unconvincing. Our previous studies[1] with several aza- and diazacholesterols revealed that 22-azacholesterol (1a) represented the most potent inhibitor of cholesterol side-chain cleavage found to date and that 20-azacholesterol (3a) was somewhat less inhibitory in this respect. This observation was particularly noteworthy in light of the current view[5] that 22-(*R*)-hydroxylation appears to be much more significant than 20α-hydroxylation in the side-chain cleavage reaction. It should be pointed out, however, that the pathway from cholesterol to pregnenolone is still unclear and reactions other than hydroxylation at C-20 and C-22 may be of greater importance.[6] This paper represents a continuation of our structure-activity relationship studies in this series and attempts to ascertain the stereochemical



1a, X = NHR; Y = H
b, X = NHR'; Y = H
2a, X = H; Y = NHR
b, X = H; Y = NHR'



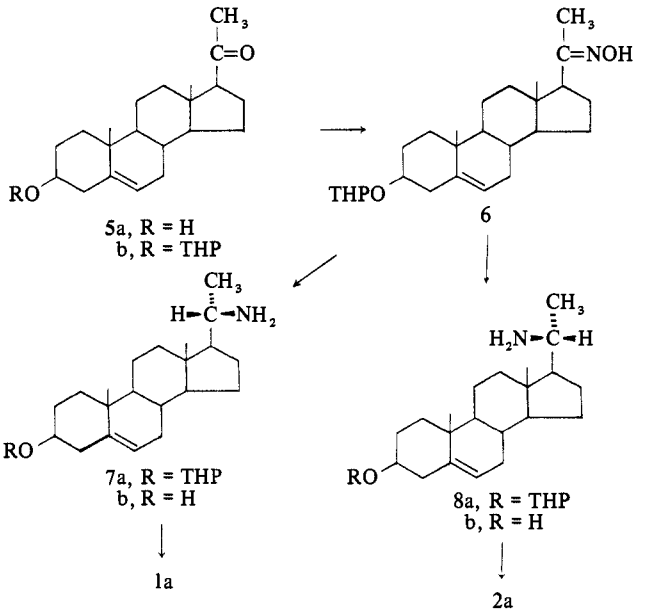
3a, Z = $CH_3$-N-$CH_2R$
b, Z = $CH_3$-N-$CH_2R'$
4a, Z = $CH_3$-N-$CH_2R$
b, Z = $CH_3$-N-$CH_2R'$

R = $(CH_2)_2CH(CH_3)_2$; R' = $(CH_2)_2N(CH_3)_2$

requirements for inhibition of the cleavage reaction.

20α- and 20β-22-azacholesterols (1a and 2a) were readily prepared from pregnenolone (5a) *via* the 3-tetrahydropyranyl (THP) ether 5b. Conversion of 5b to the 20-oxime 6 and subsequent reduction with Na in 1-propanol gave a 1:1 mixture of the two epimeric amines 7a and 8a. By retaining the THP ether protecting group, these epimers could be readily separated on deactivated neutral alumina (activity II). Subsequent cleavage of the 3-THP protecting group with mild acid furnished the corresponding amines 7b and 8b. Reductive alkylation of these amines with isovaleraldehyde and $NaBH_4$ in dry MeOH afforded the desired 20α- (1a) and 20β-azacholesterols (2a) in almost quantitative yield.

The epimeric 22,25-diazacholesterols (1b and 2b) were prepared by SN2 displacement of the 20-tosyloxypregnane derivatives as part of another study in our laboratory.[7] 17α-20-Azacholesterol (4a) was prepared *via* SN2 displacement



5a, R = H
b, R = THP

6

7a, R = THP
b, R = H

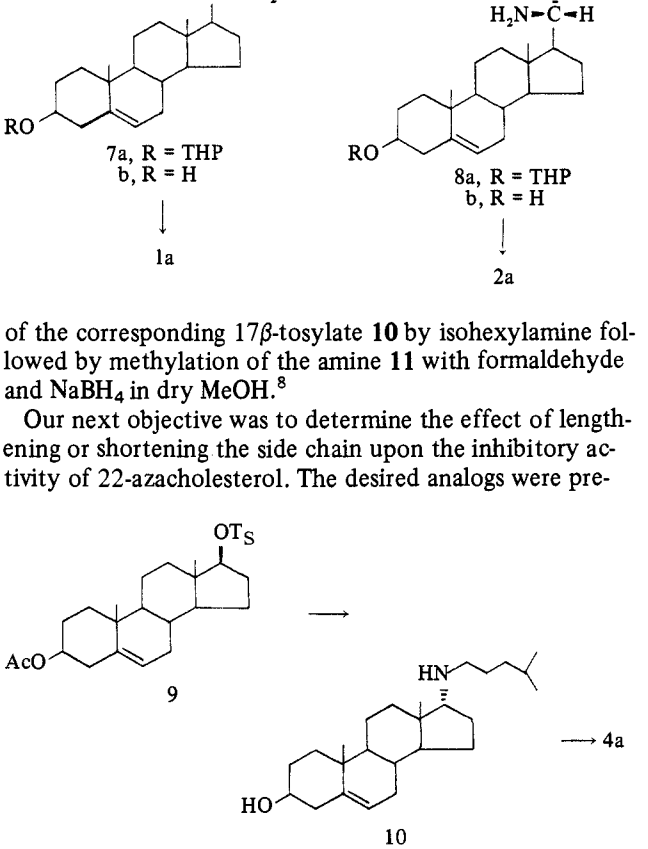
8a, R = THP
b, R = H

1a

2a

of the corresponding 17β-tosylate 10 by isohexylamine followed by methylation of the amine 11 with formaldehyde and $NaBH_4$ in dry MeOH.[8]

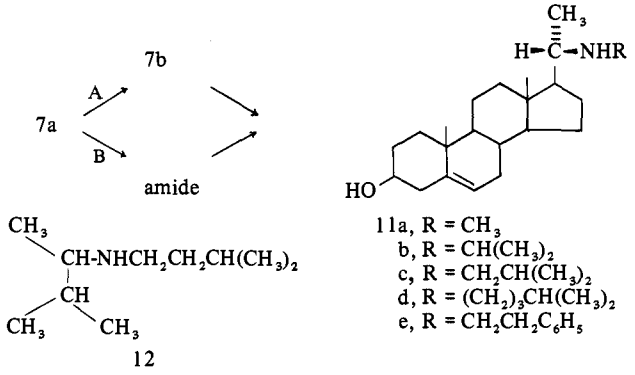
Our next objective was to determine the effect of lengthening or shortening the side chain upon the inhibitory activity of 22-azacholesterol. The desired analogs were pre-



9

10

4a




pared by two general methods. For method A, 20α-aminopregn-5-en-3β-ol (**7b**) served as the starting material. Reductive alkylation with the appropriate aldehyde or ketone afforded compounds **11a-c**. Method B involved acylation of **7a** with the appropriate acid chloride and subsequent LAH reduction of the resulting amide to give **11d** and e.

In order to ascertain the necessity of the steroid nucleus for inhibition, the nonsteroidal secondary amine (**12**) simulating the side chain of 22-azacholesterol was synthesized. This amine was readily prepared by $NaBH_4$ reduction of the Schiff base derived from isopropyl methyl ketone and isoamylamine.

Assays for cholesterol side-chain cleavage activity were performed according to the method of Doering.[9] An acetone powder preparation of the mitochondrial fraction of bovine adrenal was used as the enzyme source. The powder was reconstituted in 0.1 *M* phosphate buffer (pH 7.4) to give a protein concentration of 5-8 mg/ml as determined by the method of Lowry, *et al.*[10] By employing cholesterol-7α-*t*,-26-$^{14}C$ as substrate, one of the products of the cleavage reaction, isocaproic-$^{14}C$ acid, was removed by heating *in vacuo* at 140°. The enzymic activity was then calculated from the changes in the isotope ratios. The inhibitory activity of the test compounds was determined at a final concentration of 0.1 m*M* or less. In all determinations, controls were run in the absence of inhibitor and the per cent inhibition was the average of at least two experiments.

## Results and Discussion

Table I shows the inhibitory activity of the epimeric aza- and diazacholesterols. In the 22-azacholesterol series, the 22α-epimer (**1a**) has the same configuration as cholesterol at C-20 and was clearly far superior to the 20β-epimer (**2a**) as an inhibitor of the cleavage reaction. This specificity was decreased when C-25 was replaced with a nitrogen atom (**1b** *vs.* **2b**). On the other hand, the inhibitory potency of the less active 20-azacholesterols was largely unaffected by changing the configuration at the C-17 position (**3a** and **3b** *vs.* 4a and **4b**).

Table I. Inhibitory Activity of Epimeric Aza Analogs of Cholesterol on the Conversion of Cholesterol to Pregnenolone

| Compound | Concentration, μM | % inhibition[a] |
|---|---|---|
| 20α-22-Azacholesterol (1a) | 100 | 97.3 ± 4.0 |
| | 10 | 86.1 ± 1.1 |
| | 3 | 58.0 ± 10.4 |
| | 1 | 23.2 ± 12.3 |
| 20β-22-Azacholesterol (2a) | 100 | 56.0 ± 5.7 |
| | 10 | 12.7 ± 11.4 |
| 20α-22,25-Diazacholesterol (1b) | 100 | 70.5 ± 11.5 |
| 20β-22,25-Diazacholesterol (2b) | 100 | 47.5 ± 5.3 |
| 17β-20-Azacholesterol (3a) | 100 | 68.6 ± 8.8 |
| | 10 | 43.7 ± 12.3 |
| 17α-20-Azacholesterol (4a) | 100 | 91.0 ± 5.2 |
| | 10 | 25.9 ± 2.7 |
| 17β-20,25-Diazacholesterol (3b) | 100 | 38.1 ± 11.5 |
| | 10 | 6.9 ± 0.9 |
| 17α-20,25-Diazacholesterol (4b) | 100 | 53.1 ± 10.6 |
| | 10 | 15.7 ± 8.9 |

[a] Error limits are standard deviations.

It is noteworthy that in the 20α series replacing C-25 with N caused a marked decrease in inhibitory activity in the cholesterol side-chain cleavage reaction. This is the antithesis of the structural requirements for inhibition of cholesterol biosynthesis in this series. 25-Azacholesterol and 20,25-diazacholesterol are extremely potent inhibitors of cholesterol biosynthesis, whereas 20- and 22-azacholesterol are essentially devoid of this property.[11]

Table II outlines the inhibitory activity of the side-chain-modified analogs of 22-azacholesterol. These data clearly indicate that increasing or shortening the chain length by one methylene has little effect on inhibitory activity. Moreover, the slight increase in inhibitory activity of 22-aza-24a-homocholesterol (**11d**) suggests that it may be possible to incorporate larger alkyl or aryl substituents into the side chain. The high inhibitory activity of the aryl analog **11e** lends support to this hypothesis, and further work along this line is now in progress.

In addition to alterations in stereochemistry, other departures from the cholesterol template leading to a decrease in the inhibitory activity included (1) shortening of the side chain by more than one methylene, (2) replacement of the 22-N with oxygen, and (3) removal of the steroid nucleus.

## Experimental Section†

**Pregnenolone 20-Oxime 3-THP Ether (6).** A soln of pregnenolone 3-THP ether (5b, 27 g, 68 mmoles), hydroxylamine · HCl (17.2 g, 250 mmoles), and NaOH (54.0 g) in EtOH (500 ml) and $H_2O$ (250 ml) was heated to gentle reflux and stirred for 2 hr. Solvent was removed under reduced pressure, and the residue was washed with $H_2O$, filtered, and dried ($Na_2SO_4$). Recrystn of the crude product from EtOH afforded pure 6 (18.9 g, 70%) as colorless cryst product, mp 196-198°, [α]D −38.8°. Ir and nmr were as expected. *Anal.* ($C_{26}H_{41}NO_3$) C, H.

**20-Aminopregn-5-en-3β-ol 3-THP Ether (7a and 8a).** To a refluxing soln of oxime 6 (16.6 g, 40 mmoles) in dry *n*-PrOH (800 ml) was added 28 g of metallic Na in small pieces over a period of 3 hr. The mixt was refluxed until all of the Na had dissolved. The soln was then concd to about 200 ml under reduced pressure and poured into $H_2O$ (1000 ml). The resulting mixt was extd with $Et_2O$ and dried ($Na_2SO_4$), and removal of the solvent afforded a mixt of the two epimeric amines (15 g, 93.6%). A portion of these amines was recrystd from dil EtOH, mp 136-138°. Ir and nmr were as expected. *Anal.* ($C_{26}H_{43}NO_2$) C, H.

These epimers were readily sepd on a column (4 × 60 cm) made up of deactivated neutral alumina (600 g, activity II). The column was first eluted with hexane (1000 ml). Subsequent elution with $CHCl_3$–hexane (7:3) gave pure 20β-amine **8a** (6.94 g). An analytical sample was obtd by recrystn from hexane, mp 156-157°, [α]D −28.9°. *Anal.* ($C_{26}H_{43}NO_2$) C, H. Further elution with pure $CHCl_3$ afforded a mixt of amines (300 mg) and this was followed by the 20α-amine 7a (6.95 g) when $CHCl_3$–MeOH (19:1) was used for elution. An analytical sample was obtd by recrystn from hexane, mp 117-119°, [α]D −50.4°. *Anal.* ($C_{26}H_{43}NO_2$) C, H.

†The nmr spectra were obtd with a Varian A-60A spectrometer. Optical rotations were measured in $CHCl_3$ on a Perkin-Elmer 141 polarimeter. Infrared spectra were recorded on a Perkin-Elmer 337 spectrophotometer. The melting points were measured on a Thomas-Hoover apparatus and are corrected. Where analyses are indicated by symbols of the elements, analytical results obtd for those elements were within ±0.4% of the theoretical values. Analyses were performed by Spang Microanalytical Lab., Ann Arbor, Mich., and Midwest Microlab, Ltd., Indianapolis, Ind.

Table II. Inhibitory Activity of 22-Azacholesterol Analogs on the Conversion of Cholesterol to Pregnenolone

| Compound | Concentration, $\mu M$ | % inhibition[a] |
|---|---|---|
| 22-Azacholesterol (1a) | 100 | 97.3 ± 4.0 |
| | 10 | 86.1 ± 1.1 |
| | 3 | 58.0 ± 10.4 |
| | 1 | 23.2 ± 12.3 |
| 20α-Methylaminopregn-5-en-3β-ol (11a) | 100 | 82.3 ± 7.5 |
| | 10 | 27.3 ± 3.9 |
| 22-Aza-23,24-bisnorcholesterol (11b) | 100 | 69.5 ± 8.8 |
| | 10 | 23.7 ± 16.2 |
| 22-Aza-24-norcholesterol (11c) | 100 | 100 ± 0.0 |
| | 10 | 86.2 ± 6.9 |
| | 1 | 31.8 ± 7.4 |
| 22-Aza-24a-homocholesterol (11d) | 100 | 100 ± 0.0 |
| | 10 | 95.0 ± 5.8 |
| | 1 | 60.5 ± 7.4 |
| | 0.1 | 28.8 ± 19.9 |
| 20α-Phenylethylamino-pregn-5-en-3β-ol (11e) | 100 | 100 ± 0.0 |
| | 10 | 87.9 ± 11.3 |
| | 1 | 14.3 ± 9.4 |
| | 0.1 | 4.65 ± 4.0 |
| *N*-Isoamyl-2,3-dimethyl-propylamine (12) | 100 | 32.6 ± 5.3 |
| | 10 | 6.9 ± 9.8 |
| 22-Oxacholesterol[b] | 100 | 47.5 ± 2.4 |
| | 10 | 0 ± 0.0 |
| 22-Oxa-24-norcholesterol[b] | 100 | 15.3 ± 4.3 |
| | 10 | 0 ± 0.0 |
| 22-Oxa-25-azacholesterol[b] | 100 | 81.4 ± 16.8 |
| | 10 | 21.2 ± 8.3 |

[a] Error limits are standard deviation. [b] These compounds were kindly supplied by Dr. J. M. Kraemer of E. Merck AG, Darmstadt.

**20β-Aminopregn-5-en-3β-ol (7b).** A soln of amine 7a (3.5 g, 8.7 mmoles) in 0.2 *N* EtOH-HCl was stirred for 2 hr, poured into $H_2O$, and neutralized with $K_2CO_3$. The resulting ppt was filtered and recrystd from aqueous EtOH to give pure 7b, mp 171–172°, lit.[12] 172°. In the same manner, **20β-aminopregn-5-en-3β-ol** (8b) was obtd from amine 8a, mp 222°, lit.[12] 220°.

***N*-Isohexyl-17α-aminoandrost-5-en-3β-ol (10).** A soln of tosylate[8] 9 (3.0 g, 6.1 mmoles) and isohexylamine (25 ml) was refluxed under $N_2$ with stirring for 6 days. The excess amine was distd and the residual oil dild with ice-$H_2O$ (400 ml). The mixt was extd with $Et_2O$ and the $Et_2O$ ext washed with $H_2O$, dried ($Na_2SO_4$), and evapd to dryness under reduced pressure. The residue was dissolved in *i*-PrOH-HCl soln and dild with $Et_2O$. The HCl salt was collected by filtration. Liberation of free base gave 246 mg (10.7%) of crude **10**, mp 64–65°. Recrystn from $Me_2CO$ afforded analytical sample, mp 110–111°, [α]D −87.3°. Ir and nmr were as expected. *Anal.* ($C_{25}H_{43}NO$) C, H.

***N*-Methyl-*N*-isohexyl-17α-aminoandrost-5-en-3β-ol (17α-20-Azacholesterol (4a).** Formaldehyde soln (37%, 0.6 ml) was added with stirring to a soln of amine **10** (150 mg, 0.48 mmole) in abs MeOH (5 ml), and the reaction mixt was stirred at room temp for 2 hr. $NaBH_4$ (500 mg) was added with stirring in small portions at ice-bath temp. The mixt was allowed to come to room temp (30 min) and then poured into ice-$H_2O$ (200 ml). The mixt was extd with $Et_2O$, and the ext washed and dried in the usual manner. Removal of the solvent afforded a residue which when recrystd from $Me_2CO$ gave pure 4a (74.5 mg, 48.5%), mp 85–86°, [α]D −79.7°. Ir and nmr were as expected. *Anal.* ($C_{26}H_{45}NO$) C, H.

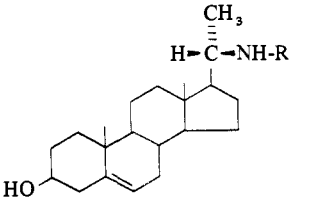
***N*-Isoamyl-20α-aminopregn-5-en-3β-ol (1a). General Method A.** A soln of 20α-aminopregn-5-en-3β-ol (7b, 500 mg, 1.58 mmoles) and isovaleraldehyde (680 mg, 7.9 mmoles) in abs MeOH (50 ml) was refluxed for 2 hr on a steam bath. The reaction mixt was allowed to cool to 10–20° in an ice bath, and then $NaBH_4$ (1.5 g) was added in small portions with stirring over a period of 30 min. Stirring was continued for 2 hr whereupon $Me_2CO$ was added dropwise to decompose the excess $NaBH_4$ and the mixt was poured into ice-$H_2O$. The ppt was collected by filtration, washed with $H_2O$, dried, and recrystd from $Me_2CO$ to give pure 1a (590 mg, 99%), mp 128–129°, lit.[11] 120–121°. Ir and nmr were identical in every respect with the authentic sample. Other homologs listed in Table III were prepared in a similar manner.

***N*-Isoamyl-20β-aminopregn-5-en-3β-ol (2a)** was prepared from 20β-aminopregn-5-en-3β-ol (**8b**) and isovaleraldehyde in the same manner described in method A in quantitative yield, mp 151–152° ($Me_2CO$), [α]D −64.7°. Ir and nmr were as expected. *Anal.* ($C_{26}H_{45}NO$) C, H.

***N*-Isohexyl-20α-aminopregn-5-en-3β-ol [20α-22-Aza-24a-homocholesterol (11d)]. General Method B.** To a soln of 20α-aminopregn-5-en-3β-ol 3-THP ether (7a, 500 mg, 1.22 mmoles) in $C_6H_6$ (20 ml) and triethylamine (500 mg) was added dropwise with stirring a soln of 4-methylvaleryl chloride (840 mg, 6.1 mmoles) in $C_6H_6$ (10 ml). The mixt was stirred at room temp for 24 hr and then poured into ice-$H_2O$ (200 ml). The organic phase was sepd and washed successively with $H_2O$, 5% $NaHCO_3$ soln, and $H_2O$. The $C_6H_6$ soln was dried ($Na_2SO_4$) and filtered, and the solvent removed to give crude amide (600 mg), ir $\nu_{max}^{KBr}$ 1650 $cm^{-1}$ (CONH). This crude amide was taken up in hot dioxane (25 ml) and added dropwise to a slurry of LAH (500 mg) in dioxane (75 ml) at the reflux temp. The mixt was refluxed with stirring for 24 hr whereupon the excess hydride was decomposed by the successive dropwise addn of 75% aqueous dioxane (40 ml), 25% NaOH soln (5 ml), and $H_2O$ (5 ml). The inorganic salts were removed by filtration and washed with hot dioxane. The filtrate was evapd to dryness, and the residue dissolved in $Et_2O$. Treatment with gaseous HCl formed the HCl salt and simultaneously cleaved the protecting THP ether. The salt pptd and was collected by filtration. Liberation of the free base with $K_2CO_3$ and recrystn from $Me_2CO$ afforded pure 11d (404 mg, 80%), mp 113–114°, [α]D −26.6°. Ir and nmr were as expected. *Anal.* ($C_{27}H_{47}NO$) C, H.

***N*-Isoamyl-2,3-dimethylpropylamine (12).** A soln of isopropyl methyl ketone (8.6 g, 100 mmoles) and isoamylamine (8.7 g, 100 mmoles) in abs MeOH (20 ml) was heated on a steam bath for 2 hr. Removal of the solvent afforded a colorless oil whose ir spectrum showed the presence of a peak at 1640 $cm^{-1}$ for C=N, and absence of peaks for $NH_2$ and C=O. The crude oil was taken up in dry MeOH (100 ml) and $NaBH_4$ (9.5 g) was added portionwise at ice-bath temp. The reaction mixt then poured into ice-$H_2O$ and extd with $Et_2O$. The ext was dried ($Na_2SO_4$), and the solvent re-

Table III. 22-Azacholesterol Analogs




| No. | R | Method | Crystn solvent | Mp, °C | [α]D, deg | Formula | Analyses |
|---|---|---|---|---|---|---|---|
| 1a | $CH_2CH_2CH(CH_3)_2$ | A | $Me_2CO$ | 128–129 | | $C_{26}H_{45}NO$ | Known[a] |
| 11a | $CH_3$ | A | $Me_2CO$-$H_2O$ | 222 | | $C_{22}H_{37}NO$ | Known[b] |
| 11b | $CH(CH_3)_2$ | A | $Me_2CO$-$H_2O$ | 151–152 | −21.9 | $C_{24}H_{41}NO$ | C, H |
| 11c | $CH_2CH(CH_3)_2$ | A | $Me_2CO$-$H_2O$ | 128–130 | −34.5 | $C_{25}H_{43}NO$ | C, H |
| 11d | $CH_2CH_2CH_2CH(CH_3)_2$ | B | $Me_2CO$ | 113–114 | −26.6 | $C_{27}H_{47}NO$ | C, H |
| 11e | $CH_2CH_2C_6H_5$ | B | $Me_2CO$ | 120–121 | −35.8 | $C_{29}H_{43}NO$ | C, H |

[a] See ref 11. [b] See ref 13.

moved to give crude **12** as an oil. Fractional distn of this oil gave pure **12** (900 mg), bp 158–160°. Nmr was as expected. *Anal.* ($C_{10}H_{23}N$) C, H.

**Enzyme-Inhibition Studies. Materials and Methods.** All chemical solvents were reagent grade and redistd prior to use. Glucose 6-phosphate and NADPH were purchased from Sigma. Glucose-6-phosphate dehydrogenase was obtd from Boehringer, Mannheim. Cholesterol-7α-*t* and -26-$^{14}C$ were purchased from New England Nuclear Corp., 0.25 *M* sucrose in 0.01 *M* potassium phosphate buffer (pH 7.4) was used as homogenizing medium.

**Preparation of Acetone Powder.** Bovine adrenal glands were obtd approx 30 min after slaughter, brought to the laboratory in ice, and processed immediately. All operations were performed at 0–4°. The glands were trimmed to remove fat and connective tissues and cut in half longitudinally. The medulla was carefully dissected from the cortex, and the cortical region was scraped off the capsule and homogenized with 5 μl of homogenizing medium per mg of adrenal gland wet weight in a Waring blender for 1 min. The homogenate was then passed through four layers of gauze, and the filtrate was centrifuged at 1200*g* for 20 min at 0° in a Sorvall refrigerated centrifuge. The nuclear pellet was discarded and the resulting supernatant centrifuged at 15,000*g* for 15 min at 0°. The mitochondrial pellet so obtd was washed with supplemented sucrose and recentrifuged at 15,000*g* for 15 min. The washed mitochondrial pellet was resuspended with 0.25 volume of the original homogenizing medium in a glass homogenizer. The homogenate was then transferred dropwise to 10 volumes of redistd $Me_2CO$ at −25 to −30°. The $Me_2CO$ soln was agitated vigorously with a magnetic stirrer during the transfer. After 10 min, the sedimented protein was sepd by decanting the $Me_2CO$. The protein was washed once with fresh cold $Me_2CO$ (−25°). The acetone powder so obtd was dried *in vacuo* in the dark over $CaCl_2$ and KOH for 2 days at −23°.

**Reconstitution of Acetone Powder.** This was accomplished essentially as described by Doering.[9] The enzyme was reconstituted in 0.1 *M* potassium phosphate buffer, pH 7.4, and used immediately. The protein concentration was determined by the method of Lowry, *et al.*[10]

**Incubation Procedure.** All incubations were performed in the dark using a Dubnoff metabolic shaker. Substrate cholesterol and cofactor were prepared according to Doering's procedure.[9] The inhibitors (azasteroids) were dissolved in 20 μl of dimethylformamide (DMF) and added to the incubation mixt prior to the addn of substrate. In all experiments, two to four controls were run in the presence of 20 μl of DMF and absence of inhibitors. Incubation flasks (10 ml, conical) were packed in ice prior to incubation and contain 0.53 ml of double-distd $H_2O$, 0.2 ml of cofactor, 0.2 ml of enzyme prepn, and 3 units of glucose-6-phosphate dehydrogenase. The flasks were preincubated without substrate and in the presence of inhibitor for 10 min at 37.5°, and then substrate cholesterol was added directly into the incubation mixt. Zero-time and 20-min samples were taken and immediately added to a counting vial containing 10 drops of formic acid (97+%) and carrier steroids. The vials were placed in a vacuum oven and heated to 140° with the aid of a very slight vacuum to remove one of the cleavage products, isocaproic-$^{14}C$ acid.

**Determination of Enzymatic Activity and Inhibition.** Bray's dioxane scintillation fluid (6 ml) was added to each vial. The vials were refrigerated overnight to remove any possible chemiluminescence effect. Each vial was then counted in a Beckman LS-200. The amt of cholesterol metabolized in the control or inhibition experiment was readily calculated by comparison of the isotope ratios ($^3H/^{14}C$) at 20 min *vs.* zero time. In all experiments, one set of incubation flasks contained 3 μ*M* 22-azacholesterol as the standard.

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