between prednisolone clearance and additive concentration (3, 25). Increasing the additive concentration in the perfusate would drive the phase transition to completion, and further increases in the additive concentration would cause proportionately smaller increases in prednisolone clearance. In contrast, the relationship between griseofulvin clearance and additive concentration appears to be linear (Fig. 6), further suggesting that different barriers limit the absorption rate of griseofulvin and prednisolone.

If the effect of the additives listed in Table IV is due to increased membrane fluidity, it may be possible to determine whether the intestinal absorption of any particular compound is rate limited by the cell membranes of the intestinal epithelium. The additives would only increase the absorption rate of those substances whose absorption was membrane rate limited. The effects of one or more of the additives on the absorption of several drugs were observed (3, 17, 18). Prednisolone and closely related prednisone are unique in that they are the only drugs studied whose absorption rate was increased by the additives. Thus, the epithelial cell membrane may not be an important rate-limiting barrier to the absorption of many drugs. *In vitro* and *in situ* studies with rat intestine led to a similar conclusion (26).

In summary, the short and medium chain length fatty acids increase the intestinal absorption rate of prednisolone while they decrease the absorption rate of griseofulvin. The proposed explanation for these effects is that the absorption rate of griseofulvin is limited by the intestinal blood flow rate or an aqueous stagnant layer while the absorption of prednisolone is controlled by the intestinal epithelium. The absorption-altering effects of the fatty acids were attributed to their ability to reduce intestinal blood flow or to increase the thickness of the stagnant layer while simultaneously increasing the permeability of the intestinal epithelium.

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Synthesis and Preliminary Anti-Inflammatory Evaluation of 17β-Amino-3β-methoxy-5-androstene Hydrochloride and Related Derivatives

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Received May 24, 1976, from the *Department of Pharmacy, The Queen's University of Belfast, Belfast BT9 7BL, Northern Ireland.* Accepted for publication December 17, 1976. (Publication delayed at the request of the authors.)

Abstract □ To study the anti-inflammatory properties of 17β-aminosteroids, some 17β-aminoandrostene hydrochlorides based on the 3β-methoxy-5-androstene nucleus were prepared. The syntheses were accomplished *via* a two-stage amination of 3β-methoxy-5-androsten-17-one, involving reduction of an intermediate 17-imine or, for the synthesis of 17β-amino-3β-methoxy-5-androstene, the 17-oxime. The compounds were examined for anti-inflammatory activity in the rat cotton pellet model of inflammation. All tested aminosteroids displayed significant activity. Two compounds also were screened in an adjuvant-induced arthritis model of inflammation and displayed activity.

Keyphrases □ 17β-Aminoandrostenes, substituted—synthesized, evaluated for anti-inflammatory activity in rats □ Anti-inflammatory activity—various substituted 17β-aminoandrostenes evaluated in rats □ Structure–activity relationships—various substituted 17β-aminoandrostenes evaluated for anti-inflammatory activity in rats

Over the past two decades, considerable interest has developed in the synthesis and biological evaluation of aminosteroids (1). The compounds produced have various biological properties including antimicrobial, hypocholesterolemic, hypotensive, and local anesthetic.

Anti-inflammatory activity was established for a series of 16β-amino-17α-hydroxy-20-ketopregnenes using the cotton pellet and foot edema models of inflammation (2). The activity associated with these aminosteroids was not influenced markedly by structural alterations that normally enhance the anti-inflammatory activity of corticosteroids.

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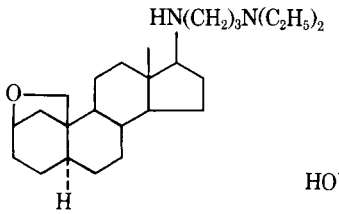
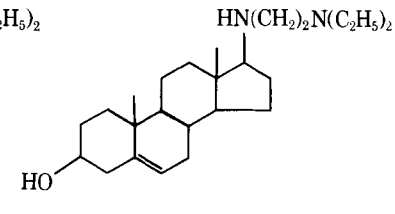


Ia



Ib

Filderman and Kovacs (3) studied the anti-inflammatory properties of the azasteroidal glycoside tomatine and noted activity in several inflammation models, including the mouse peritoneal capillary permeability test, a model in which corticosteroids are ineffective. Tomatidine, the aglycone of tomatine, did not exhibit significant anti-inflammatory activity in any of the experimental models.

Several series of aminosteroids, based on various androstane nuclei of differing A- and B-ring functionalities, recently were reported to show anti-inflammatory activity in both intact (4–6) and adrenalectomized rats (7). The compounds, examples of which include 17β-[*N*-(2-diethylaminopropyl)amino]-5α-androstan-2β,19-epoxide (I*a*) and 17β-[*N*-(2-diethylaminoethyl)amino]-3β-hydroxy-5-androstene (I*b*), are characterized by the possession of a 17β-amine function.

The present study investigated the anti-inflammatory activity of androstenes possessing a 17β-amine function, including examination for corticosteroid-like activity. This report describes the synthesis and preliminary anti-inflammatory evaluation of a series of 17β-aminoandrostenes based on the 3β-methoxy-5-androstene nucleus.

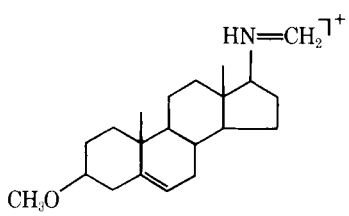
DISCUSSION

Synthesis—3β-Methoxy-5-androsten-17-one[1] (II) was utilized as the starting material for the synthesis of the title compounds. Acid-catalyzed condensation of this steroid (II) with the appropriate amines following an established procedure (8) yielded the corresponding 17-iminosteroids (III*a*–III*e*), which were smoothly reduced to the desired 17β-aminosteroids (IV*a*–IV*e*) with lithium aluminum hydride in dioxane (Scheme I). The reduction of 17-iminosteroids with lithium aluminum hydride previously was reported to proceed to the 17β-amino epimer (8).

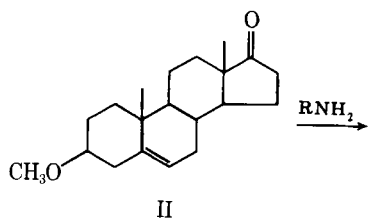
The intermediate 17-iminosteroids (III*a*–III*e*) were oils or oily solids for which satisfactory elemental analyses could not be obtained. The IR spectra of these compounds were consistent with the assigned structures. In each case, reduction to the desired aminosteroid and hydrochloride formation gave a crystalline product (IV*a*–IV*e*·HCl) whose structure was confirmed by spectral data and elemental microanalyses.

The 17β-aminosteroids IV*a*–IV*e*·HCl (Scheme I) were further characterized by mass spectrometry. The mass spectrum of each compound showed a weak ion signal with an *m/e* value corresponding to the free amine and a strong base peak at *m/e* 316 corresponding to Fragment A, resulting from fission of the amine side chain alpha to the C-17 nitrogen atom (9).

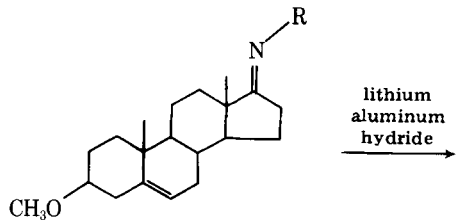
17β-Amino-3β-methoxy-5-androstene hydrochloride (VI·HCl) was



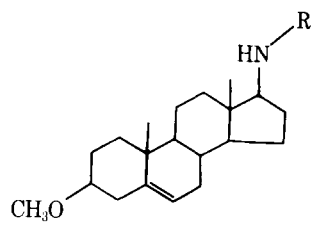
Fragment A



II



III*a*: R = $CH_2CH_2NH_2$, · 2HCl
III*b*: R = CH_2CH_2OH, · HCl
III*c*: R = $CH_2CH_2OCH_3$, · HCl
III*d*: R = $CH_2CH_2CH_3$, · HCl
III*e*: R = $CH_2CH(CH_3)OH$, · HCl

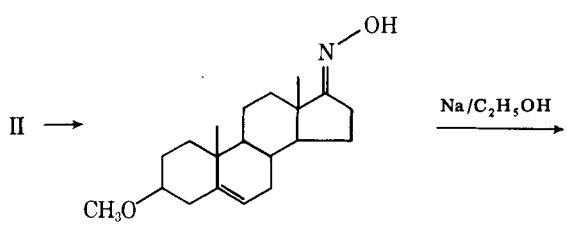


IV*a*–IV*e*

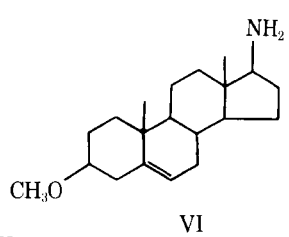
Scheme I

prepared by reduction of oxime V, which was synthesized from II by an established route (10) (Scheme II). Reduction of V with lithium aluminum hydride in dioxane proved unsuitable for the synthesis of the 17β-amine VI, the reaction giving a low yield of the desired compound with, in all cases, several other basic products[2]. Sodium in ethanol reduction of V according to an established procedure (11) and subsequent hydrochloride formation gave a satisfactory yield of the desired VI·HCl.

Biological—The title compounds were examined for anti-inflammatory activity in the rat cotton pellet model of inflammation (12). Male albino rats[3], 100–120 g, were used. Sterilized cotton-wool pellets of known weight (10 mg) were implanted subcutaneously in groups of five rats under ether anesthesia. Four pellets were placed in each animal, one in each groin and one in each axilla. The test compound, as a solution in 5% (w/v) gum acacia, was administered orally to each animal in the group



V



VI

Scheme II

[1] Compound II, mp 142° [lit. (13) mp 142°], was prepared from 3β-hydroxy-5-androsten-17-one 3-tosylate according to the method of Heyl *et al.* (14). Satisfactory analytical data were obtained for all samples prepared.

[2] S. C. Griggs and J. King, to be published.

[3] Wistar strain.

Table I—Anti-Inflammatory Studies: Rat Cotton Pellet Model of Inflammation

Compound[a]	Mean Weight of Granulation Tissue Produced per Pellet, mg ± *SEM*	Mean Inhibition of Granulation Tissue Formation, %
Controls		
Gum acacia (5% w/v)	27.00 ± 4.48	0
Prednisolone	13.35 ± 2.27	50.6
IV*a*·2HCl	13.7 ± 1.6	49.3
IV*b*·HCl	11.65 ± 1.05	57.0
IV*c*·HCl	11.7 ± 2.63	57.0
IV*d*·HCl	14.1 ± 1.74	47.8
IV*e*·HCl	14.7 ± 1.47	45.6
VI·HCl	12.65 ± 2.91	53.2

[a] The dose was 1.0 mg/kg in all cases.

on 4 consecutive days, the first dose being given immediately after implantation of the pellets.

On the 5th day, the rats were killed with chloroform; the pellets were dissected, all fat and extraneous tissue were removed, and the pellets were dried overnight in a hot air oven at 60°. Two groups of control rats received oral doses of either 5% (w/v) gum acacia or prednisolone [suspended in 5% (w/v) gum acacia]. The weight of granulation tissue produced in each pellet was calculated, and the mean value for each group was compared with that of the controls (Table I).

Compounds IV*a*·2HCl and IV*b*·HCl were further assessed for their ability to prevent developing adjuvant arthritis in the rat[4]. Groups of four male inbred rats[5], 120–150 g, were injected with heat-killed *Mycobacterium tuberculosis*[6] (300 μg) suspended in liquid paraffin (0.5 ml). Injections were given intradermally to the plantar aspect of one hindpaw. The volume of each hindpaw (up to the calcaneum and astragalas bones) was measured by displacement of mercury using a plethysmograph coupled to an air-type blood pressure transducer.

The test compound, as a solution in aqueous 0.25% (w/v) hydroxyethylcellulose[7], was administered orally twice daily, at a dose and volume of 1.0 ml/100 g, to each animal in the group for the duration of the experiment. Normal animals without arthritis and arthritic animals, each dosed with hydroxyethylcellulose alone, were included as control groups. A positive control group received indomethacin. The arthritis was allowed to develop for 18 days, and the paw volumes were remeasured. The changes in paw volumes during the experiment were calculated and summed for each group.

The percentage inhibition of developing adjuvant-induced arthritis (Table II) was calculated from:

$$\text{percentage inhibition} = \frac{\Delta V_a - \Delta V_t}{\Delta V_a - \Delta V_c} \times 100 \qquad \text{(Eq. 1)}$$

where ΔV_a is the increase in combined paw volume of arthritic control animals, ΔV_t is the increase in combined paw volume of arthritic animals dosed with the test compound, and ΔV_c is the increase in paw volume of nonarthritic control animals.

All aminosteroids tested displayed at least significant antigranuloma activity ($p > 0.02$) in the rat cotton pellet model. Prednisolone, at the same dose level as the test compounds, produced a similar inhibition of granuloma formation (Table I). Compounds IV*a*·2HCl and IV*b*·HCl were further screened in an adjuvant-induced arthritis model of inflammation. Both compounds displayed anti-inflammatory activity (Table II).

The results clearly establish the 3β-methoxy-5-androstene nucleus as a suitable basis for the synthesis of 17β-aminosteroids with anti-inflammatory activity.

The activity displayed by the compounds tested, including the aminosteroids IV*b*–IV*e*·HCl, each of which possesses a 17β-alkylamine substituent hitherto unassociated with anti-inflammatory activity, and also the primary amine VI·HCl, suggests that the nature of the 17β-alkylamine substituent may be relatively unimportant in determining this biological activity in the 17β-aminosteroids. Quantitative studies may illustrate more clearly the effect of the structure of the 17β-alkylamine substituent on the level of anti-inflammatory activity.

[4] Performed by Allen and Hanburys Ltd.

[5] PVG strain.

[6] Human strains C, DT, PN (Ministry of Agriculture and Fisheries, Weybridge, England).

[7] Natrosol.

Table II—Anti-Inflammatory Studies: Adjuvant-Induced Arthritis Model of Inflammation

Compound	Percentage Reduction in Paw Volume[a]	
	Activity of Test Compound[b]	Activity of Indomethacin[c]
IV*a*·2HCl	70	45
IV*b*·HCl	61	57

[a] Mean of two experiments. [b] The dose was 25 mg/kg po. [c] The dose was 1.0 mg/kg po.

EXPERIMENTAL[8]

The general procedure for the synthesis of the hydrochlorides of aminosteroids IV*a*–IV*e* is shown in Scheme I. A solution of II (5 g, 0.016 mole), the appropriate amine (15 ml), and *p*-toluenesulfonic acid monohydrate (0.2 g) in benzene (200 ml) was refluxed in a Dean–Stark separator until water removal was complete (~6 hr). Solvent removal under reduced pressure gave a viscous oil (~5 g). TLC[9] of the oil indicated the presence of one major component with an R_f value different from that of the starting material.

A solution of the oil in methylene dichloride–methanol (9:1) was chromatographed on silica gel[9] (200 g). Elution with the same solvent gave a pure (as evidenced by TLC) sample of the desired iminosteroid (III*a*–III*e*) as an oil. The IR spectrum of the oil showed major bands at 1675 (C=N) and 1105 (CO) cm^{-1}. A solution of the iminosteroid (4 g, 0.0125 mole) in dry dioxane (50 ml) was slowly added to a stirred slurry of lithium aluminum hydride (2 g) in dioxane (50 ml), and the reaction mixture was stirred at reflux temperature for 48 hr. After this period, aqueous dioxane (50 ml, 50% v/v) was added, and the reaction was left for a further 2 hr. The reaction mixture was then filtered, and the solvent was removed under reduced presure to give an oil. Hydrogen chloride gas was then passed through a solution of the oil in dry ether, and the resultant precipitate was filtered and dried to give IV*a*–IV*e*.

17β-[*N*-(2-Aminoethyl)amino]-3β-methoxy-5-androstene Dihydrochloride (IV*a*·2HCl)—The yield was 2.2 g (31%); recrystallized from ethanol–ether; mp >350° (dec.); $[\alpha]_D^{25}$ −42.1°; IR: 3000–2700 (N^+H_2 stretch, CH), 1600 (N^+H_2 def.), 1105, and 1030 (CO stretch) cm^{-1}; mass spectrum: *m/e* 346 (5), 316 (100), and 30 (100).

Anal.—Calc. for $C_{22}H_{38}N_2O{\cdot}2HCl$: C, 62.98; H, 9.61; N, 6.67. Found: C, 62.74; H, 9.81; N, 6.56.

17β-[*N*-(2-Hydroxyethyl)amino]-3β-methoxy-5-androstene Hydrochloride (IV*b*·HCl)—The yield was 2.4 g (36%); recrystallization from ethanol–ether; mp 265–267° (dec.); $[\alpha]_D^{25}$ −12.1°; IR: 3550 (OH), 3000–2700 (N^+H_2 stretch, CH), 1600 (N^+H_2 def.), 1105, and 1030 (CO stretch) cm^{-1}; mass spectrum: *m/e* 347 (12) and 316 (100).

Anal.—Calc. for $C_{22}H_{37}NO_2{\cdot}HCl$: C, 68.81; H, 9.94; N, 3.64. Found: C, 68.52; H, 10.01; N, 3.51.

17β-[*N*-(2-Methoxyethyl)amino]-3β-methoxy-5-androstene Hydrochloride (IV*c*·HCl)—The yield was 2.6 g (40%); recrystallized from ethanol; mp 300° (dec.); $[\alpha]_D^{25}$ −15.6°; IR: 3000–2700 (N^+H_2 stretch, CH), 1600 (N^+H_2 def.), 1105, and 1030 (CO stretch) cm^{-1}; mass spectrum: *m/e* 361 (3) and 316 (100).

Anal.—Calc. for $C_{23}H_{39}NO_2{\cdot}HCl$: C, 69.40; H, 10.13; N, 3.51. Found: C, 69.12; H, 10.23; N, 3.46.

17β-(*N*-Propylamino)-3β-methoxy-5-androstene Hydrochloride (IV*d*·HCl)—The yield was 2.5 g (40%); recrystallized from ethanol; mp 330° (dec.); $[\alpha]_D^{25}$ −28.09°; IR: 3000–2700 (N^+H_2 stretch, CH), 1600 (N^+H_2 def.), 1105, and 1030 (CO stretch) cm^{-1}; mass spectrum: *m/e* 345 (10), 330 (10), and 316 (100).

Anal.—Calc. for $C_{23}H_{39}NO{\cdot}HCl$: C, 72.30; H, 10.55; N, 3.67. Found: C, 72.15; H, 10.54; N, 3.61.

17β-[*N*-(2-Hydroxypropyl)amino]-3β-methoxy-5-androstene Hydrochloride (IV*e*·HCl)—The yield was 2.4 g (37%); recrystallized

[8] All melting points were obtained using an electrothermal capillary melting-point apparatus and are uncorrected. Elemental analyses were performed by the Microanalytical Laboratories, Chemistry Department, The Queen's University of Belfast. Optical rotations were obtained using a Bellingham and Stanley polarimeter and, unless otherwise stated, refer to 2% (w/v) solutions in methanol. IR spectra were recorded using a Perkin-Elmer 257 grating spectrophotometer and, unless indicated otherwise, refer to pressed potassium bromide disks. Mass spectra were obtained using an AEI MS902 spectrometer operating at 70 ev. The PMR spectrum was obtained using a Varian A-60D spectrometer with chloroform-*d* as the solvent and tetramethylsilane as the internal reference.

[9] TLC was performed using 0.08-cm (0.03-in.) layers of Kieselgel G (Type 60) (E. Merck, Darmstadt, West Germany). Methanol (5% v/v) in methylene dichloride was used as the developing solvent. Column chromatography was performed using silica gel M.F.C., 100–200 mesh (Hopkins and Williams, Essex, England).

from ethanol; mp 285–287° (dec.); IR: 3550 (OH), 3000–2700 (N^+H_2 stretch, CH), 1600 (N^+H_2 def.), 1105, and 1030 (CO stretch) cm^{-1}; mass spectrum: *m/e* 361 (4) and 316 (100).

Anal.—Calc. for $C_{23}H_{39}NO_2$·HCl: C, 69.40; H, 10.13; N, 3.51. Found: C, 69.31; H, 9.97; N, 3.61.

3β-Methoxy-5-androsten-17-one 17-Oxime (V)—A solution of II (5 g, 0.016 mole), sodium acetate (9 g), and hydroxylamine hydrochloride (3 g) in 5% (v/v) aqueous ethanol (80 ml) was refluxed for 4 hr. After sitting at room temperature overnight, the crystalline precipitate was collected and washed with water, giving 4 g (78%) of V. Recrystallization from methanol gave the analytical sample, mp 218–219°; $[\alpha]_D^{25}$ +11.6°; IR: 3500 (OH), 1660 (C=N), 1105, and 1030 (CO stretch) cm^{-1}; PMR ($CDCl_3$): δ 0.92 (s, 3H, C-13 methyl), 1.03 (s, 3H, C-10 methyl), 3.36 (s, 3H, C-3 methoxyl), and 5.35 (broad, 1H, C-6 proton) ppm.

Anal.—Calc. for $C_{20}H_{31}NO_2$: C, 75.66; H, 9.84; N, 4.42. Found: C, 75.69; H, 9.97; N, 4.51.

17β-Amino-3β-methoxy-5-androstene Hydrochloride (VI·HCl)—Sodium (12 g, freshly cut) was added (during 1 hr) in small increments to a refluxing soluton of V (2 g, 0.006 mole) in absolute ethanol (100 ml). After sodium addition, the reaction was stirred at the reflux temperature for 2 hr. The hot mixture was then diluted with warm (60°) water (1 liter) and allowed to stand at room temperature for 24 hr. The resultant solid was dissolved in dry ether, and hydrogen chloride gas was passed through the ethereal solution to give 1.2 g (45%) of VI·HCl. Recrystallizations from ethanol gave the analytical sample, mp 294–296° (dec.); $[\alpha]_D^{25}$ −19.1°; IR: 3200–2800 (N^+H_3 stretch, CH), 1610 (asym. N^+H_3 def.), 1515 (sym. N^+H_3 def.), 1105, and 1030 (CO stretch) cm^{-1}; mass spectrum: *m/e* calc. for $C_{20}H_{33}NO$ (M^+ − HCl): 303.2562. Found: *m/e* 303.2562.

Anal.—Calc. for $C_{20}H_{33}NO$·HCl·C_2H_5OH: C, 68.44; H, 10.44; N, 3.63. Found: C, 68.24; H, 10.40; N, 3.69.

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Steady-State Urinary Excretion Method for Determining Bioequivalence of Conjugated Estrogen Products

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Abstract □ The steady-state excretion of conjugated estrogens in the urine of postmenopausal women dosed with conjugated estrogens tablets was studied using a modification of a previously published method. The procedure was used to quantitate the estrogens both before and during conjugated estrogens replacement therapy. The method, which is relatively specific, involves enzyme hydrolysis of urine samples, a number of classical extraction and purification steps, and analysis of the silylated estrogens on a 2.7-m, 1.7% diethylene glycol succinate column using flame-ionization detection. The results indicate that steady-state urinary estrogen excretion levels were obtained within 17 days of dosing. Furthermore, the urinary estrogen excretion profile was significantly different from the composition of the estrogens in the dosage form.

Keyphrases □ Estrogens, conjugated—GLC analyses, steady-state urinary excretion in postmenopausal women □ Excretion, urinary—conjugated estrogens at steady state in postmenopausal women □ GLC—analyses, conjugated estrogens in urine of postmenopausal women

Conjugated estrogen mixtures have been used therapeutically since 1942. However, there is no detailed information concerning the human plasma or urinary levels of these estrogens after administration, primarily because of the lack of sufficiently sensitive and specific analytical methods.

BACKGROUND

Many recent reports focused on the analysis of estriol and other estrogens in pregnancy urine to follow or monitor fetal health (1–5). The urinary estrogen excretion levels in nonpregnant women (6, 7), in postmenopausal women (8–11), and in men (12, 13) were studied. Other investigators studied the types of conjugation of steroids and separated

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