

## Resolution of steroid 2,4-dinitrophenylhydrazone isomers by thin-layer chromatography\*

Geometrical isomerism of 2,4-dinitrophenylhydrazones of various ketones and aldehydes has been known for many years. Such isomerism of steroid derivatives has apparently not been encountered, although the possibility has been suggested<sup>1</sup>. Recently, in the separation of dinitrophenylhydrazones of steroids of biological origin multiple spots were detected on the thin-layer chromatograms of supposedly pure steroid derivatives. The finding prompted an investigation of these reaction products of steroid ketones with dinitrophenylhydrazine. Progesterone was selected initially as a "model" compound.

### *Experimental*

The progesterone used was re-crystallized several times from *n*-hexane and methanol-water, and had a constant melting point of 128–130° (uncorr.). It was shown to be chromatographically pure in the paper partition systems cyclohexane-propylene glycol, cyclohexane-90% acetic acid and cyclohexane-85% methanol, and in the thin-layer systems methanol-methylene chloride (1:99) and cyclohexane-ethyl acetate (1:3).

2,4-Dinitrophenylhydrazone derivatives were prepared according to the general method of REICH<sup>2</sup> using well established conditions. The dinitrophenylhydrazine was freshly recrystallized as the hydrochloride prepared from Eastman reagent grade material by recrystallization from ethanol-conc. HCl (5:1) and ethanol-conc. HCl (19:1).

The products, 4-pregnene-3,20-dione-3-dinitrophenylhydrazone (progesterone-mono-DNPH) and 4-pregnene-3,20-dione-3,20-bis-dinitrophenylhydrazone (progesterone-bis-DNPH) were freed from contaminants by chromatography on neutral alumina columns<sup>2</sup> and silica gel thin-layer plates, and recrystallized from absolute ethanol and benzene-methanol. Progesterone-mono-DNPH showed a  $\lambda_{\max}$  in chloroform at 388 m $\mu$  (*E* 20,100) while progesterone-bis-DNPH showed a  $\lambda_{\max}$  at 380 m $\mu$  (*E* 49,050). These compounds had melting points of 215–216.5° and 284–286° (uncorr.), respectively, agreeing with literature values<sup>3</sup>.

These steroid derivatives were chromatographed in a wide variety of thin-layer solvent systems on Silica Gel G and alumina plates to demonstrate geometrical isomerism (Table I).

Whereas most thin-layer systems showed only one symmetrical spot for each compound, certain systems resolved these areas into two closely running zones, of which the less polar predominated in all cases. Notable among these systems were benzene-chloroform and benzene-methylene chloride. Where separation of two isomers occurred, the less polar was orange and the more polar yellow. Upon elution, the difference in colour vanished. Spraying the plates with 0.01 *N* NaOH in 80% aqueous methanol showed no difference in colour between the two zones, in contrast to findings with keto-acid DNPH derivatives<sup>4</sup>. Cleavage of residues of chloroform extracts of these areas with hydrochloric acid-acetone<sup>5</sup> gave good yields of progesterone.

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TABLE I

*R<sub>F</sub>* VALUES OF STEROID DERIVATIVES IN VARIOUS TLC SYSTEMS (SILICA GEL)

<i>System</i>	<i>Progesterone- mono-DNPH</i>	<i>Progesterone- bis-DNPH</i>	<i>Pregnenolone- 20-DNPH</i>
Acetone-benzene (1:4)	0.72	0.75	0.48
Acetone-benzene (1:19)	0.56	0.70	0.18
Chloroform-benzene (3:1)	0.15, 0.25	0.29, 0.40	0.07
Acetone-chloroform-benzene (1:5:5)	0.58	0.68	0.26
Ether-chloroform (3:1)	0.72	0.80	0.50
Chloroform	0.36	0.53	0.09
Methylene chloride	0.42, 0.45	0.67	0.13
Methanol-chloroform (1:199)	0.55	0.69	0.22
Benzene-acetic acid (9:1)	0.52	0.67	0.37
Benzene-acetic acid (19:1)	0.42	0.57, 0.61	0.22
Chloroform-acetic acid (98:2) *	0.49	0.67	0.17
Chloroform-pyridine (98:2) *	0.40, 0.43	0.64	0.20
Benzene-ethyl acetate (1:2)	0.70	0.82	0.40

\* These systems showed two solvent fronts due to breakdown of the mobile phase.

rone as the only detectable ketone in all cases. While the different forms of the dinitrophenylhydrazones were fairly stable in the dry state, standing for several days in chloroform solution resulted in rearrangement of the polar forms to the less polar forms, demonstrated by thin-layer chromatography in benzene-chloroform (1:3). No such rearrangement occurred with the less polar isomers, indicating ultimate equilibration to the less polar forms. This is in contrast with findings with keto-acid derivatives, where each isomer may be resolved into the two forms<sup>4,6</sup>.

Ultraviolet spectroscopy in chloroform showed identical spectra for the progesterone-mono-DNPH isomers ( $\lambda_{\max}$  388–390  $m\mu$ ) and for the progesterone-bis-DNPH isomers ( $\lambda_{\max}$  380  $m\mu$ ). Infrared spectra, while essentially similar, did show differences for the isomeric forms, noticeably in the 1500–1535  $cm^{-1}$ , 1300–1310  $cm^{-1}$ , and fingerprint regions for progesterone-bis-DNPH and the 1600–1630  $cm^{-1}$ , 1500–1540  $cm^{-1}$ , 1420–1450  $cm^{-1}$ , and fingerprint regions for progesterone-mono-DNPH.

The rapid rearrangement of the more polar isomer during attempts to recrystallize it for melting point determinations did not permit the use of standard techniques. Samples of each isomer of progesterone-bis-DNPH were prepared by allowing drops of concentrated chloroform eluates of freshly chromatographed material to evaporate spontaneously on cover slips.

Material which appeared crystalline under the microscope was carefully heated on a hot stage and the melting points observed. The more polar isomer had a melting point of 274.5–276° (uncorr.) while the less polar form had a m.p. of 282–283.5 (uncorr.). The latter melting point agrees with that of material crystallized from absolute ethanol, and shows that the crystals were of material completely rearranged to the less polar form.

### Discussion

The lack of further resolution of the progesterone-bis-DNPH, as well as the failure to resolve 5-pregnen-3 $\beta$ -ol-20-one-20-DNPH (Table I) would indicate that for progesterone the 3-keto derivative only is capable of isomerism. The data of

GÖNDÖS *et al.*<sup>7</sup>, showing isomerism of 17-ketosteroid-17-oximes, indicate that the apparent non-existence of C-20 isomerism may be a peculiarity of the 20-ketone, possibly due to the steric hindrance exerted by the steroid nucleus. This is supported by the resolution of 17 $\beta$ -acetoxy-4-androsten-17 $\beta$ -ol-3-one-3-DNPH, prepared by acetylating the testosterone-DNPH, into two isomers with  $R_F$  values of 0.28 and 0.33 in the system benzene-chloroform (1:1). Further work is being done in this area to elucidate this, among other questions.

This demonstration of isomerism in the C<sub>21</sub> steroid-DNPH derivatives indicates the need for caution in interpreting chromatograms of such derivatives to avoid mistaking the isomeric forms of the derivative of one compound from biological samples for two dissimilar derivatives. As the application of this reaction for steroidal identification is widespread, involving steroids from such diverse sources as human pregnancy blood<sup>8</sup> and botanical extracts<sup>9</sup>, such a need to distinguish between isomeric forms of the desired steroid derivative and products of unknown ketones is apparent.

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