Synthesis and Antimicrobial Properties of 17β-Amino-4-aza-5α-androstane and Derivatives

W. E. SOLOMONSˣ **and N. J. DOORENBOS**

Abstract □ To develop further structure–activity relationships relative to antimicrobial activity in 4-azasteroids, 17β-amino-4-aza-5α-androstane and derivatives were synthesized. While using the Leuckart-Wallach reductive amination to place a β-amine function in the 17-position, the 5-double bond of the 4-aza-5-en-3-one system was reduced stereospecifically and a dimethylaminoethyl group was eliminated from the 4-nitrogen in the same system. These observations should give insight into the mechanism of the Leuckart-Wallach reaction. Antimicrobial studies showed that the 17β-amino grouping almost completely abolishes activity.

Keyphrases □ 17β-Amino-4-aza-5α-androstane and derivatives—synthesis, screened for antimicrobial activity □ Azasteroids—synthesis of 17β-amino-4-aza-5α-androstane and derivatives, screened for antimicrobial activity □ Antimicrobial activity—4-azasteroids synthesized and screened □ Structure–activity relationships—4-azasteroids and antimicrobial activity

Previous studies showed that certain 4-azasteroids possess high antimicrobial activity (1). A study of structure–activity relationships among these steroids strongly indicated that polar functional groups in the vicinity of the 17-position greatly decreased antimicrobial potency (2–4). Since these findings, potent compounds with ester and ether groups in the 17- and 20-positions were synthesized (1, 5, 6). However, no basic amine or quaternary nitrogen functional groups have been substituted in this region of the molecule. To establish structure–activity relationships in this region of the molecule, the synthesis of 17β-amino-4-aza-5α-androstane and its derivatives was undertaken.

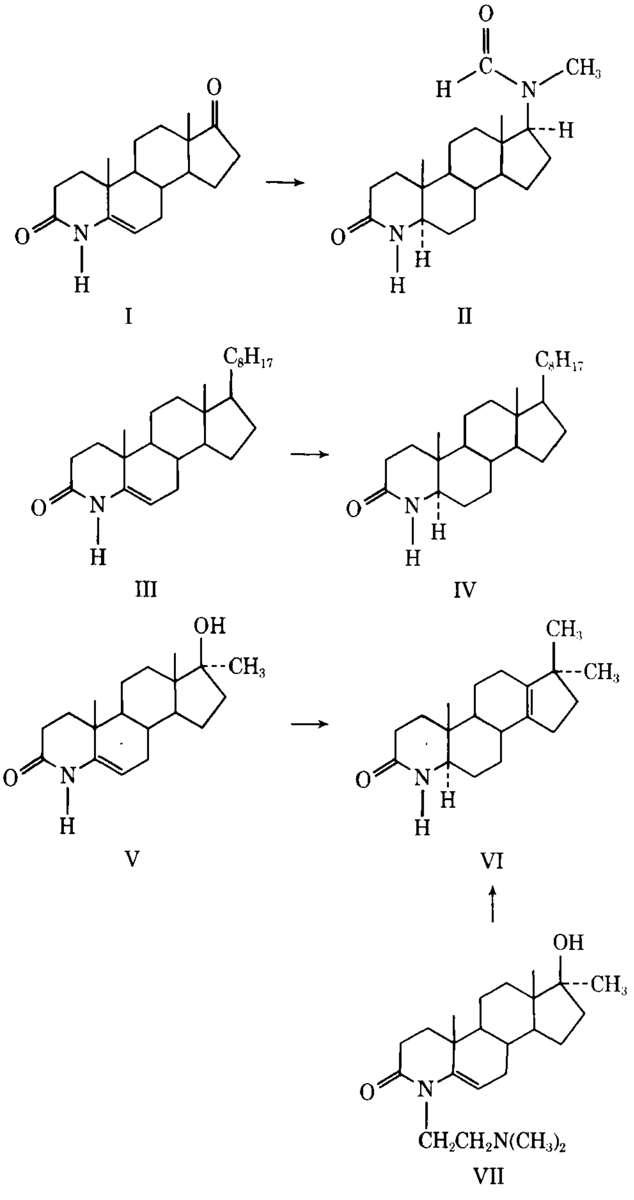
SYNTHESIS

While synthesizing the title compounds, a novel reaction was observed. This reaction proved to be of practical value in obtaining the target compounds. Scheme I outlines this reaction and the studies made of it.

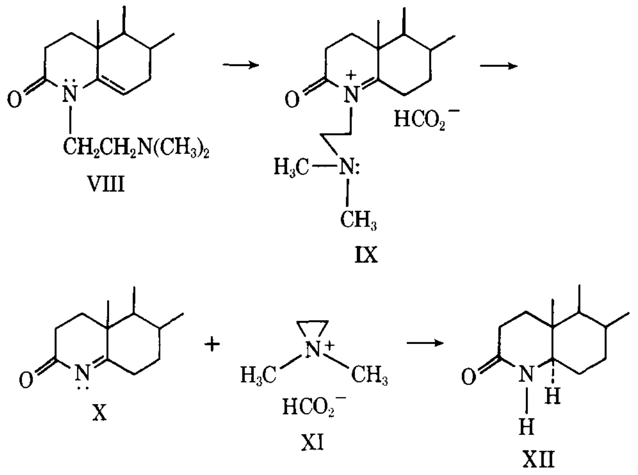
Compound I was produced by ozonolysis of androst-4-en-3,17-dione to 3,5-*seco*-4-norandrostan-5,17-dion-3-oic acid, followed by ring closure in concentrated ammonium hydroxide. The Leuckart-Wallach reductive amination of I was attempted to determine the stability of the 4-aza-5-en-3-one system under the rigorous conditions of this reaction. The formation of 17β-amine groups from 17-keto steroids by this reaction has been thoroughly studied (7–9). The product obtained from I was shown by IR, UV, NMR, and elemental analysis to have Structure II. It has been rigorously demonstrated (7–9) that this reaction gives an amide group with 17β-stereochemistry from 17-keto steroids.

The question of the configuration of the 5-position was answered by reacting Compound III under the same conditions as I. Melting point, mixed melting point, TLC, and optical rotation data proved IV to be the same as the known 4-aza-5α-cholestan-3-one (10), usually produced by catalytic hydrogenation of III. This method proved to be superior to catalytic hydrogenation (with stable molecules such as III) because of shorter reaction times and a cleaner product. Low-pressure hydrogenation of gram quantities of III usually required long reaction times (as much as 2 weeks in some instances) and large quantities of catalyst and gave a product contaminated with starting material.

On the other hand, attempts to convert more complex molecules such as V and VII to the corresponding saturated lactams by formic acid reduction gave rearrangement products. The Wagner-Meerwein rearrangements in the D-ring were not surprising (11). The elimination of the 4-dimethylaminoethyl group of VII was unexpected, but it can be rationalized if the reduction of the 5-double bond occurs according to the mechanisms for the Leuckart-Wallach reductive amination proposed either by Leonard and Sauers (12) or more recently by Lukasiewicz (13). Both mechanisms require the formation of a positive charge on the nitrogen, so the mechanism depicted in Scheme II is a possibility. The compounds obtained from V and VII were identical in physical properties and possessed superimposable NMR spectra. The successful completion of the reactions (Scheme I) indicated the route of



Scheme I

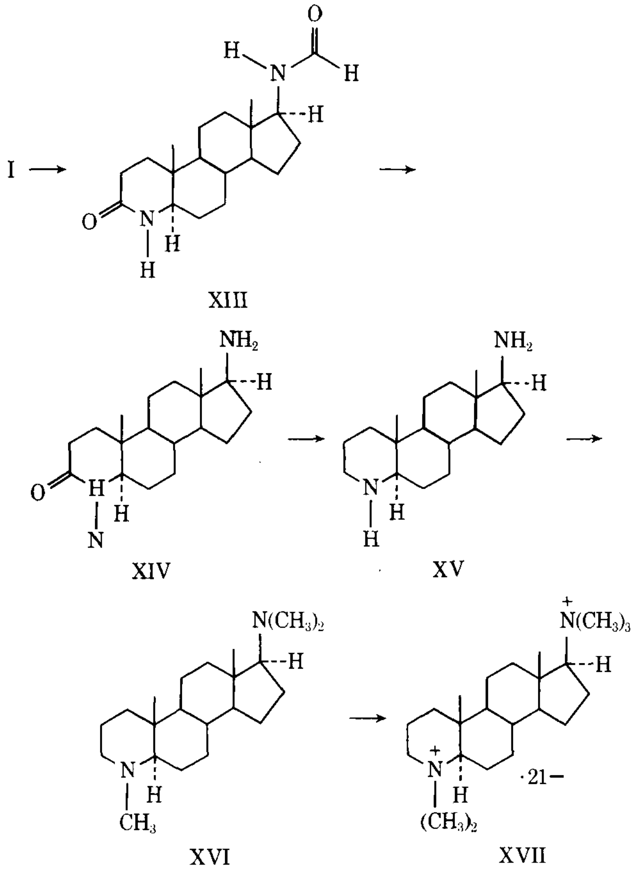


Scheme II

synthesis (Scheme III) to be taken for the title compounds. The Leuckart–Wallach reductive amination of I with formic acid and formamide gave XIII, which was converted to XIV by a simple acid-catalyzed hydrolysis. Lithium aluminum hydride reduction of XIV gave XV, one of the target compounds, 17β-amino-4-aza-5α-androstane. Two derivatives were prepared from XV, 17β-dimethylamino-4-methyl-4-aza-5α-androstane (XVI) and the corresponding bisquaternary salt XVII.

Another compound, 4,20-diaza-5α-cholestane, was similarly synthesized (Scheme IV) and may be considered as either an androstane or a cholestane.

The Leuckart–Wallach reaction has been used under various conditions and with slightly varying reagents (14). The reaction from I to XVIII is a variation utilizing the substrate, I, isohexylamine, and formic acid. The IR spectrum of XVIII showed replacement of the 17-ketone with the amide, but the UV spectrum showed incomplete reduction of the 5-double bond. The reduction was completed by heating XVIII with formamide and formic acid, producing XIX. Compound XIX was converted to XX by lithium aluminum hydride reduction in tetrahydrofuran.



Scheme III

BIOLOGICAL

A twofold serial dilution assay was used to determine the approximate antimicrobial potency of Compounds XV, XVI, XVII, and XX. For comparison, ND-502 (4-methyl-4-aza-5α-cholestane), a 4-azasteroid of known activity, was tested by the same procedure.

The cell cultures were obtained from the Department of Biology, University of Mississippi. The strains of *Staphylococcus aureus* and *Escherichia coli* are identified by the numbers K-257 and ATCC-4157, respectively. *Saccharomyces cerevisiae, Aspergillus niger,* and *Bacillus cereus* cultures have been part of University stock cultures for many years and are typed periodically by students using microscopic and biochemical methods.

The first tube of each series contained 100 μg of sample; therefore, succeeding tubes contained 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 μg. The level of activity of the compounds was determined by choosing the last tube in which there was no noticeable growth of the organism. No statistical analyses were employed. To ensure that the cells were viable under test conditions, tubes containing no steroid were used as blanks.

The biological data are presented in Table I. The growth medium for the bacteria was nutrient broth; for the fungi, it was mycophil broth.

EXPERIMENTAL[1]

4-Aza-5-androstene-3,17-dione (I)—A solution of 50.0 g (0.174 mole) of 4-androstene-3,17-dione in 75 ml of ethyl acetate and 300 ml of acetic acid was ozonized with 2.9 equivalents of ozone at ice bath temperature. After addition of 20 ml of 30% hydrogen peroxide and 20 ml of water and stirring for 12 hr, the solution was mixed with 500 ml of ether and washed with 500 ml of water. After extracting the water phase with 3 × 125 ml of ether, combining the ether fractions, washing with 3 × 300 ml of water, and extracting with 300 ml of 5 *M* sodium hydroxide, the basic solution was made acidic to blue litmus by slowly adding water-phosphoric acid (50:50 v/v) with cooling. After extracting the oily precipitate with 3 × 200 ml of *n*-butanol, washing the extract with 3 × 200 ml of water, and evaporating the solvent, 26.2 g (49%) of a viscous oil was obtained. The IR spectrum (liquid film) showed major bands at 3700–3050 (broad OH, carboxyl group), 1740 (17-ketone), and 1720 (5-ketone and carbonyl of the carboxyl group) cm^{-1}.

A solution of 33.0 g (0.107 mole) of 3,5-*seco*-4-norandrostane-5,17-dione-3-oic acid thus obtained in 350 ml of concentrated ammonium hydroxide was heated under nitrogen for 22 hr at 170° in a steel pressure vessel. After sitting at room temperature for 12–20 hr, the crystalline precipitate was collected by filtration and washed with concentrated ammonium hydroxide, giving 20.5 g (66%) of I. Recrystallization from *sec*-butanol gave I, mp 346–348°; $[\alpha]_D^{24}$ +56.6° (chloroform); UV_{max} (95% ethanol): 235 nm (ϵ 13,500); IR: 3190, 3050 (NH), 2910 (CH), 1750 (17-ketone), 1680 (C=C), and 1670 (lactam carbonyl) cm^{-1}; NMR ($CDCl_3$): δ 8.68 (broad, NH), 5.00 (broad vinyl), 1.18 (s, 3, C-19 methyl), and 0.96 (s, 3, C-18 methyl).

Anal.—Calc. for $C_{18}H_{25}NO_2$: C, 75.22; H, 8.77; N, 4.87. Found: C, 75.37; H, 8.97; N, 4.93.

17β-(*N*-Methylformamido)-4-aza-5α-androstan-3-one (II)—A mixture of 2.0 g (6.95 mmoles) I, 2 ml (0.039 mole) of 90% formic

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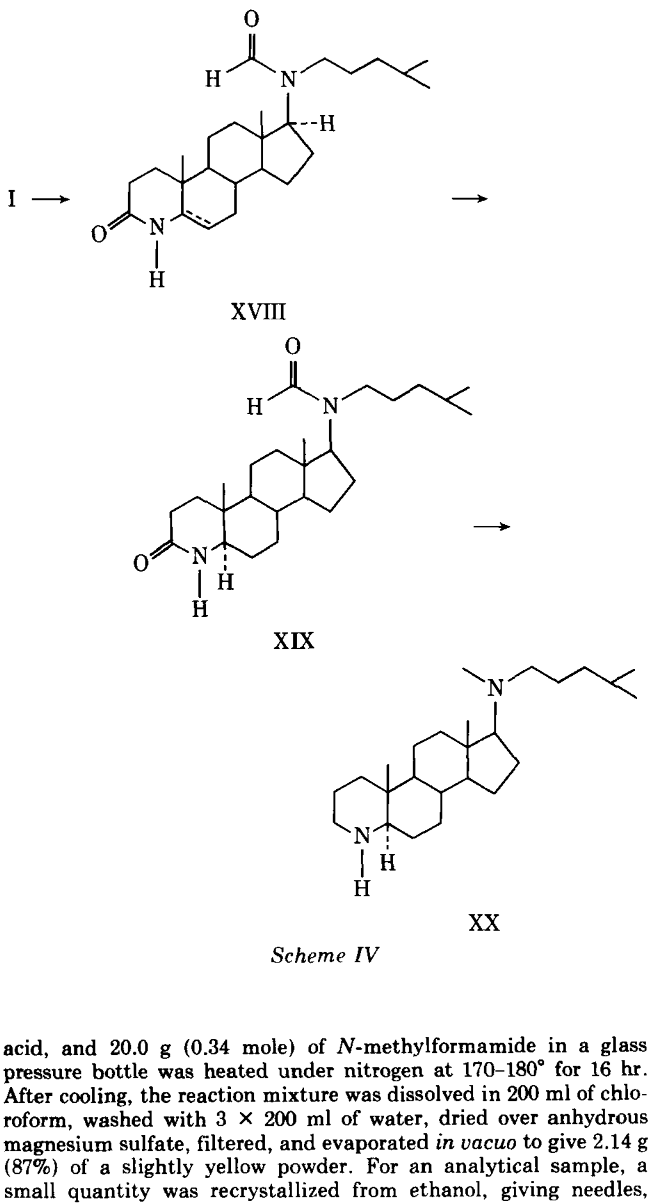
[1] All melting points were determined on a Thomas-Hoover capillary melting-point apparatus and the elemental analyses were obtained from Midwest Microlab, Inc., Indianapolis, Ind., or from Alfred Bernhardt Mikroanalytisches Laboratorium, West Germany. The wavelength of absorption in the UV spectra was determined on a Perkin-Elmer model 202, and the absorbance was determined on a Beckman model DU spectrophotometer. The IR spectra were recorded on a Perkin-Elmer model 257 spectrophotometer, and all spectra were determined in potassium bromide unless indicated otherwise. For the NMR spectra, sample solutions in most instances were 40 mg of sample/0.5 ml of chloroform-*d*. Tetramethylsilane was the internal reference. Ozone was generated by a Welsbach model T-816 ozone generator. Specific rotations were determined on a Perkin-Elmer model 141 automatic polarimeter as 2% solutions in chloroform.



Scheme IV

acid, and 20.0 g (0.34 mole) of *N*-methylformamide in a glass pressure bottle was heated under nitrogen at 170–180° for 16 hr. After cooling, the reaction mixture was dissolved in 200 ml of chloroform, washed with 3 × 200 ml of water, dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo* to give 2.14 g (87%) of a slightly yellow powder. For an analytical sample, a small quantity was recrystallized from ethanol, giving needles, mp 287–288.5°; $[\alpha]_D^{25}$ +12.0° (chloroform); UV_{max} (95% ethanol): transparent at 235 nm; IR: 3465, 3405, 3265, 3170, 3055 (NH and H_2O), and 1648 (amide and lactam carbonyl) cm^{-1} and absence of 17-ketone absorption; NMR ($CDCl_3$): δ 8.20 (s, 1, formyl), 6.41 (broad, 1, NH exchanged with D_2O), 3.0–4.0 (m, 2–3, *N*-methines), 2.93 (s, 3, *N*-methyl), 0.93 (s, 3, C-19 methyl), and 0.76 (s, 3, C-18 methyl).

Anal.—Calc. for $C_{20}H_{32}N_2O_2$: C, 72.25; H, 9.70; N, 8.43. Found: C, 72.07; H, 9.77; N, 8.18.

4-Aza-5α-cholestan-3-one (IV)—A mixture of 2.0 g (5.0 mmoles) 4-aza-5-cholesten-3-one (III), 20.7 g (0.351 mole) *N*-methylformamide, and 2.0 ml (0.039 mole) 90% formic acid in a glass pressure bottle was heated under nitrogen for 16 hr at 170–185°. The cooled reaction mixture was dissolved in 200 ml of chloroform, washed with 3 × 100 ml of 10% potassium carbonate and 3 × 100 ml of distilled water, dried over magnesium sulfate, filtered, and evaporated *in vacuo* to give 1.9 g (95%) of a pale-yellow solid. The UV spectrum taken in 95% ethanol showed the absence of enamine lactam absorption at 235 nm. Recrystallization from acetone (distilled from potassium permanganate and from sodium hydroxide pellets) gave fine, colorless needles, mp 245–249°; $[\alpha]_D^{20}$ +44.4° (chloroform). Reported values (10) are: mp 240–251°, after one recrystallization; mp 250–252°, after several recrystallizations; and $[\alpha]_D^{25}$ +45°. The IR spectrum gave major bands at 3170, 3050 (NH), and 1672 (lactam carbonyl) cm^{-1}. Preparation of the same compound by low-pressure hydrogenation with platinum oxide gave a compound having a melting point of 246–253° and a mixed melting point with the above material of 246–249°. TLC of these compounds, separately and mixed spotting of 40–80 μg on a silica gel thin-layer sheet with development in benzene–methanol–ethyl acetate (84:5:11), was performed. Iodine vapors were used for detection. The material obtained by hydrogenation and the mixture gave two spots: one corresponding to a small amount of starting material and one to the product. The product of reduction with *N*-methylformamide and formic acid corresponded to the major product from hydrogenation and gave only one spot.

17-Dimethyl-4-aza-18-nor-5α-androstan-13-en-3-one (VI)—*From 17β-Hydroxy-17α-methyl-4-aza-5-androsten-3-one (V) (15)*—After heating a mixture of 2.00 g (6.6 mmoles) of V, 20.0 g (0.444 mole) of formamide, and 3 ml (0.059 mole) of 90% formic acid in a glass pressure bottle under nitrogen for 16 hr at 175–185°, the cooled reaction mixture was dissolved in 200 ml of chloroform and washed with 2 × 150 ml of water, 2 × 100 ml of 20% sodium carbonate, and 3 × 150 ml of water. It was then dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo*, yielding 1.82 g of a hard, tan solid. The UV spectrum taken in 95% ethanol showed the absence of enamine lactam absorption at 235 nm, and the IR spectrum indicated that dehydration of the tertiary alcohol had occurred. Two crystallizations from acetonitrile gave colorless prisms, mp 237–240°; IR: 3262, 3150, 3040 (NH), 1684 (lactam carbonyl), and 1631 (13,14-double bond) cm^{-1}; NMR ($CDCl_3$): δ 7.13 (broad, 1, NH exchanged with D_2O), 0.96 (s, 6,17-dimethyl), 0.89 (s, 3, C-19 methyl), and no vinyl proton absorption.

Anal.—Calc. for $C_{19}H_{29}NO$: C, 79.39; H, 10.17; N, 4.87. Found: C, 79.46; H, 10.20; N, 4.79.

From 4-Dimethylaminoethyl-17β-hydroxy-17α-methyl-4-aza-5-androsten-3-one (VII) (16)—After heating a mixture of 2.20 g (5.4 mmoles) of VII, 45.2 g (1.00 mole) of formamide, and 8 ml (0.156 mole) of 90% formic acid at 170–180° for 72 hr, the cooled reaction mixture was rinsed into a separator with methanol and shaken with 200 ml of cold 10% sodium carbonate. This mixture was extracted with 3 × 80 ml of chloroform, and the chloroform extracts were combined, washed with 3 × 150 ml of water, dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo*, yielding 2.19 g of an oil which solidified. After drying 24 hr in the presence of phosphorus pentoxide at 54° *in vacuo*, the substance weighed 1.51 g. The IR spectrum indicated absence of the hydroxyl function, and the UV spectrum indicated reduction of the 5-double bond. Recrystallization twice from acetonitrile gave 424 mg of prisms, mp 239–242°. The IR spectrum of this compound was the same as that of the substance obtained above, and the NMR spectra of the two substances were superimposable.

Anal.—Calc. for $C_{19}H_{29}NO$: C, 79.39; H, 10.17; N, 4.87. Found: C, 79.43; H, 10.35; N, 4.65.

The NMR spectrum of the starting material clearly showed the presence of the 17β-hydroxyl group, the vinyl proton of the 5-double bond, the dimethylaminoethyl side chain, and the 17α-methyl and angular methyl groups.

17β-Formamido-4-aza-5α-androstan-3-one (XIII)—A mixture of 5.0 g (17.4 mmoles) of I, 3 ml (0.059 mole) of 90% formic acid, and 20 g (0.444 mole) of formamide in a glass pressure bottle was heated under nitrogen at 170–180° for 16 hr. After cooling, the reaction mixture was taken up in 200 ml of chloroform and washed with 2 × 130 ml of water, 3 × 130 ml of 10% potassium carbonate, and 3 × 130 ml of water. It was then dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo* to give 5.33 g (96%) of a white solid. A portion of this material (1.8 g) was heated in acetonitrile and filtered; the filtrate was evaporated slowly (5 days) and filtered to give 127 mg (2.3%) of white powder, mp 295–304° dec.; UV_{max} (95% ethanol): no absorption at 235 nm; IR: 3250, 3218, 3020 (NH), 1673, and 1658 (lactam and amide carbonyls) cm^{-1}.

Anal.—Calc. for $C_{19}H_{30}N_2O_2$: C, 71.66; H, 9.50; N, 8.80. Found: C, 71.45; H, 9.42; N, 8.88.

17β-Amino-4-aza-5α-androstan-3-one (XIV)—After refluxing 1.12 g (3.5 mmoles) XIII, 16 ml methanol, and 8 ml (0.096 mole) of concentrated hydrochloric acid for 3 hr, adding 150 ml of water, and extracting with 3 × 100 ml of chloroform, 50 ml of 32% potassium carbonate was added to the acidic water phase. The basic solution was extracted with 3 × 95 ml of chloroform, and the chloroform extracts were combined, washed with 3 × 150 ml of water, dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo* to give 0.75 g (73%) of a hard, pale-yellow solid, mp 225–240°. After subliming 0.31 g of this substance a distance of 2.6 cm three times at 0.005 mm and a maximum temperature of 215°, 210 mg (49% calculated as if the entire sample were similarly processed) of a white solid was recovered, mp 204–240°; $[\alpha]_D^{25}$ +47.3° (chloroform); IR: 3417, 3267, 3187, 3047 (NH), and 1653 (lactam carbonyl) cm^{-1}; NMR ($CDCl_3$): δ 7.08 (broad, NH of lactam), 1.25 (s, NH_2), 0.92 (s, C-19 methyl), and 0.67 (s, C-18 methyl).

Anal.—Calc. for $C_{18}H_{30}N_2O$: C, 74.44; H, 10.41; N, 9.64. Found: C, 74.14; H, 10.35; N, 9.59.

TLC[2] of this substance by spotting 40 μg and developing with chloroform–methanol–concentrated ammonium hydroxide (85:14:1) gave a single well-defined spot that was detected by iodine vapors.

17β-Amino-4-aza-5α-androstane (XV)—When 4.0 g (0.105 mole) of lithium aluminum hydride was added to a solution of 1.60 g (5.5 mmoles) XIV in 150 ml of dry tetrahydrofuran in a 300-ml three-necked flask fitted with a nitrogen inlet and outlet, condenser, and mechanical stirrer, the mixture was refluxed for 36 hr. After decomposing the excess lithium aluminum hydride by dropwise addition of 40–50 ml of saturated sodium sulfate, the mixture was filtered and the filtrate was evaporated *in vacuo,* giving 1.30 g (86%, quite crude) of a yellow semisolid. Purification was accomplished by subliming 0.92 g through a distance of 2.6 cm to a cold finger at an initial temperature of 95° and 0.005 mm. The first material collected, an oil, was removed before collecting a white solid (0.56 g) while raising the temperature to 156°. This substance recrystallized from acetonitrile, giving 0.31 g (28% calculated as if the whole sample were similarly processed) of pearly white leaflets, mp 156.5–157.5°; $[\alpha]_D^{23}$ +38.2° (chloroform); IR: 3335, 3235 (NH), and 1580 (NH_2 bending vibrations) cm^{-1} and absence of carbonyl absorption; NMR ($CDCl_3$): δ 1.23 (s, NH_2, exchanged with D_2O), 0.99 (s, C-19 methyl), and 0.68 (s, C-18 methyl).

Anal.—Calc. for $C_{18}H_{32}N_2$: C, 78.20; H, 11.67; N, 10.13. Found: C, 78.11; H, 11.50; N, 10.11.

17β-Dimethylamino-4-methyl-4-aza-5α-androstane (XVI)—A solution of 0.50 g (1.8 mmoles) XV, 5.0 ml (0.098 mole) of 90% formic acid, and 5.0 ml (0.063 mole) of formalin was refluxed for 6 hr and warmed at 40–45° for an additional 12 hr. After cooling, concentrated ammonium hydroxide was added dropwise until the solution turned red litmus blue. The precipitate was collected and dried *in vacuo* at 50° for several hours to give 0.52 g of product.

Purification Method A—The solid was recrystallized from acetonitrile, yielding 0.40 g (69%) of a fluffy, white powder, mp 149–151°; IR: 2960, 2930, 2850, 2830, 2805, 2755, and 2695 cm^{-1} (Bohlmann bands, CH stretching at 3 and 5, hydrogens adjacent and *trans,* diaxial to the nonbonded pair of electrons on nitrogen), and no NH stretching; NMR ($CDCl_3$): δ 2.28 (s, 17β-dimethylamino), 2.20 (s, 4-*N*-methyl), 0.98 (s, C-19 methyl), and 0.81 (s, C-18 methyl).

Anal.—Calc. for $C_{21}H_{38}N_2$: C, 79.18; H, 12.02; N, 8.79. Found: C, 79.11; H, 11.93; N, 8.78.

Purification Method B—A procedure similar to Method A was used for 0.42 g (1.9 mmoles) XV. After workup and drying *in vacuo,* 0.57 g of crude material was obtained, which was sublimed through a distance of 2.6 cm at 0.005 mm and a maximum wax bath temperature of 150°, giving a white solid, 0.51 g (85%), identical to that obtained with Method A.

Anal.—Calc. for $C_{21}H_{38}N_2$: C, 79.18; H, 12.02; N, 8.79. Found: C, 79.00; H, 11.95; N, 8.57.

17β-Dimethylamino-4-methyl-4-aza-5α-androstane Bismethiodide (XVII)—After refluxing 0.5 g (1.6 mmoles) of XVI in 70 ml of acetonitrile and 7.0 g (0.049 mole) of methyl iodide for 48 hr, the solution was reduced to one-half its volume and stored at −15° for 2 days, yielding prisms. These prisms were rapidly collected and dried for several days at 54° *in vacuo* in the presence of phosphorus pentoxide. Recrystallization from acetonitrile gave 317 mg (34%) of colorless prisms, mp 279.5° (after drying powdered material at 100°, 0.025 mm, for 48 hr) with liberation of a gas. The IR spectrum gave major bands at 3640–3100 (broad doublet), 2930, 2839 (CH), and 1615 cm^{-1}.

Anal.—Calc. for $C_{23}H_{44}I_2N_2$: C, 45.85; H, 7.36; I, 42.13; N, 4.64. Found: C, 45.63; H, 7.20; I, 42.02; N, 4.40.

Table I—Inhibitory Concentration (Micrograms per Milliliter)

Microorganisms	Steroids[a]				
	XV	XVI	XVII	XX	ND-502
S. cerevisiae	50	50	—	—	12.5
A. niger	—	—	—	—	50.0
Staph. aureus	—	—	—	—	12.5
B. cereus	—	—	—	—	25.0
E. coli	—	—	—	—	—

[a] No inhibition at 100 μg/ml.

17β-(*N*-Isohexylformamido)-4-aza-5α-androstan-3-one (XIX)—In a glass pressure bottle, 2.0 g (6.95 mmoles) of I, 6.0 g (0.059 mole) of isohexylamine, and 2.74 ml (0.053 mole) of 90% formic acid were heated under nitrogen at 175–180° for 16 hr. After cooling, the reaction mixture was taken up in 200 ml of chloroform and washed with 3 × 100 ml of water, 2 × 100 ml of 20% potassium carbonate, and 3 × 100 ml of water. It was then dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo. N*-Formylisohexylamine formed during the reaction and was removed from the product by distillation at 0.025 mm and a maximum temperature of 200° (wax bath), giving 2.86 g of a hard glass which lacked 17-ketone absorption at 1740 cm^{-1} in the IR spectrum. However, the UV spectrum showed only partial reduction of the 5-double bond. This solid was added to 20.0 g (0.444 mole) of formamide and 3 ml (0.059 mole) of 90% formic acid, heated under nitrogen at 170–180° for 16 hr, cooled, and worked up as before, including the high vacuum distillation, giving 2.40 g of a substance which showed no UV absorption at 235 nm. Recrystallization twice from ethyl acetate yielded 0.53 g (19%) of fine, shiny leaflets. These leaflets were boiled in 25 ml of acetone and sufficient chloroform to effect solution. After storing at −15° for 24 hr, warming to room temperature, and filtering, 147 mg (5.2%) of white powder was collected, mp 241–243°; IR: 3171, 3060 (NH), and 1670 (very intense lactam and amide carbonyls) cm^{-1}; NMR ($CDCl_3$): δ 8.56 (s, 1, formyl), 0.95 (s, 3, C-19 methyl), 0.90 (d, 6, left half superimposed on the C-19 methyl singlet), and 0.72 (s, 3, C-18 methyl).

Anal.—Calc. for $C_{25}H_{42}N_2O_2$: C, 74.58; H, 10.51; N, 6.96. Found: C, 74.38; H, 10.40; N, 6.70.

4,20-Diaza-5α-cholestane (XX)—To a slurry of 100 ml of tetrahydrofuran and 2.0 g (0.053 mole) of lithium aluminum hydride in a 500-ml three-necked flask fitted with condenser, drying tube, dropping funnel, and mechanical stirrer was added dropwise 0.92 g (2.3 mmoles) XIX in 50 ml tetrahydrofuran. After rinsing the dropping funnel with 50 ml of tetrahydrofuran, the mixture was refluxed for 36 hr and cooled in an ice bath; the complex was decomposed by adding dropwise 1.7 ml of water followed by 2.0 ml of 20% sodium hydroxide and then 6.0 ml of water. After stirring an additional 3 hr, the solid salts were removed by decanting tetrahydrofuran three times, filtering, and concentrating the filtrate *in vacuo* to give 1.04 g of an oil which solidified. Drying under vacuum in the presence of phosphorus pentoxide and recrystallization three times from acetonitrile gave 478 mg (56%), mp 101–102°; $[\alpha]_D^{27}$ +24.8° (chloroform); IR: 3270 (NH) cm^{-1} and absence of carbonyl absorption; NMR ($CDCl_3$): δ 2.21 (s, 3, *N*-methyl), 0.88 (s, 3, C-19 methyl), 0.83 (d, 6, isopropyl group in side chain), and 0.75 (s, 3, C-18 methyl) and a proton under the broad methylene absorption exchangeable with D_2O.

Anal.—Calc. for $C_{25}H_{46}N_2$: C, 80.15; H, 12.38; N, 7.48. Found: C, 80.38; H, 12.31; N, 7.58.

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Comparative Study of Seven Media for Sterility Testing

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Abstract □ A comparative study of the efficacy of sterility testing of pharmaceuticals according to two systems is described. The culture media used in the first system were: dithionite–thioglycollate (HS-T) broth, recommended by the Nordic Pharmacopoeia Board, and a peptone liver digest medium or a peptone liver digest agar. The media used in the second system were: fluid thioglycollate medium and soybean–casein digest medium, both prescribed by the USP XVIII, and Sabouraud liquid medium or Sabouraud dextrose agar. The supplemental use of one of the two latter media has been recommended, because the USP XVIII media were considered inadequate. A total of 180 cultures of fastidious bacteria, yeasts, and molds was investigated. The first system was found superior to the second.

Keyphrases □ Sterility testing—comparison between seven media divided into two systems, 180 cultures of bacteria, yeasts, and molds □ Culture media for sterility testing—comparison between seven media divided into two systems, 180 cultures of bacteria, yeasts, and molds □ Medium for sterility testing—two systems compared using 180 cultures of bacteria, yeasts, and molds

For sterility testing of pharmaceutical products, the FDA, USP XVI, and USP XVII prescribed fluid thioglycollate medium and Sabouraud liquid medium. A collaborative study performed by 12 laboratories in the United States and Canada showed that soybean–casein digest medium is superior to Sabouraud liquid medium (1). Accordingly, USP XVIII and the First Supplement to NF XIII replaced Sabouraud liquid medium with soybean–casein digest medium. In Germany, the Subcommittee for Microbiology, a Division of the Pharmaceutical Committee of the Confederation of the Pharmaceutical Industry, recommended the use of three media: fluid thioglycollate medium, soybean–casein digest medium, and Sabouraud liquid medium for sterility testing. The Nordic Pharmacopoeia Board recommended dithionite–thioglycollate (HS-T) broth[1].

In 1972, the author developed peptone liver digest medium and peptone liver digest agar to replace Sabouraud liquid medium and Sabouraud dextrose agar. Dithionite–thioglycollate (HS-T) broth and both peptone liver digest medium and peptone liver digest agar contain neutralizing constituents and supplementary mineral salts. To find out which media are most reliable for sterility testing of pharmaceuticals, the efficacy of these seven media was investigated.

MATERIALS AND METHODS

Microorganisms[2]—To simulate low levels of contamination which might possibly occur in a contaminated pharmaceutical preparation being tested for sterility, the inoculum was diluted so that every vessel (test tube or petri dish) of medium contained about 50–200 colony-producing units as determined by a plate count. When petri dishes were used, the inoculum was suspended in buffer solution and 0.1 ml was spread out on the surface of the solidified medium which had been poured the day before.

The following microorganisms were employed:

I. 18 strains belonging to 15 species of the genus *Bacillus*.
II. 22 strains belonging to 12 species of the genus *Clostridium*.
III. 48 strains belonging to 40 species of the following 22 genera: *Bacteroides, Comamonas, Corynebacterium, Desulfovibrio,*

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[1] This medium was developed recently by Dr. Clausen, University of Oslo, Oslo, Norway.

[2] The 180 cultures used included the following: 78 from the American Type Culture Collection, 31 from Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; 14 from the National Collection of Industrial Bacteria, Aberdeen, Scotland; 10 from the National Collection of Type Cultures, London, England; four from the Institute of Food Microbiology, Chiba University, Chiba, Japan; one from the Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Ill.; one from the Commonwealth Mycological Institute, Kew, England; and 41 cultures from other sources.