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Chirality Determination of (19R)-, (19S)- and (19RS)-[19-^{3}H,^{2}H,^{1}H]-3β-hydroxyandrost-5-en-17-one's+

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The synthesized title compounds were converted to their corresponding 4-en-3-one's which in turn were oxidized with selenium dioxide and hydrogen peroxide. The resulting seco acid lactones were oxidized by the Kuhn-Roth method to yield samples of acetic acid. The acetates were analyzed for chirality by the malate-synthetase fumarase procedure.

For studies of the biosynthesis of estrogens,[1] we required (19R)-, (19S)-, and (19RS)-[19-^{3}H,^{2}H,^{1}H]-3β-hydroxyandrost-5-en-17-one's. We have developed two routes for the syntheses of (19R) and (19S) compounds.[2] The principle underlying both synthetic routes was based on the reduction of a [19-^{3}H]-10β--formyl androgen to [19-^{3}H,*H]-19-hydroxy-androgen [*H=^{2}H or ^{1}H] with high diastereoisomeric purity. Subsequently, the [19-^{3}H,*H]-alcohol was to be hydrogenolyzed to a methyl group with the stereospecific introduction of the appropriate third atom of isotopic hydrogen.

Exploratory studies showed that reduction of deuteriated (*1a*) and protiated (*1b*) 19-aldehydes with ^{1}H and ^{2}H-Haubenstock's[3] reagents respectively gave the corresponding (19S)-alcohol (*2a*) and (19R)-alcohol (*3a*) with 90—-—95% stereoisomeric selectivity. The purities of the alcohols were determined by proton NMR of the derived (*2b*) and (*3b*). The alcohols were then converted[4,5] to their 19-iodides in the retention mode[2] without loss of diastereoisomeric purity.[2] Treatment of the iodides with $LiEt_3BH^*$ (H* = ^{1}H or ^{2}H) (superhydride) gave the 19-methyl products.

Based on these observations, we have undertaken the syntheses of chiral [19-^{3}H,^{2}H,^{1}H]-androgens (Scheme 1).

Reduction of aliquots of [19-^{3}H]-aldehyde (*1c*) (Scheme 1) with [^{1}H] and [^{2}H], Haubenstock's reagents gave, after the cleavage of the ketals, the (19R)-

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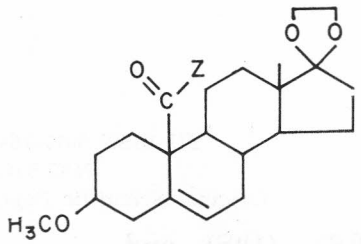
+ Dedicated to Professor Mihailo Lj. Mihailović on the occasion of his 60th birthday.

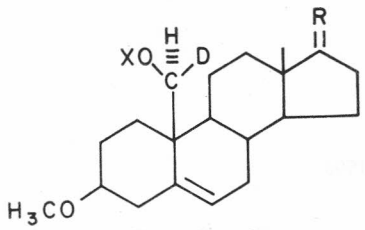
* Post Doctoral Fellow (1980—1981)

** Post Doctoral Fellow (1982—1983)



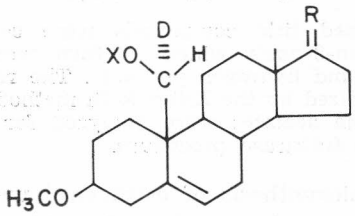
1.

a. Z = ^{2}H
b. Z = ^{1}H
c. Z = ^{3}H



2.

a. R = $[CH_2O]_2$; X = H
b. R = O ; X = Ac



3.

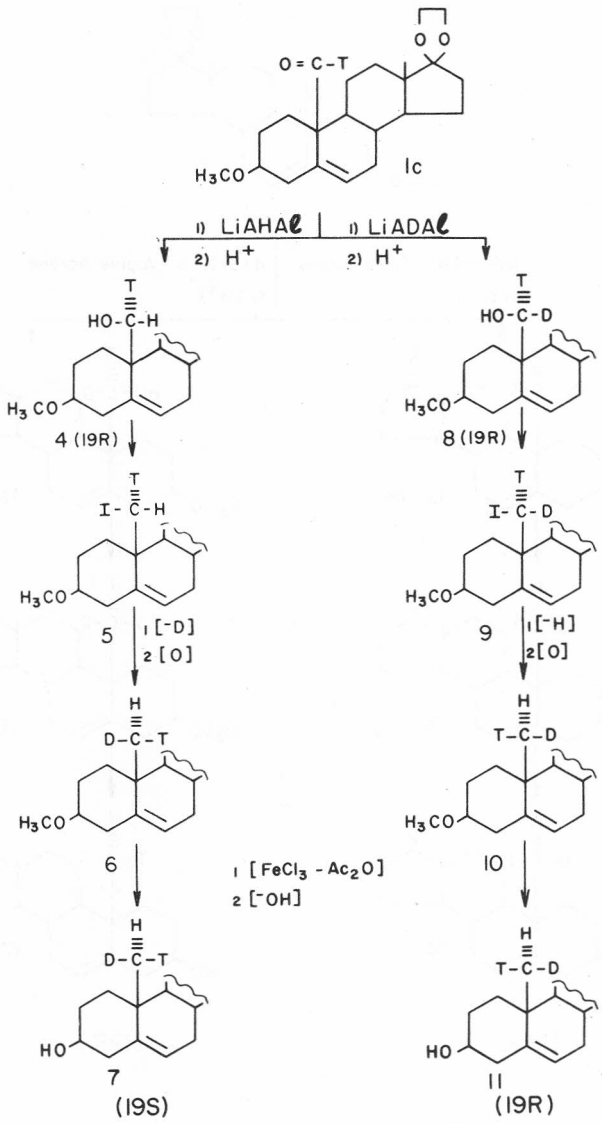
a. R = $[CH_2O]_2$; X = H
b. R = O ; X = Ac

-[19-^{3}H,^{1}H] (*4*) and (19R)[19-^{3}H,^{2}H]-(*8*) alcohols, respectively. The alcohols (*4*) and (*8*) were converted[4,5] to (19R) 19-iodides (*5*) and (*9*). The (19R)-[19-^{3}H,^{1}H]--iodide (*5*) was hydrogenolyzed with deuteriated Superhydride to give (*6*). Following oxidation of the 17-hydroxyl and cleavage of the 3-methyl ether, the required 3β-hydroxy (*7*) was obtained. Hydrogenolysis of (*9*) with protiated reagent gave (*10*), which was processed as above to yield (*11*).

The second synthesis (Scheme 2) also used [19-^{3}H]-aldehyde (*1c*) as starting material. Samples of the aldehyde were reduced with (R)-[^{2}H] and (S)-[^{2}H]--Alpine-boranes[6] to give (19S)-(12) and (19R)-[19-^{3}H,^{2}H] (15)-alcohols, respectively.[2] The alcohols were then converted to 19-iodides (*13*) and (*16*) and these were hydrogenolyzed with protiated Superhydride to give after processing (*14*) and (*17*), respectively.

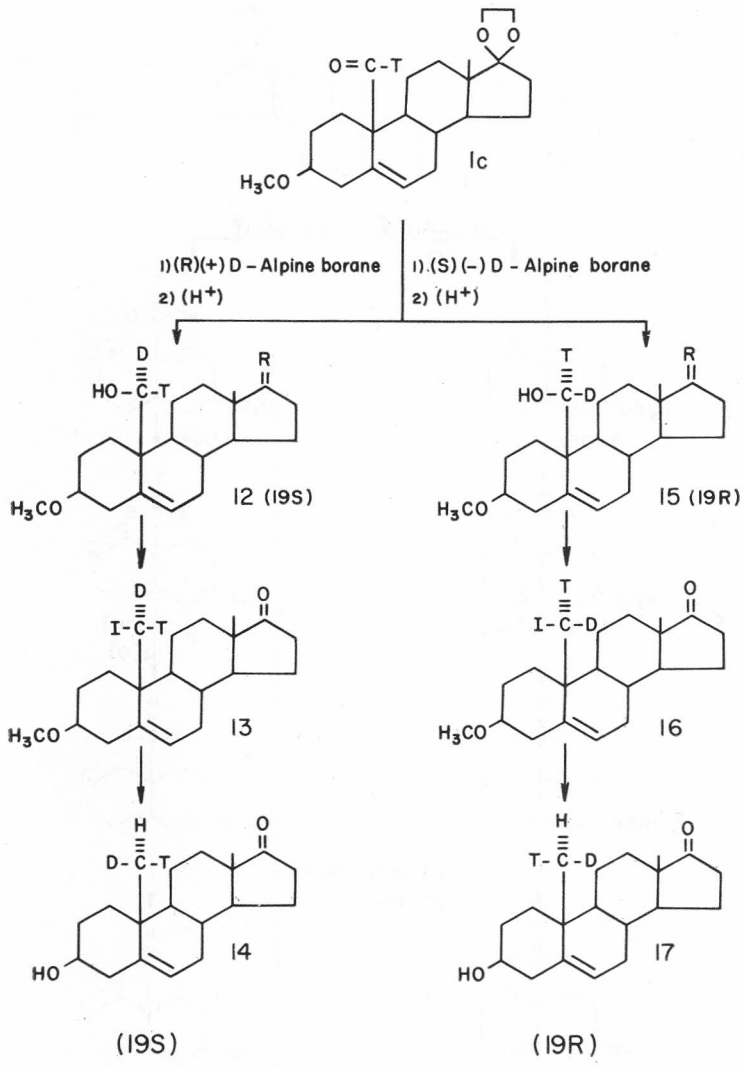
For the synthesis of (19RS) [19-^{3}H,^{2}H,^{1}H]-3β-hydroxy-androst-5-en-17-one, the (19)-tritiated-10β-formyl- (*1c*) was reduced with an equimolar mixture of (R) and (S)-Alpine boranes. The resulting (19RS) [19-^{3}H,^{1}H]-alcohol was then converted to the (19RS) [19-^{3}H,^{1}H]-iodide which in turn was hydrogenolyzed with deuteriated Superhydride. Following the conventional processing, the required (19RS) [^{3}H,^{2}H,^{1}H]-androgen was obtained.

SCHEME I



As can be seen, at this point, we have successfully completed the transformation of the [19-^{3}H]-10β-formyl substrate into androgens containing three isotopic hydrogens at C-19. However, we had no information whether the 10β-methyls of the four compounds (7), (*11*), (*14*) and (*17*) are chiral. It may be recalled that the chiralities and the diastereoisomeric purities of the 19-alcohols (4, 8, 12, 15) and iodides (5, 9, 13, 16) were confirmed by NMR. In contrast,

SCHEME 2



the course of hydrogenolysis of the iodides was totally obscure and no information was available whether the reaction proceeds with retention, inversion, or racemization. Of added concern was the hydrogenolysis of (5) with deuteriated superhydride. Should this reaction involve a significant deuterium isotope effect, it could enhance the possibility of racemization. Obviously the C-19 chiralities of the products had to be determined. The route we have chosen was to oxidize the chiral compounds by the Kuhn-Roth method and analyze the recovered samples of acetic acids derived from C-19(10) and

C-18(13) by the malate synthetase fumarase procedure.[7,8,9] Unfortunately, at this point we encountered major complications in the application of the Kuhn-Roth oxidation.

From the outset, we were concerned with the possibility of exchange of protons of the acetic acid generated in the course of the Kuhn-Roth (KR)--oxidation. To evaluate the reaction, model K.R oxidations of 3β-methoxy-androst-5-en-17-one were carried out in D_2O, under a variety of conditions (see Table I). At the termination of the reaction, phosphoric acid was added and the acetic acid was steam-distilled. The distillate was made alkaline (phenolphthaleine) and concentrated to a residue. The resulting sodium acetate was dried in vacuo, then acetonitrile, α-bromo-p-phenylacetophenone and dicyclohexano-18-crown-6-ether were added and the mixture was refluxed (30 min). The cooled reaction was filtered, the filtrate was concentrated to a residue and fractionated by TLC. The recovered ester $C_6H_5 \cdot C_6H_4 \cdot CO \cdot CH_2 \cdot O \cdot \cdot CO \cdot CH_3$ was crystallized and its proton NMR recorded. The CH_3 signal of the ester $C_6H_5 \cdot C_6H_4 \cdot CO \cdot CH_2 \cdot O \cdot CO \cdot CH_3$ was integrated and compared to the CH_2 signal which was used as reference (2H) (Table I). The results indicate that, irrespective of conditions employed, a large exchange of the methyl protons of the acetate occured, resulting in complete or significant loss of chirality.

TABLE I

Reaction Conditions*					$C_6H_5 \cdot C_6H_4—CO—CH_2—O—CO—CH_3$	
3β-methoxy androst-5-en--17-one (mg)	CrO_3 (g)	D_2O (mL)	Temp. °(C)	Time (h)	Integrated Me-Signal (atoms ^1H)	^1H-exchanged (%)
83	0.5	10	100—105	16	2.42	19.3
85	5.0	15	85—90	6	2.14	28.7
83	1.3	3.2	75—80	1.66	2.45	18.5
27.3	2.65	6.6	103	22	2.12	29.3

The CrO_3 was twice evaporated from (5 mL) D_2O.
* At room temperature; essentially no acetic acid was obtained.

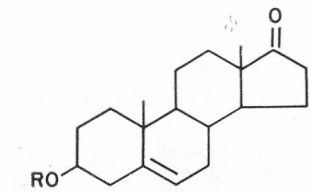
Under the circumstances, a different approach was explored. The Kuhn--Roth oxidation was to be carried out on an oxidatively modified steroid[10] whereby an oxygen atom will be inserted in the vicinity of C-10. It was anticipated that insertion of an oxygen atom in the vicinity of C-10 will facilitate the Kuhn-Roth oxidation of the steroid. This will then expedite the »release« of acetic acid and thus minimize the proton exchange. Two approaches were then evaluated. The 3-methylether (*18a*) was treated with $SeO_2—H_2O_2$ in *t*-butanol[10] to give the 5α,6β-diol (*19*). The diol was submitted to Jones' oxidation to give lactol (*20*) and acid (*21*) which were resolved via partitioning with aqueous $NaHCO_3$. The acid (*21*) was oxidized by the Kuhn-Roth procedure (entry 1, Table II).

TABLE II

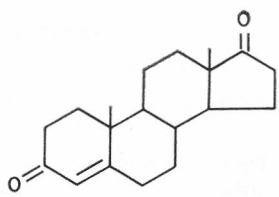
Compound	Reaction Conditions* (mg)	CrO_3 (g)	D_2O (mL)	Temp. (°C)	Time (h)	$C_6H_5 \cdot C_6H_4—CO—CH_2—O—CO—CH_3$ Integrated Me-Signal (atoms ^1H)	^1H-Exchanged (%)
21	20	2	6	90	2	2.8	7
24	190	1	4	100	24	2.3	23.3
24	100*	1	5	r. t.	60	2.85—3.0	~0—5
24	200*	4	10	r. t.	72	2.85—3.0	~0—5
24	150	2	6	50—60	45	2.1	33

* Reaction was carried out in a sealed vial.

The route which proved satisfactory and was finally adopted is as follows. The 3-methoxy ether (*18a*) was treated with $FeCl_3/Ac_2O/EtOAc$[11] and the resulting acetate (*18b*) was saponified. The obtained (*18c*) was oxidized (Jones' reagent) and isomerized (MeOH—HCl) to yield (*22*). The Δ^4-3-ketone (*22*) was treated with $SeO_2—H_2O_2$ in t-butanol[10] to give acid (*23*) which on workup



18 a. R = CH_3
b. R = Ac
c. R = H



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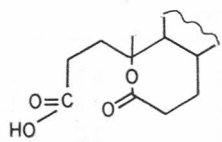

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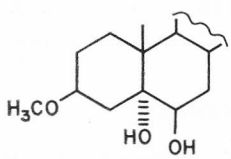

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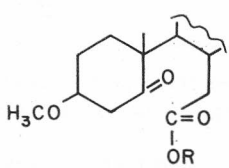

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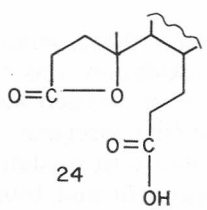

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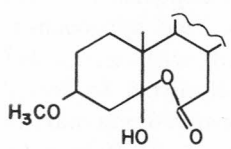

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rearranged to (*24*). The lactone-acid (*24*) was submitted to Kuhn-Roth oxidation (Table II). The results (Table II) indicate that oxidation of (*24*) at room temperature (60—72 h) proceeds with little exchange of the hydrogen atoms of the angular methyl group(s) of the steroid (Table II, entry 3,4). The results of experiments at r.t. were reproducible and usually between zero and up to 5% of the methyl hydrogen atoms of the steroid were exchanged.

We could now turn our attention to the question of chirality of the 10β-methyls of the synthesized products. The required malate synthetase was isolated from Baker yeast[9]. Fresh Baker yeast (454g) was suspended in 330 mL of standard buffer (0.005M Tris HCl, 0.001M $MgCl_2$; pH 8.0 at 0 °C). The cells were disrupted in a French Press at 20000 psi. The obtained mixture was centrifuged (8500 rpm; 1h) and the supernatant was collected (~300 mL). Following the addition of ca. 15 mL of 1M $MgCl_2$ and the adjustment of pH to 8.1 with 2M Tris base, the mixture was immersed in a water bath (50 °C) for (30 min) (no stirring) and then cooled to 5—10 °C in an ice bath. The cooled liquid was centrifuged (8500 rpm; 1h) and the supernatant collected (ca. 250 mL). Enzyme grade $(NH_4)_2SO_4$ (90g) was slowly added (40 min) to the magnetically stirred and cooled (in an ice bath) supernatant. The cooling and stirring was continued for an additional 1.5 h, then the preparation was centrifuged (8500 rpm; 30 min). The supernatant was decanted, and the pellet was suspended in ice cold standard buffer (80 mL). The mixture was dialyzed against standard buffer (2L) for 17 h. The dialyzed solution (136 mL) was centrifuged (15000 rpm; 1h). The supernatant (130 mL) was pumped onto a DEAE cellulose column (2.5 cm × 15 cm) at the rate of (1 mL per min) in the cold room. The column was eluted (1.2 mL per min) with standard buffer and fractions (12 mL per ~10 min) were collected. Fractions 1, 2 and 3 contained malate synthetase and were devoid of fumarase. The eluates 1, 2 and 3 were combined, then Cγ-gel (10 mL) was added with stirring. After stirring (5 min), the mixture was stored at 5 °C (15 min), then centrifuged (15000 rpm; 5 min). The supernatant was discarded and the pellet was suspended in standard buffer (10 mL), spinned again and the supernatant was removed.

The pellet was suspended in 10 mL of 0.05M sodium phosphate buffer (pH 7.8) containing 0.01M $MgCl_2$ and refrigerated (10 min) with occasional swirling. The suspension was centrifuged (15000 rpm; 5 min) and the supernatant saved. The operation was repeated twice more each time using 10 mL of the buffer. The combined supernatant was then concentrated in a Diaflo chamber (60 mL capacity) equipped with a PM-10 filter under 50 psi pressure of N_2 at 4 °C. To the concentrate (10 mL), dithiothreitol (DTT) (1.6 mg) was added and aliquots of the mixture (2 mL) were placed in vials. The vials were sealed and kept frozen until used.

Two preparations of malate synthetase were carried out:

Analysis of Malate Synthetase

Malate Synthetase Activity:	41.7 iu/mL	15 iu/mL
Protein Concentration:	49.02 mg/mL	38.5 mg/mL
Sp. Activity:	0.85 iu/mg	0.33 iu/mg

No fumerase activity was detected

Determination of the C-19-chirality of Steroids
Determination of Chirality

The samples of [19-^{3}H,^{2}H,^{1}H]-androgens were first pre-oxidized as described above (18 → 22 → 23 → 24) then submitted to Kuhn-Roth oxidation. The obtained tritiated sodium acetate was mixed with [2-^{14}C]-sodium acetate to give [^{3}H:^{14}C]-acetate with an isotope ratio of about (4 : 1).

To the [^{3}H:^{14}C]-sodium acetate, the following were added sequentially:

Malate production buffer*	5.65 mL
Disodium ATP	50.00 mg
Phosphotransacetylase	0.50 mg (100 i.u.)
Acetate kinase	0.025 mL (25 i.u.)
Malate synthetase	1.00 mL (15 i.u.)
Water to adjust the volume to	10 mL

The reaction was initiated by addition of coenzyme A (7.7 mg) and the incubation was carried out for 18h at room temperature.

The reaction was terminated with 2N HCl [0.5 mL] (release CO_2) and the mixture was applied to a column of Dowex 50W × 8 (2.5 × 6 cm (200—400 mesh; H^+-form). The column was eluted with water (170 mL) at a rate of (1 mL per min). To remove excess glyoxalate, 1 mL of a solution of 10 mM 2,4-dinitrophenylhydrazine in 2N HCl was added to the eluate and the mixture was stored (30 min) at room temperature. The solution was then pumped (1 mL per min) onto a column of Dowex 4G — 1 × 8 (1.7 × 12 cm) (200—400 mesh; formate form) in the cold room. The column was first washed with water (140 mL) and then eluted with a gradient of formic acid 1N formic acid (300 mL) and 3N formic acid (300 mL) The column was eluted at the rate of 12 mL per 10 min. A total of 30 fractions (12 mL each) were collected and aliquots (200 μl) of the fractions were counted. Fractions (12—16) which contained malate were combined and taken to dryness. The residue was taken up in water (5 mL) and again taken to dryness. The operation was repeated once more.

Incubation with Fumerase

The obtained (dry) malate was dissolved in 0.05M phosphate buffer (pH 7.4; 3 mL). An aliquot (1 mL) of the solution was incubated with fumarase (10 μl) (3h at room temperature). The reaction mixture was then frozen, lyophilized and the »distillate« collected. The residue was dissolved in water (0.5 mL) and again lyophilized. The residue of lyophilization was dissolved in water (1 mL).

Aliquots (100 μl) of the initial malate, malate recovered from incubation with fumarase and water from first lyophilization, were counted. As reference (RS)[^{3}H;2-^{14}C]-acetic acid and (S)-[^{3}H;2-^{14}C]-acetic acid were used.

The results are summarized in Table III.

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* Sodium glyoxalate (70 mg), EDTA-sodium salt (132 mg), $MgCl_2 \cdot 6H_2O$ (360 mg) and $NaHCO_3$ (2.97 g) were dissolved in water (150 ml) and pH adjusted to 9.7 (conc. NaOH) water was then added to make up to (200 mL).

TABLE III

Chiral Analysis of Acetic Acid Derived From 19-Chiral Methyl Androgens

Entry	Origin of CHDTCOONa	$^3H/^{14}C$ of malate		F-value*	Chirality
		Initial	Equilibrated with fumarase		
1	(RS) acetate	5.20	2.57	49	Racemic (RS)
2	'S'-acetic acid (authentic)	1.45	0.42	29	'S'
3	(19RS)[19-^{3}H,^{2}H,^{1}H]18a	3.73	1.88	50	Racemic (RS)
4	(19RS)[19-^{3}H,^{2}H,^{1}H]18a**	6.30	3.08	49	Racemic (RS)
5	7	4.15	1.36	33	'S'
6	11	3.11	1.96	63	'R'
7	14	3.85	1.31	34	'S'
8	17	4.45	2.88	65	'R'

* $F = \frac{T/^{14}C \text{ (equlibrated malate)}}{T/^{14}C \text{ (initial malate)}} \times 100$

** Prepared via reduction with (RS)-Alpine-borane

It is apparent that hydrogenolyses of the 19-iodides proceeded stereospecifically with inversion.

Acknowledgment. — This work was supported by a National Institutes of Health grant HD 14906. We thank Prof. Heinz G. Floss for kindly determining the chirality of the (RS)-acetate derived from the (19RS)-sample prepared via reduction with an equimoler mixture of (R) and (S)-Alpine borane.

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IZVOD

Određivanje hiraliteta kod (19R)-, (19S)- i (19RS)-[19-^{3}H,^{2}H,^{1}H]-3β-hidroxiandrost-5-en-17-ona

Eliahu Caspi, Chiu Chin Chang, Karl H. Busch i Thangavel Arunachalam

Sintetizovani (19R)-, (19S)- i (19RS)-[19-^{3}H,^{2}H,^{1}H]-3β-hidroksiandrost-5-en-17-oni pretvoreni su u odgovarajuće 4-en-3-one, koji su tada oksidovani pomoću selen-dioksida i vodonik-peroksida. Postali laktoni seko-kiselina oksidovani su Kuhn-Roth-ovom metodom dajući uzorke sirćetne kiseline. Hiralitet acetata analiziran je postupkom pomoću metil-sintetaze i fumaraze.