Synthesis and Antimicrobial Activity of 4-Aza-5α-sitostane and the 4-Methyl Derivative

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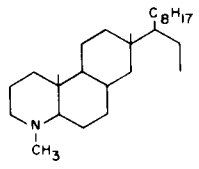
Synthesis of 4-aza-5α-sitostane (IX), and 4-methyl-4-aza-5α-sitostane (XII) were accomplished through a set of reactions involving oxidative opening of ring A of α,β-unsaturated ketone, ring closure, followed by catalytic hydrogenation and lithium aluminum hydride reduction, respectively. The antimicrobial activity for X and XII is reported.

Introduction

Interest in azasteroids has been increasing because of their potential value as pharmacodynamic and chemotherapeutic agents. 4-Azasteroids with antimicrobial (1-4), hypotensive (5), antiinflammatory (5,6), and hypocholesterolemic (5,7) activities have been prepared by Doorenbos and his co-workers. The most active antimicrobial 4-aza-steroid found was in the cholestane series, namely, 4-methyl-4-aza-5α-cholestane (ND-502); approximately 1 mcg./ml. being effective against Gram-positive bacteria, yeast and molds (4,8,9).



ND-502

Previous structure-activity relationship studies indicated that 4-aza-5α-androstanes and pregnanes, with a hydroxy or carbonyl on C_{17} and C_{20} positions, respectively, and their 4-methyl derivatives, exhibited little antimicrobial activity (10). Since 4-aza-5α-cholestanes possess high antimicrobial activity, it was not known whether the poor activity of these androstanes and pregnanes is due to the presence of the oxygen functional groups which are subject to microbial degradation, or to the lack of the hydrocarbon group at C_{17} in the cholestane series which contributes more lipophilic properties to the molecule.

Doorenbos (11) found that 4-aza-22-oxa-5α-cholestane possessed antimicrobial activity equivalent to that of 4-methyl-4-aza-5α-cholestane, suggesting that the oxygen functions, *per se*, do not lower the antimicrobial activity. Furthermore, it was recently reported that 17β-isopentyloxy-4-aza-5α-androstane and its 4-methyl derivative also possess antimicrobial activity (12). It was also found that activity decreased as the length of the hydrocarbon side chain was decreased. This suggests that the presence of a long hydrocarbon chain C_{17} is essential for the antimicrobial activity.

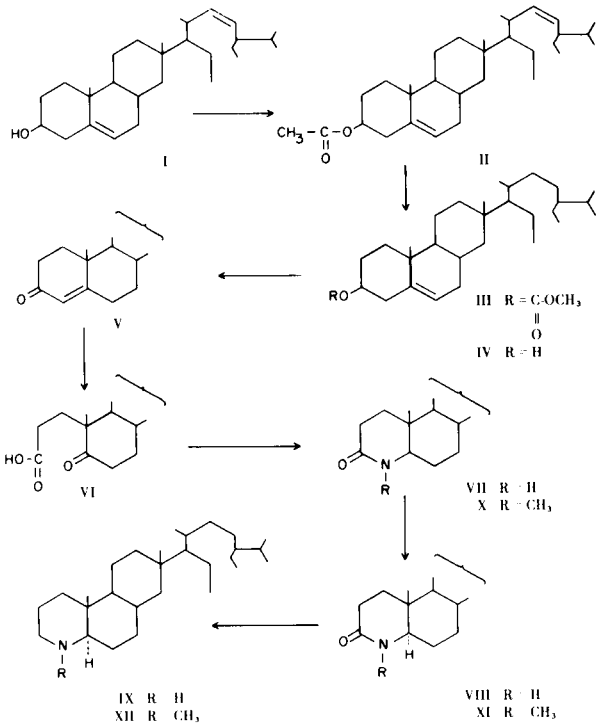
To evaluate structure-activity relationships further, the synthesis and antimicrobial properties of 4-aza-5α-sitostane (IX) and its 4-methyl derivative (XII) are reported here. These studies will demonstrate the effect of C_{24}-α-ethyl group on the hydrocarbon side chain of C_{17} on the antimicrobial activity.

Discussion

Synthesis

Commercial samples of β-sitosterol failed to yield pure products of IX and XII (Scheme I) as they contained a high percentage of campesterol, the C_{24}-α-methyl homolog of β-sitosterol, and other impurities. Attempts to purify these commercial samples of β-sitosterol by fractional crystallization and regeneration from derivatives did not eliminate the closely related phytosterols.

SCHEME I



Pure IV was obtained by selective hydrogenation at C_{22}-C_{23} double bond of stigmasteryl acetate (11) with palladium catalyst followed by hydrolysis. Selective hydrogenation was confirmed by the loss of absorption in the infrared spectrum at 10.30 μ characteristic for C=C trans double bond at C_{22}. This semisynthetic sample of IV was about 97% pure as shown by gc and mass spectral analysis, and was used as a starting material for the synthesis of IX and XII. β-Sitostenone (V) was prepared by the Oppenauer oxidation of IV and was purified by column chromatography using acid-washed alumina (see Experimental) according to the method described by Fujimoto (13). Ozonolysis of V, following the method described by Turner (14) for the ozonolysis of cholestenone, yielded 3,5-seco-4-norsitostan-5-on-3-oic acid (VI) but in poor yields. The reaction of VI with ammonium hydroxide and methylamine in a pressure vessel gave VII and X, respectively. The enamine lactams VII and X absorbed in the ultraviolet spectrum at 234 nm and 236 nm, respectively, which is characteristic of such substances (15,16). Hydrogenation of VII and X in glacial acetic acid solution with platinum catalyst gave VIII and XI. The hydrogenation of steroidal-5-enes is known to be sterospecific with hydrogen being introduced from the α-face (7,15). The reduced lactams VIII and XI were reduced wtih lithium aluminum hydride to obtain the final product IX and XII.

Biological (17)

A two-fold serial dilution assay was used to determine the approximate antimicrobial activity of 4-aza-5α-sitostane (IX) and the 4-methyl derivative (XII). For comparison, 4-methyl-4-aza-5α-cholestane, a 4-aza steroid of known activity (1,3,4), was used as a reference in this procedure.

The first tube of each series contained 100 mcg. of sample; succeeding tubes contained 50, 25, 12.5, 6.25, 3.12, 1.56 mcg./ml. The level of activity of the compounds was determined by choosing the lowest concentration in which there was no noticeable growth of the organism. Samples in which activity was found were allowed to stand for an additional 48 hours before the final readings were determined. Blanks containing no steroid were used to ensure that the cells were viable under the test conditions. The growth medium for the bacteria was bacto nutrient broth; for the fungi, it was mycophil broth. The biological data for duplicate assays are presented in Table I.

As is the case with most antimicrobial azasteroids (3), both IX and XII showed no inhibitory activity against Gram-negative bacteria represented by *Escherichia coli* in this screening. However, they did show activity against other microorganisms. It appears that these 4-aza-5α-sitostane derivatives are less effective as antimicrobial agents than the reference compound 4-methyl-4-aza-5α-cholestane.

Table I

Minimal Inhibitory Concentrations of Steroids Determined by Serial Dilution Method
(Expressed in Micrograms per Milliliter)

Microorganisms	Steroids (μg./ml.) IX	XII	4-Azasteroid (a)
Staphylococcus aureus	12.5	12.5	6.25
Escherichia coli	(b)	-------	------
Candida albicans	50	25	25
Saccharomyces cerevisiae	12.5	12.5	3.125
Aspergillus niger	12.5	12.5	3.125

(a) 4-Methyl-4-aza-5α-cholestane (ND-502). (b) Organisms were not inhibited.

EXPERIMENTAL

All melting points are uncorrected and were determined on a Thomas-Hoover melting point apparatus. Uv spectra were recorded by a Perkin-Elmer Model 202 Spectracord using 95% ethanol solutions. Ir spectra were determined using Perkin-Elmer spectrophotometers (Model 137 and 137G). The ir spectra were determined using potassium bromide pellets unless otherwise specified.

Nmr spectra were determined on a Varian A-60A spectrometer using tetramethylsilane as the internal standard. Chemical shifts are recorded as (δ) values (ppm) and cps values. Microanalyses were determined by Galbraith Laboratories, Inc., Knoxville, Tennessee and Midwest Microlab., Inc., Indianopolis, Indiana. Ozone was generated by a Welsbach T-816 ozone generator. Thin layer chromatography (18) on silica gel was performed using one of the following systems: System No. 1, benzene-methanol-ethyl acetate (85:10:5) and System No. 2, chloroform-methanol-ammonia (85:14:1). The steroid sample was dissolved in chloroform or methanol for spotting. Steroids were detected on the developed chromatogram by iodine vapors.

5,22-Stigmastadien-3β-ol, (Stigmasterol) (I)

The sample of stigmasterol (19) (m.p. 168-169°) obtained for this study, showed the following analysis by gas chromatography: Campesterol, 1.6%; β-Sitosterol, 4.1%; Stigmasterol, 86.7%.

White needles, m.p. 168-170° [lit. (20) m.p. 168-169°], were obtained upon recrystallization methanol-ethyl acetate (1:1); ir: 2.90 μ (OH), 6.01 μ (C=C) and 10.30 μ (-CH=CH- *trans* double bond out of plane deformation at C_{22}-C_{23} which differentiates this compound from the structurally similar sitosterol).

5,22-Stigmastadien-3β-ol Acetate (Stigmasterol Acetate) (II).

Stigmasterol, 41.2 g. (0.1 mole) (I) was added to 400 ml. of acetic acid containing 120 ml. of acetic anhydride and about 0.4 g. of *p*-toluenesulfonic acid was added. The mixture was allowed to reflux for 1 hour. The reaction mixture was mixed with crushed ice and the precipitate which formed was extracted with ether. The extract was washed twice with water, once with 10% sodium hydroxide, then with water and dired over anhydrous magnesium sulfate. The solvent was evaporated under vacuum and the white residue was recrystallized from methanol to obtain white shining plates, 40.9 g. (90%), m.p. 140-141° [lit. (21) m.p. 140.141°]; ir: 5.75 μ (C=O stretching), 8.03 μ (C-O stretching), 5.99 μ as a shoulder (C=C stretching of the C_5-C_6 doubld bond), and 10.3 μ (-CH=CH- *trans* double bond); nmr (deuteriochloroform): δ 0.708 (s, C_{18}-methyl), 5.33 (broad m, 1, olefinic proton at C_6), 5.06 (broad m, 2, olefinic CH=CH group at C_{22} of the side chain), 4.4 (broad m, 1, 3α-H at C_3), and 1.95 (s, $3CH_3$-CO-O group).

5-Stigmasten-3β-ol Acetate (β-Sitosterol Acetate) (III).

A mixture of 45.5 g. (0.1 mole) of II and 1.5 g. of 10% palladium on charcoal in 150 ml. of ethyl acetate was hydrogenated at room temperature and atmospheric pressure until 1.2 equivalents of hydrogen had been absorbed (an additional 0.5 g. of 10% palladium on charcoal was added during the course of the hydrogenation). After removing the catalyst, evaporating the solvent *in vacuo*, and recrystallizing the residue from methanol, 38.9 g. (85%) of white shining plates was obtained, m.p. 125-127° [lit. (22) m.p. 125-126°]; ir: 5.75 μ (C=O stretching), 8.00 μ (C-O stretching) and there was no absorption at 10.3 μ (indicating absence of -CH=Ch- *trans* double bond at C_{22}-C_{23}).

5-Stigmasten-3β-ol (β-Sitosterol) (IV).

A solution of 45.6 g. (0.1 mole) of III was allowed to reflux with 25% aqueous alcoholic sodium hydroxide for 1 hour, cooled, and ice water was added. The precipitate was extracted with ether, washed with water and dried over anhydrous sodium sulfate. The solvent was evaporated *in vacuo* and the white solid residue was crystallized from methanol to obtain white needles in 85% yield, m.p. 133-135° [lit. (22) m.p. 136-137°], which showed no depression in melting point when mixed with an authentic sample (23); ir (Nujol): 2.99 μ (OH stretching vibration polymeric association) and 6.01 μ (C=C stretching); nmr (deuteriochloroform); δ 0.71 (s, C_{18}-methyl), 1.05 (s, C_{19}-methyl), 5.33 (broad m, 1, olefinic proton at C_6), and 1.7 (broad s, 1, OH proton which exhanged with deuterium oxide).

4-Sitosten-3-one (β-Sitostenone) (V).

A mixture of 206.3 g. (0.5 mole) of IV, and 190 g. (0.93 mole) of aluminum isopropoxide in 1200 ml. dry acetone (freshly distilled) and 1000 ml. of dry benzene was refluxed for 72 hours. The mixture was cooled, treated with 200 ml. of water and 700 ml. of 20% sulfuric acid. The mixture was shaken vigorously and transferred to a separatory funnel where it was diluted further with 200 ml. of water and shaken for several minutes. The yellow aqueous layer was extracted with several 50 ml. portions of benzene. The combined benzene extracts were washed thoroughly with water and dried over anhydrous sodium sulfate for 24 hours. The solvent was evaporated *in vacuo* and a yellow oily residue obtained which solidified upon cooling, m.p. 77-82°, 165.0 g. (80%). The product (16.8 g.) was dissolved in hexane and chromotographed on 650 g. of acid washed alumina after elution with hexane, 3.0 g. of a yellow oil separated, which had a peculiar odor. Further elution with hexane-benzene (1:1) gave fractions containing small amounts of materials with melting ranges, first at 150-160°, followed by one at 120-122°. With benzene-hexane (8:3.5) a white solid, m.p. 158-160° eluted which was identified as β-sitostenone (10 g.) [lit. (13) m.p. 157-159°]; uv max 242 nm (log ε 4.24); ir (chloroform): 5.99 μ (C=O of α,β unsaturated ketone) and 6.20 μ (C=C of Δ^4-3 ketone); nmr (deuteriochloroform): δ 0.71 (s, C_{18}-methyl), 1.2 (s, C_{19}-methyl), and 5.58 (s, 1, olefinic proton at C_4).

3,5-Seco-4-norsitostan-5-on-3-oic Acid (VI).

To a solution of 23.6 g. (0.057 mole) of V, 225 ml. of glacial acetic acid and 125 ml. of ethyl acetate was added along with 0.067 mole of ozone at ice-bath temperature. After addition of 100 ml. of water, 10 ml. of 30% hydrogen peroxide was added, and the mixture was stirred for 24 hours, then extracted several times with ether. The combined ether extract was washed five times with 200 ml. portions of water then extracted with two 200 ml. portions of 10% sodium hydroxide. The basic solution was cooled and acidified with 10% hydrochloric acid. After extracting the oily precipitate with three 250 ml. portions of ether, washing with three 250 ml. portions of water, drying over anhydrous magnesium sulfate, evaporating the solvent *in vacuo*, 7.4 g. (30%) of a pale yellow oil was obtained. Attempts to crystallize the oil from different solvents were unsuccessful; ir (chloroform); 3.25 μ (carboxyl OH) and 5.88 μ (C=O carboxylic acids). Neutralization equivalent Calcd: 433.66. Found: 436.

4-Aza-5-sitosten-3-one (VII).

A solution containing 4.3 g. (0.01 mole) of the viscous oil VI, 25 ml. of ethanol and 200 ml. of concentrated ammonium hydroxide was heated in a pressure vessel, which was evacuated after replacing the air with nitrogen, at 150° for 8 hours. The reaction vessel was then allowed to cool. The white solid precipitate ob-

tained was filtered, washed with water and dried to yield 750 mg. (25%) of a white solid, m.p. 222-225°. After crystallization from methanol, a white crystalline solid was obtained, m.p. 222-225°. [lit. (13) m.p. 216-222°]; uv max 234 nm (log ϵ 3.92); ir: 2.95 μ (NH), 3.15 μ (NH, H-bonding), 6.02 μ (lactam C=O), and 5.96 μ (C=C); nmr (carbon tetrachloride): δ 0.71 (s, C_{18}-methyl), 1.13 (s, C_{19}-methyl), 4.86 (broad m, 1, olefinic proton at C_6), and 8.00 (broad m, 1, NH which exchanges with deuterium oxide).

The compound showed one spot on tlc, with R_f 0.72, using system No. 1, and R_f 0.89 using system No. 2.

Anal. Calcd. for $C_{28}H_{47}NO$: C, 81.30; H, 11.45; N, 3.39. Found: C, 80.93; H, 11.64; N, 3.34.

4-Aza-5α-sitostan-3-one (VIII).

A mixture of 412 mg. (1 mmole) of VII, 170 mg. of platinum oxide, and 75 ml. of glacial acetic acid was hydrogenated at 48 psi pressure and 70° for 5 hours. The catalyst was filtered and the solvent removed *in vacuo*. The semisolid residue was crystallized from ethanol to give 332 mg. (80%) of white crystalline needles, m.p. 265-267° dec.; uv; no absorption at 234 nm; ir (chloroform); 2.95 μ (NH), but no absorption at 5.96 μ (C=C), and at 6.02 μ (lactam C=O); nmr (carbon tetrachloride); δ 0.68 (s, C_{18}-methyl), 0.917 (s, C_{19}-methyl), and 0.61 (s, 1, N-H which exchanged with deuterium oxide). The compound showed one spot on tlc with R_f 0.61 using system No. 1 and R_f 0.87 using system No. 2.

Anal. Calcd. for $C_{28}H_{49}NO$: C, 80.90; H, 11.88; N, 3.37. Found: C, 80.95; H, 12.9; N, 3.29.

4-Aza-5α-sitostane (IX).

A slurry of 1.5 g. (39.5 mmoles) of lithium aluminum hydride in 150 ml. of dry tetrahydrofuran was added to a solution of 2.07 g. (5 mmoles) of VIII in 10 ml. of dry tetrahydrofuran. The mixture was refluxed for 36 hours. After decomposing excess lithium aluminum hydride with mehtanol followed by water, the mixture was treated with 10% sodium hydroxide solution until the precipitated salts dissolved. The mixture was extracted several times with ether and the ethereal extract was dried over anhydrous sodium sulfate. The solvent was evaporated *in vacuo* to yield an oily residue. Crystallization from methanol-ether mixture yielded a white microcrystalline powder, 1.14 g. (57%), m.p. 68-70°; ir: no peak at 6.02 μ indicating the absence of the lactam carbonyl group.

Anal. Calcd. for $C_{28}H_{51}N$: C, 83.92; H, 12.08; N, 3.49. Found: C, 84.28; H, 12.01; N, 3.45.

4-Methyl-4-aza-5-sitosten-3-one (X).

The procedure used was essentially similar to that described for the synthesis of VII, using 2.17 g. (5 mmoles) of VI dissolved in 15 ml. of ethanol and the solution was saturated with anhydrous methylamine. After working up the reaction mixture as previously described, the solid product was recrystallized from ethanol to yield 428 mg. (20%) of a white crystalline solid, m.p. 111-113°; uv max 236 nm; ir; 6.13 μ (lactam C=O) with an inflection at 6.00 μ (C=C); nmr (carbon tetrachloride): δ 0.71 (s, C_{18}-mehtyl), 1.06 (s, C_{19}-methyl), 4.83 (broad m, 1, olefinic proton at C_6) and 3.1 (s, 3N-CH_3). The compound showed one spot on tlc with an R_f 0.75 using system No. 1 and R_f 0.86 using system No. 2.

4-Methyl-4-aza-5α-sitostane-3-one (XI).

Following the procedure used for preparation of VIII, a solution of 2.13 g. (5 mmoles) of X in 60 ml. of glacial acetic acid and 170 mg. of platinum oxide as a catalyst. After working up the reaction mixture as previously described 1.83 g. (85%) of a white microcrystalline solid was obtained after recrystallization from ethanol, m.p. 205-207°, uv max: no absorption at 236 nm.: ir: 6.13 μ (lactam C=O).

Anal. Calcd. for $C_{29}H_{51}NO$: C, 81.06; H, 11.69; N, 3.26. Found: C, 81.38; H, 11.24; N, 3.28.

4-Methyl-4-aza-5α-sitostane (XII).

Using the method described for the synthesis of IX, a mixture of 2.15 g. (5 mmoles) of XI in 200 ml. of anhydrous ether and 1.00 g. of lithium aluminum hydride was refluxed for 15 hours. After working up the reaction mixture as previously described 124 mg. (60%) of an oil was obtained; ir (chloroform); no absorption in 4.0-6.7 μ region indicating the absence of the lactam carbonyl group. The hydrochloride salt of the base, prepared by treatment of an ethereal solution of the base with hydrochloric acid, was separated as a white crystalline solid 2.00 g. (48%), m.p. 282.284° dec.

Anal. Calcd. for $C_{29}H_{54}ClN$: C, 77.02; H, 12.04; N, 3.10. Found: C, 77.20; H, 12.20; N, 3.08.

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