Assignment by ^{13}C-NMR Spectroscopy of Configuration at C-5 in 17α-Ethylestran-17β-ol, an Impurity in the Anabolic Steroid Ethylestrenol

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Abstract □ The stereochemistry at C-5 in 17α-ethylestran-17β-ol, found as an impurity in ethylestrenol, was assigned by ^{13}C-NMR spectroscopy. The hydrogen atom attached to C-5 is in the α-configuration. Resonance assignments were confirmed by partial deuteration, off-resonance ^{13}C-(^{1}H)-decoupling, and comparison with model compounds.

Keyphrases □ 17α-Ethylestran-17β-ol—impurity in ethylestrenol, stereochemistry at C-5 assigned □ Ethylestrenol impurity—stereochemistry at C-5 assigned □ Stereochemistry—at C-5 of 17α-ethylestran-17β-ol, impurity in ethylestrenol □ Anabolic steroids—ethylestrenol impurity, stereochemistry at C-5 assigned

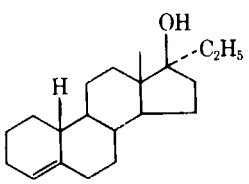
Anabolic steroids are used medically for conditions characterized by wastage of protein and bone (1). Because of their particular properties, they are abused nonmedically to increase muscle development and to improve athletic performance (2). Numerous undesirable side effects have been reported (1), but it is not clear whether such side effects are due to the drugs themselves or to impurities.

As part of a screening procedure to isolate and identify impurities in anabolic steroids by GLC, the drug ethylestrenol (17α-ethylestr-4-en-17β-ol) (I) was examined. An impurity that may be present (up to 2%) (3) is 17α-ethylestran-17β-ol (II*a*); the stereochemistry of this compound at C-5 was determined by ^{13}C-NMR spectroscopy. It is reasonable to expect that II*a* will have some hormonal activity of its own (4).

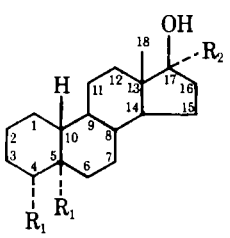
To assist in the elucidation of the ^{13}C-NMR spectrum of II*a*, 17α-ethyl-5α-estran-17β-ol-4,5-d_2 (II*b*) was synthesized from I, and 17α-ethyl-5α-estran-17β-ol-4,5,20,20,21,21,21-d_7 (II*c*) was synthesized from 17α-ethinyl-17β-hydroxyestr-4-ene[1] (III).

EXPERIMENTAL

GLC—A solution of I in acetone (1 mg/ml) was chromatographed on 3% OV-7 on Chromosorb W at 210°. The conditions were: nitrogen carrier gas, 50 ml/min; and flame-ionization detector gases, hydrogen, 45 ml/min, and air, 500 ml/min. Under these conditions, II*a*, present at a level of about 0.7%, separated from I. Retention times were: I, 656 sec; and II*a*, 562 sec. A mass spectrum of II*a* (obtained by GLC–mass spectrometry) showed a molecular ion at *m/e* 290 and many features similar to those of the mass spectrum of I but 2 *m/e* units higher, which suggested that the impurity was the dihydro derivative of I.



I



II*a*: R_1 = H, R_2 = C_2H_5
II*b*: R_1 = D, R_2 = C_2H_5
II*c*: R_1 = D, R_2 = C_2D_5

Synthesis of II*a*—Ethylestrenol (200 mg) was dissolved in 200 ml of ethanol and hydrogenated at atmospheric pressure over 10% palladium-on-charcoal (10 mg) for 1 hr. After the catalyst had been removed by filtration, the ethanolic solution was evaporated to dryness, whereupon the product crystallized. Recrystallization from acetone–water gave II*a*, mp 62–63° (86%). The GLC retention time, the mass spectrum, and the IR spectrum of II*a* were identical with those of the impurity isolated from I.

Anal.—Calc. for $C_{20}H_{32}O$: C, 82.1; H, 11.8. Found: C, 82.7; H, 11.7.

Synthesis of II*b*—This synthesis utilized a method similar to that for II*a*, except that deuterium was used in place of hydrogen. The mass spectrum of II*b* showed that at least two atoms of deuterium were incorporated, and the height of the $(M + 1)^+$ peak relative to the M^+ peak suggested that a partial incorporation of a third atom of deuterium occurred. The melting point of II*b* was 62–63°; a mixture of II*a* and II*b* gave the same value, indicative of no configurational heterogeneity.

Synthesis of II*c*—Compound III (200 mg) was reacted with deuterium as for I. The mass spectrum of II*c* showed that at least seven atoms of deuterium were incorporated.

^{13}C-NMR Spectroscopy—^{13}C-NMR spectra were recorded[2] in deuterochloroform with complete proton decoupling. Chemical shifts are expressed with respect to tetramethylsilane with an accuracy of ±0.05 ppm.

DISCUSSION

Three methods were used to assign the ^{13}C-NMR spectra: comparison with model compounds (5, 6) corrected for substituent effects (7), analysis of multiplet splitting in off-resonance decoupled spectra, and measurement of deuterium substitution effect on chemical shifts and resonance intensities.

The ^{13}C-NMR spectrum of II*a* was assigned by comparison with the model compounds 5α- and 5β-androstane (5, 8), 5α-androstane-17α-ol, 5α-androstane-17β-ol (9), 5α-cholestane (5), and *trans-anti-trans*-perhydrophenanthrene (6). 5α- and 5β-Androstane differ in chemical shifts mainly at the resonances for C-19, C-9, C-7, and C-5. Assignments were verified by studying the multiplet structures in off-resonance decoupled spectra as well as by deuterium substitution in II*b* and II*c*.

Off-resonance decoupling should lead to the following multiplet structures for the various carbon resonances in II*a*: singlets, 13 and 17; doublets, 5, 8, 9, 10, and 14; triplets, 1, 2, 3, 4, 6, 7, 11, 12, 15, 16, and 20; and quartets, 18 and 21.

Deuterium substitution is useful for the assignment of possibly ambiguous resonances. Its effects are twofold. There is a substantial decrease in intensity for the substituted carbon because of the extreme lengthening of the spin-lattice relaxation time, T_1, as well as splitting of the resonance into a multiplet pattern because of ^{2}H–^{13}C-spin–spin coupling. In addition, the chemical shift of the deuterated carbon usually differs significantly from that of its protonated analog (10–15). Thus, depending on the signal-to-noise ratio of the ^{13}C-spectrum, the resonance of a deuter-

[1] Organon Ltd., West Hill, Ontario, Canada.

[2] Varian CFT-20 and XL-100 NMR spectrometers, Georgetown, Ontario, Canada.

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Table I—^{13}C-Chemical Shifts of IIa–IIc and the Influence of Deuteration and Off-Resonance Decoupling

Chemical Shifts[a]			Multiplet Structure[b]	Intensity upon Deuterium Substitution[c]	Assignment[d]
IIa	IIb	IIc			
83.52	83.56	83.43	s	d	17
49.86	49.85	49.86	d	—	9
48.55	48.55	48.55	d	—	14
47.46	47.35	47.37	d	d	10
46.52	46.54	46.52	s	—	13
43.34	43.33, 43.27	43.34, 43.31, 43.21	d	d	5
42.29	42.29	42.30	d	—	8
34.56	34.45	34.59, 34.49, 34.43	t	d	4†
34.03	33.93	34.00, 33.94, 33.83	t	d	6†
33.70	33.74	33.69	t	—	16
31.62	31.63	31.63	t	—	7
30.93	30.91	30.94	t	—	1
30.35	30.32	30.33	t	—	12
28.87	28.88	28.54	t	d	20
26.84	26.85, 26.74	26.79	t	d	2*
26.45	26.44, 26.30	26.44, 26.39, 26.35	t	d	3*
25.40	25.40	25.42	t	—	11
23.47	23.48	23.49	t	—	15
14.45	14.45	14.48	q	—	18
7.79	7.83	7.08	q	d	21

[a] In parts per million downfield from the internal standard tetramethylsilane; accuracy of chemical shifts is 0.05 ppm. [b] Multiplet structure in off-resonance decoupled spectrum. [c] If no change was observed, a − sign is indicated; a "d" symbolizes a decrease in intensity. Data are for IIc. [d] The * and † assignments may be reversed.

ated carbon can be completely absent from the spectrum or appear slightly shifted from its usual position with an observable ^{13}C–^{2}H-coupling. With partial deuteration, a resonance from the residual protonated carbon may appear with the usual chemical shift but reduced intensity. The chemical shifts and linewidths of resonances due to carbons adjacent to the position of deuterium incorporation may be affected also (10, 14, 15).

Reduction of the double bond in ethylestrenol in the presence of deuterium gas should lead to deuterium incorporation in IIa at positions 4 and 5 as well as at the adjacent carbons 3, 6, and 10 because of allylic migration. With III, reduction in the presence of deuterium gas should affect C-20, C-21, C-4, and C-5 as well as C-3, C-6, and C-10.

Table I gives the chemical shifts and assignments of the 17α-ethyl-5α-estran-17β-ols studied. The configuration at position 5 is assigned as 5α based on a chemical shift observed at 49.9 ppm attributed to C-9 (in accord with the calculated chemical shift for this position: 5α, 49.9; and 5β, 35.4 ppm). The 5β-isomer is not expected to show any resonance at such a low field. The chemical shifts of C-7 and C-5 are also more in accord with those calculated for the 5α-isomer (C-7, calculated = 32.6, observed = 31.6 ppm; C-5, calculated = 41.9, observed = 43.3 ppm) than for the 5β-isomer (C-7, calculated = 27.1; C-5, calculated = 36.0 ppm).

To assign the remaining resonances in ethyl-5-estran-17β-ol, the multiplet structures of the off-resonance decoupled spectra were evaluated. Two singlets at 46.52 and 83.52 ppm were attributed to C-13 and C-17. Carbon-17 also showed an upfield shift of 0.1 ppm in IIc as a result of deuterium substitution at C-20. The assignments are in good agreement with the values calculated for these carbons (C-13, 45 ppm; C-17, 82 ppm). Two quartets were assigned to C-18 and C-21. Carbon-21 showed a very broad resonance in IIc and was shifted upfield ~0.7 ppm as a result of the deuterium substitution.

The doublets were attributed to C-5, C-8, C-9, C-10, and C-14. Carbon 9 was unperturbed by deuteration, as were C-14 and C-8. The assignments for C-9, C-14, and C-8 (Table I) are in good agreement with the values calculated for these carbons (49.9, 48.8, and 41.4 ppm, respectively). The doublets assigned to C-5 and C-10 both showed decreased intensities in IIb and IIc because of partial deuterium substitution at these carbons. The chemical shift calculated for C-5 of the 5α-isomer (41.9) is in reasonable agreement with the assigned value of 43.3 ppm, whereas the calculated value for the 5β-isomer is 36.0 ppm. The assignment of C-10 (47.5 ppm) corresponds well with that reported for the same carbon in perhydrophenanthrene (48.2 ppm).

Triplet structures in the off-resonance decoupled spectra of C-1, C-2, C-7, C-11, C-12, C-15, and C-16 should be unperturbed by deuteration in IIb and IIc, whereas C-3, C-4, and C-6 should be affected by deuterium incorporation directly at these carbon atoms. Carbon-1 was assigned by comparison with the same carbon in perhydrophenanthrene (30.1 ppm). Carbons-2 and 3 were assigned at 26.5 and 26.8 ppm, respectively, and can be compared to similar positions in perhydrophenanthrene at 27.1 and 26.8 ppm. Both C-2 and C-3 appeared to be affected by deuteration in IIb and IIc, showing decreased resonance intensities and upfield isotope shifts of ~0.1 ppm because of interactions with deuterium on neighboring (vicinal and geminal) carbons.

Carbons-4 and 6 were assigned at 34.56 and 34.03 ppm, respectively, by comparison with perhydrophenanthrene (35.0 and 34.6 ppm); both showed decreased intensities for the residual proton-bearing carbons with upfield shifts of 0.1 ppm because of the presence of neighboring deuterium-substituted carbons. With such small differences in chemical shift between C-2 and C-3 and C-4 and C-6, absolute assignments are difficult and those in Table I may be reversed.

Carbon-7 was assigned by calculating the chemical shift after removal of the 19-CH_3 group from 5α-androstane (calculated = 32.6 ppm, observed = 31.6 ppm). Carbon-13 was calculated in a manner similar to that used for C-7 (calculated = 26.3, observed = 25.4 ppm). Carbon-12 was assigned based on calculation of a γ-substituent effect on 5α-androstane-17β-ol (calculated = 34.4, observed = 30.35 ppm). Carbon-15 was assigned at 23.47 ppm by comparison with a similar position in 5α-cholestane (24.2 ppm) and 5α-androstane-17β-ol (23.4 ppm). Carbon-16 (33.7 ppm) was assigned by calculating either the effect of a hydroxyl substituent at C-17 in 5α-cholestane (calculated = 34.3 ppm) or a β- and γ-alkane substituent effect in 5α-androstane-17β-ol (calculated = 34.6 ppm).

In summary, the chemical shifts assigned to the various carbon atoms of IIa are in complete agreement with the structure of 17α-ethyl-5α-estran-17β-ol and with the hypothetical ^{13}C-spectrum based on contemporary knowledge of the influence of substituents on ^{13}C-chemical shifts.

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COMMUNICATIONS

Lack of Influence of Rabbit Nictitating Membrane on Miosis Effect of Pilocarpine

Keyphrases □ Pilocarpine hydrochloride—miosis induction, effect of rabbit nictitating membrane □ Nictitating membrane, rabbit—effect on miosis induction by pilocarpine hydrochloride □ Ophthalmic cholinergics—pilocarpine hydrochloride, effect of rabbit nictitating membrane on miosis induction

To the Editor:

The rabbit frequently is used in ophthalmological research even though it possesses some ocular anatomical dissimilarities to the human. There are differences in the normal tear volume and the tear turnover rate between humans and rabbits (1, 2). A prominently different anatomical feature of the rabbit is the nictitating membrane, a so-called third eyelid. This epithelially covered sheet of cartilaginous tissue retracts into the nasal canthus secondarily to retractions of the globe with contraction of the retractor bulbi muscles (3). Because this membrane is well vascularized and occupies part of the precorneal space, it conceivably could influence the dynamics of instilled material in the eye (3, 4).

Ten New Zealand albino rabbits of either sex, 1.5–2.5 kg, were used in the present study. A 50-μl dose of 2% pilocarpine hydrochloride[1] was administered to the right eye by slightly pulling away the lower eyelid from the globe and allowing the measured drop to fall onto the cornea and collect into the lower conjunctival sac. After a few seconds, the eyelid was carefully returned to its normal position. Pupil diameters were measured under controlled lighting conditions using a micrometer caliper held at a constant distance between the animal's and the observer's eyes.

At the completion of the first experiment, in which the control response to the drug was recorded, the nictitating membranes were surgically removed at the base from the test eyes of all rabbits. After 1 week, the operated animals were handled in the same manner as before and were treated with the same lot of drug as in the first experiment. Pupil size measurements were made in the usual manner for comparison with the previous week's results. The nictitating membrane had no apparent effect on the response parameters of pilocarpine-induced miosis following topical dosing of an aqueous solution (Table I).

These results are surprising for a number of reasons. First of all, the nictitating membrane can be moved approximately halfway across the cornea. In so doing, the membrane occupies a significant portion of the precorneal space. Therefore, one would expect a reduction in the fluid volume in the normal rabbit eye and a concomitant loss of drug available for absorption. However, the movement of the membrane across the eye is accompanied by a retraction of the eye into the orbit. Thus, with its movement, there conceivably could be no net change in the volume of fluid that the rabbit eye can hold.

Second, the membrane is well vascularized and there are many lymphatic nodules in the connective tissue (3). Therefore, the presence of the membrane might provide a competitive depot to channel drug away from the corneal absorption process. Third, tear secretions from the nictitans and Harderian glands containing oil and mucus empty into the nictitating membrane (3). The removal of the membrane carries with it the contribution of the nictitans glandular secretions to the composition of the tears and its possible effect on steady-state tear volume and/or turnover rate. It may be that some of these processes are operative but in effect cancel one another.

An alternative explanation could be that a relative lack of movement of the nictitating membrane occurs throughout drug absorption. We observed that, after dosing, the rabbits may close their eyelids for 15–30 sec but that the membranes remain retracted. Quantitatively, Chrai *et al.* (2) showed that 1 min after dosing a 50-μl aqueous drop, only 28.2% of the original instilled volume remained. Consequently, pilocarpine hydrochloride absorption may be rapid relative to the nictitating membrane activity. Thus, our experiments indicate that the membrane in its fully retracted state has no effect on the response to pilocarpine solution as observed under these

Table I—Miosis Induced by 50 μl of 2% Pilocarpine Hydrochloride Solution in Albino Rabbit Eyes before and after Removal of the Nictitating Membrane

Area under Curve, mm/mm × hr	Peak Intensity[a]	Total Duration, hr	Baseline Diameter, mm
Before Removal of Nictitating Membrane (n = 10)			
0.9194[b] ±0.2058	0.4275 ±0.0445	4.38 ±1.02	4.975 ±0.611
After Removal of Nictitating Membrane (n = 8[c])			
0.9388 ±0.3057	0.3911 ±0.0641	4.32 ±0.96	5.181 ±0.575

[a] Peak intensity = $[(\text{pupil diameter})_{t_0} - (\text{pupil diameter})_{t_{max}}]/(\text{pupil diameter})_{t_0}$. [b] Mean ± *SD*. [c] One rabbit suffered from debilitating diarrhea and another died; therefore, only eight rabbits were used after removal of the nictitating membrane.

[1] Isopto Carpine 2%, Alcon Laboratories, Fort Worth, TX 76101.

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