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THE REACTION OF 21-HYDROXYPREGNENOLONE AND Δ^5-PREGNENE-3β,17α,21-TRIOL-20-ONE WITH 2,4-DINITROPHENYLHYDRAZINE[1]

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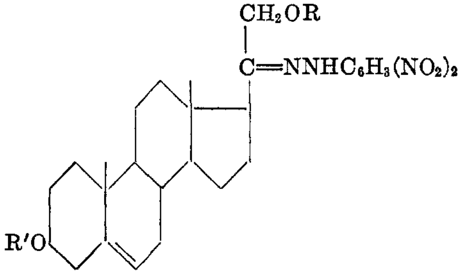
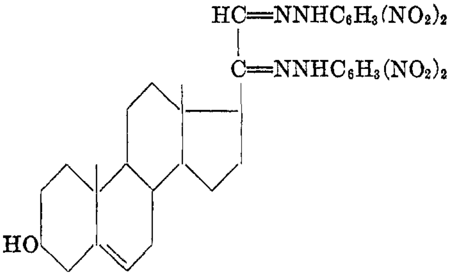
The reaction of steroid ketones with 2,4-dinitrophenylhydrazine has recently been discussed (1). In the present work this reaction is extended to two steroids having a ketol side chain.

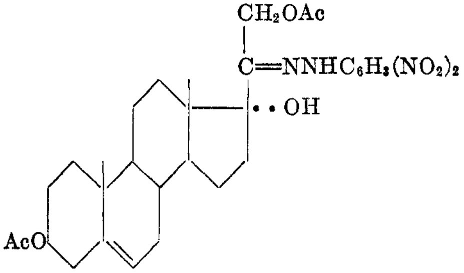
In alcoholic solution containing a small amount of conc'd hydrochloric acid, 21-hydroxypregnenolone rapidly consumed one mole of dinitrophenylhydrazine to give the dinitrophenylhydrazone I which was characterized by its diacetate III. On prolonged standing at room temperature, however, the osazone IV was obtained as a brown precipitate. This compound also resulted, when the reaction mixture was refluxed for two hours. Since only 2.27 moles of dinitrophenylhydrazine instead of 3 moles were utilized, it was apparent that the osazone formation was not quantitative.



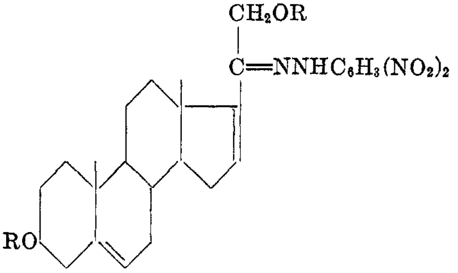
I R = R' = H
II R = $COCH_3$, R' = H
III R = R' = $COCH_3$



IV



V



VI R = H
VII R = $COCH_3$

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21-Acetoxypregnenolone was found to behave like the free ketol, *i.e.*, the corresponding dinitrophenylhydrazone II was isolated in almost quantitative yield. Under the conditions employed by us the 21-acetoxy group was hydrolyzed only to a slight extent (*cf.* 2). The formation of II proved that the keto group in 21-acetoxypregnenolone is not so unreactive as is generally assumed (3, 4). Accordingly, the semicarbazone could be prepared with semicarbazide acetate or hydrochloride in the presence of pyridine.

It is noteworthy that the infrared spectrum of the diacetate III showed two bands in the ketone region, one at 5.77 μ (3-acetoxy group), the other at 5.68 μ (21-acetoxy group). The latter is in the same position as the band characteristic for the acetoxy group in 20,21-ketol acetates (5).

The reaction of Δ^5-pregnene-3β,17α,21-triol-20-one with dinitrophenylhydrazine in alcoholic hydrochloric acid proceeded with the consumption of one mole of reagent and resulted in a mixture of dinitrophenylhydrazones which could be separated only partly by chromatography. One of them was red and showed a maximum at 387 mμ indicating that it was α,β-unsaturated. Obviously dehydration had occurred with the formation of VI. The orange acetate VII exhibited a maximum at 381 mμ. A second, yellow dinitrophenylhydrazone gave an acetate with an absorption maximum at 357 mμ which would be expected from compound V containing the 17-hydroxyl group.

The formation of the unsaturated dinitrophenylhydrazone VI is surprising, since neither allopregnane-3β,17α-diol-20-one nor 17α-hydroxyprogesterone undergo dehydration, when reacted with dinitrophenylhydrazine at room temperature. At elevated temperatures, however, the former gives a quantitative yield of Δ^{16}-pregnene-3β-ol-20-one dinitrophenylhydrazone, the latter a mixture of the bisdinitrophenylhydrazones of $\Delta^{4,16}$-pregnadiene-3,20-dione (1) and 17α-hydroxyprogesterone (1).

In a recent publication by Gornall and Macdonald (6) a colorimetric method for the quantiative determination of corticosteroids has been described. It involves reaction with dinitrophenylhydrazine and development of a red-brown chromogen on subsequent treatment with alkali. We have used the first step of this method, under slightly varied conditions, for the preparation of dinitrophenylhydrazones and have found that none of the steroid ketones investigated reacted quantitatively. The amount of dinitrophenylhydrazine utilized by a single keto group ranged from 0.45 to 0.85 mole. In the case of allopregnane-3β,17α-diol-20-one, dinitrophenylhydrazone formation was accompanied by dehydration as described above. 21-Acetoxypregnenolone gave a mixture of dinitrophenylhydrazone I and osazone IV. Surprisingly, the osazone IV was also obtained in good yield from Δ^5-pregnene-3β,17α,21-triol-20-one diacetate. It is suggested that either the saturated dinitrophenylhydrazone, corresponding to V, or the unsaturated dinitrophenylhydrazone VI or both are primary reaction products. Their further conversion to the osazone IV would bear analogy to the formation of 21,21-dimethoxypregnane-3α-ol-11,20-dione from pregnane-3α,17α,21-triol-11,20-dione and Δ^{16}-pregnene-3α,21-diol-11,20-dione (7). Thus only two moles of dinitrophenylhydrazine would be required to produce the osazone IV.

EXPERIMENTAL[3]

All *dinitrophenylhydrazones*, if not mentioned otherwise, were prepared and purified according to "Method A 2" described previously (1).

21-Hydroxypregnenolone dinitrophenylhydrazone (I). A suspension of 50 mg. of 21-hydroxypregnenolone in ethanol and 10 drops of conc'd hydrochloric acid was filled up to 5 cc. and allowed to stand overnight. Then 3 cc. of the clear solution was added to a solution of 29.0 mg. of dinitrophenylhydrazine (1.82 moles) in 4.5 cc. of abs. ethanol and 7 drops of conc'd hydrochloric acid. After 24 hours, 1.07 moles of the reagent had been utilized. The crude reaction product was chromatographed and yielded, in addition to a small amount of osazone IV (eluted with benzene-chloroform 7:3) (see below), the yellow dinitrophenylhydrazone I (eluted with chloroform and chloroform-ethanol 9:1). The latter, after recrystallization from ethanol, showed the double m.p. 243–246° and 248–250°; maximum at 372 mμ (ϵ 24,855).

Anal. Calc'd for $C_{27}H_{36}N_4O_6$: N, 10.93. Found: N, 10.82.

When the reaction was carried out in chloroform-acetic acid ("Method B 1")(1), only 0.14 mole of reagent was utilized.

The *diacetate III* was prepared in the usual manner, chromatographed, eluted with benzene-chloroform 9:1, and recrystallized from benzene-hexane and chloroform-ethanol; double m.p. 142° and 165–166°; maximum at 364 mμ; infrared bands at 5.68 and 5.77 μ (chloroform).

Anal. Calc'd for $C_{31}H_{40}N_4O_8$: N, 9.39. Found: N, 9.52.

21-Acetoxypregnenolone dinitrophenylhydrazone (II). This compound was prepared by the addition of 36.1 mg. of 21-acetoxypregnenolone to a solution of 29.1 mg. of dinitrophenylhydrazine (1.52 moles) in 3.5 cc. of abs. ethanol and 7 drops of conc'd hydrochloric acid. The substance dissolved readily and yellow crystals precipitated after 10 minutes. The mixture was allowed to stand overnight (reagent utilized 1.04 moles) and was worked up in the usual manner. Chromatography and elution with benzene-chloroform 7:3 gave a 94% yield of the dinitrophenylhydrazone II which melted, after two recrystallizations from chloroform-ethanol, at 202–203°; maximum at 365 mμ.

Anal. Calc'd for $C_{29}H_{38}N_4O_7$: N, 10.10. Found: N, 10.24.

In chloroform-acetic acid 6:1 at room temperature or in a boiling mixture of abs. ethanol-acetic acid 6:1, 21-acetoxypregnenolone reacted only with 0.06 and 0.08 mole of dinitrophenylhydrazine respectively.

Δ^5-Pregnene-3β-ol-20-one-21-al bisdinitrophenylhydrazone (IV). A mixture of 10.0 mg. of 21-acetoxypregnenolone, 16.7 mg. of dinitrophenylhydrazine (3.15 moles), 3 cc. of abs. ethanol, and 6 drops of conc'd hydrochloric acid was refluxed for 2 hours. A brown precipitate appeared which proved to be the osazone IV. The reagent utilized amounted to 2.27 moles. Chromatography of the crude reaction product gave some dinitroaniline (eluted with benzene; m.p. 179–182°; maximum at 330 mμ) and the osazone IV (eluted with benzene-chloroform 3:2). After two recrystallizations from chloroform-ethanol, the latter melted at 280–282° (dec.); maxima at 350, 398, and 445 mμ.

Anal. Calc'd for $C_{33}H_{38}N_8O_9$: N, 16.23. Found: N, 15.85.

The same product was obtained, when the reaction was carried out at room temperature for 4 days. The reagent utilized amounted to 1.97 moles; m.p. 279–281° (dec.).

Anal. Calc'd for $C_{33}H_{38}N_8O_9$: N, 16.23. Found: N, 16.06.

A suspension of 21-acetoxypregnenolone dinitrophenylhydrazone (II) in abs. ethanol

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[3] All melting points were taken on a Kofler micro hot stage and are corrected. The ultraviolet spectra of the dinitrophenylhydrazones were taken in chloroform on a Beckman spectrophotometer Model DU, the infrared spectrum on a Perkin-Elmer spectrophotometer Model 21. The microanalyses were carried out by Huffman Microanalytical Laboratories, Wheatridge, Colo., one by Drs. Weiler & Strauss, Oxford, England. The aluminum oxide used in all experiments was acid-washed and kindly supplied by Dr. H. B. MacPhillamy, Ciba Pharmaceutical Products, Summit, N. J.

and a few drops of conc'd hydrochloric acid was allowed to stand at room temperature for 3 weeks. The precipitate, after filtration and washing with ethanol, melted at 280° (dec.) and was identical with the osazone IV described above.

21-Acetoxypregnenolone semicarbazone. This derivative was prepared according to the method of Dutcher and Wintersteiner (8) and was purified by chromatography (eluted with chloroform-ethanol 1:1). After recrystallization from dil. ethanol, it melted at 224–227°; maximum at 236 mμ (ethanol).

Anal. Calc'd for $C_{24}H_{37}N_3O_4$: N, 9.74. Found: N, 9.64.

This semicarbazone was also prepared with semicarbazide hydrochloride and pyridine according to the method of Hopper (9).

Reaction of Δ^5-pregnene-3β,17α,21-triol-20-one with dinitrophenylhydrazine. To a solution of 20.0 mg. of dinitrophenylhydrazine in 3 cc. of abs. ethanol and 6 drops of conc'd hydrochloric acid, 18.7 mg. of Δ^5-pregnene-3β,17α,21-triol-20-one[4] was added. The steroid dissolved immediately, and on standing overnight a few red crystals precipitated. More of this material was obtained during extraction with chloroform, since it was only slightly soluble in this solvent. The crystals melted at 235–240° and showed a maximum at 386 mμ (probably VI). The dinitrophenylhydrazine utilized amounted to 1.0 mole. Chromatography of the chloroform-soluble portion gave a small amount of osazone IV and fractions which could not be crystallized satifactorily. Those eluted with chloroform (maximum at 387 mμ) and chloroform-ethanol 9:1 were acetylated separately. The first acetate, after chromatography (eluted with benzene-chloroform 9:1) and crystallization from chloroform-ethanol, formed orange feathery needles of m.p. 220–225°; maximum at 381 mμ (VII).

Anal. Calc'd for $C_{31}H_{38}N_4O_8$: N, 9.42. Found: N, 9.15.

Chromatography of the second acetate proved that it contained a small amount of VII. The main product (eluted with benzene-chloroform 7:3 to 1:1) crystallized from dilute ethanol only with difficulty. The yellow prisms melted at 183–184°; maximum at 357 mμ. This compound is probably the dinitrophenylhydrazone V.

Anal. Calc'd for $C_{31}H_{40}N_4O_9$: N, 9.15. Found: N, 8.92.

Δ^{16}-Allopregnene-3β-ol-20-one acetate dinitrophenylhydrazone. A mixture of 17.2 mg. of allopregnane-3β,17α-diol-20-one 3-acetate,[5] 26.0 mg. of dinitrophenylhydrazine, 7 cc. of abs. ethanol, and 6 drops of conc'd hydrochloric acid was refluxed for 2 hours. The dinitrophenylhydrazone precipitated as an orange gel which could not be crystallized. The acetate was chromatographed (eluted with benzene) and recrystallized twice from chloroform-ethanol; light orange felty needles with double m.p. *ca.* 150° and 235–237°; maximum at 386 mμ.

Anal. Calc'd for $C_{29}H_{38}N_4O_6$: N, 10.40. Found: N, 10.50.

$\Delta^{4,16}$-Pregnadiene-3,20-dione bisdinitrophenylhydrazone. The conditions for preparation of this dinitrophenylhydrazone from 17α-hydroxyprogesterone were the same as those in the previous experiment. The reaction product proved to be a mixture of 17α-hydroxyprogesterone bisdinitrophenylhydrazone (m.p. 280–283°; maximum at 376 mμ)(1) and $\Delta^{4,16}$-pregnadiene-3,20-dione bisdinitrophenylhydrazone (m.p. *ca.* 185°)(1).

Pregnane-3α-ol-11,20-dione 20-monodinitrophenylhydrazone. This dinitrophenylhydraone[6] was prepared in the usual manner (hydrochloric acid, room temperature). The reagent utilized amounted to 1.08 moles. After recrystallization from chloroform-ethanol, the yellow leaflets melted at 237.5–240°; maximum at 365 mμ.

Anal. Calc'd for $C_{27}H_{36}N_4O_6$: N, 10.93. Found: N, 10.90.

The same dinitrophenylhydrazone was obtained, when the reaction mixture was refluxed

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[4] Prepared by saponification of the diacetate according to Heer and Miescher, *Helv. Chim. Acta*, **34**, 359 (1951); m.p. after recrystallization from benzene 223–227°.

[5] This material was obtained through the courtesy of Dr. M. Tishler, Merck & Co., Rahway, N. J.

[6] The starting material was kindly supplied by Dr. H. L. Mason, Mayo Clinic, Rochester, Minn.

TABLE I

DINITROPHENYLHYDRAZONES PREPARED IN METHANOL-HCl AT 60°

STEROID KETONE	DNPH[a] REACTED WITH STEROID	REACTION PRODUCTS
Testosterone	0.85 mole	Testosterone DNP[b]
Pregnenolone	.47 mole	Pregnenolone DNP[b]
Allopregnane-3β,17α-diol-20-one 3-acetate	.68 mole	Δ¹⁶-Allopregnene-3β-ol-20-one DNP[b]
Pregnane-3α-ol-11,20-dione	.45 mole	Pregnane-3α-ol-11,20-dione 20-mono DNP[b]
21-Acetoxypregnenolone	1.04 moles	Osazone IV and dinitrophenylhydrazone I
Δ⁵-Pregnene-3β,17α,21-triol-20-one diacetate	1.60 moles	Osazone IV

[a] DNPH = dinitrophenylhydrazine. [b] DNP = dinitrophenylhydrazone.

for 1 hour. The reagent utilized amounted to 1.02 moles indicating that the 11-keto group does not react (*cf.* 6).

Preparation of dinitrophenylhydrazones according to a modification of Gornall's method (*6*). An example is given for testosterone: a solution of 20.1 mg. of testosterone in 28 cc. of methanol was mixed with a solution of 28 mg. of dinitrophenylhydrazine in 28 cc. of methanol-conc'd hydrochloric acid 3:1 and heated for 110 minutes to 60°. After addition of 8 gm. of potassium acetate and 4 drops of acetone, the mixture was allowed to stand for several hours, diluted with 120 cc. of water, and extracted with chloroform. Chromatography of the crude product gave the dinitrophenylhydrazones of acetone and testosterone.

The dinitrophenylhydrazone obtained from allopregnane-3β,17α-diol-20-one 3-acetate crystallized in orange needles of m.p. 250.5–253° and showed a maximum at 385 mμ indicating that dehydration had occurred. On recrystallization only a gelatinous precipitate was obtained.

21-Acetoxypregnenolone gave a mixture of osazone IV and dinitrophenylhydrazone I which could readily be separated. When Δ^5-pregnene-3β,17α,21-triol-20-one diacetate was submitted to the reaction described above, the osazone IV precipitated in 55% yield. After washing with methanol, it melted at 280–281° and after recrystallization from chloroform-ethanol at 286–288°.

Anal. Calc'd for $C_{33}H_{38}N_8O_9$: N, 16.23. Found: N, 15.74.

The filtrate contained more osazone IV in addition to other dinitrophenylhydrazones which could not be separated by chromatography.

The results of these and similar experiments are summarized in Table I.

SUMMARY

The reaction of 21-hydroxy- and 21-acetoxy-pregnenolone with 2,4-dinitrophenylhydrazine under varying conditions proceeds either with formation of the normal dinitrophenylhydrazones or of Δ^5-pregnene-3β-ol-20-one-21-al bis-dinitrophenylhydrazone. The latter can also be obtained from Δ^5-pregnene-3β,17α,21-triol-20-one, when the reaction is carried out at 60°. At room temperature $\Delta^{5,16}$-pregnadiene-3β,21-diol-20-one dinitrophenylhydrazone is formed which has been characterized as its diacetate.

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