The Transformation and Resolution of 8-Aza Steroids by Fungi

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(Received 5 March 1965)

1. When ($\pm$)-8-aza-D-homo-oestrone 3-methyl ether was added to a culture of *Aspergillus ochraceus*, the laevo-rotatory enantiomer is reduced to a 17A alcohol. Thus ($\pm$)-8-aza-D-homo-oestrone 3-methyl ether has been resolved into both its components, the laevo-rotatory enantiomer being obtained by chromic acid oxidation of the alcohol, and the dextro-rotatory enantiomer as residual precursor from the fermentation. 2. Fungi have been found to demethylate the 3-methyl ethers of 8-aza steroids to the corresponding phenols. In particular, 8-aza-D-homo-oestrone 3-methyl ether was demethylated by *Aspergillus flavus* and 8-aza-D-homo-oestradiol 3-methyl ether by *Cunninghamella blakesleeana*.

The preparation of 8-aza steroids has been described by Clarkson (1965), who obtained ($\pm$)-8-aza-D-homo-oestrone 3-methyl ether (I), which he reduced with sodium borohydride to ($\pm$)-8-aza-D-homo-oestradiol 3-methyl ether (II) (see Scheme 1). This reaction is stereospecific, giving only the racemate with the β -hydroxyl group. Transformation of the oestrone and the oestradiol has been attempted with a large number of fungi, chosen mainly for their ability to 11-hydroxylate carbocyclic steroids.

As yet no unequivocal 11-hydroxylation of these compounds has been achieved, but two other reactions were observed.

8-Aza-D-homo-oestrone 3-methyl ether was reduced by *Aspergillus ochraceus* to a mixture of two 17A epimeric alcohols (see Scheme 1). The minor component of this mixture had the same R_F values on thin-layer chromatograms as the sodium borohydride reduction product (II), but the major product (V) was distinguished by differences of R_F and slight differences of infrared spectrum. As the hydroxyl group of the former has the β -configuration, that of the latter must be α . The new alcohol was optically active, having $[\alpha]_D^{21} -90^\circ$, and was oxidized by chromium trioxide to ($-$)-8-aza-D-homo-oestrone 3-methyl ether, $[\alpha]_D^{18} -86^\circ$. From the culture filtrates, residual unchanged precursor

Scheme 1.

was isolated with $[\alpha]_D^{25} + 94^\circ$. Thus $(\pm)$ -8-aza-D-homo-oestrone 3-methyl ether has been resolved into both its components, having infrared spectra in solution identical with each other and with that of the racemic form.

Fungi have been found to demethylate the 3-methyl ethers to the corresponding phenols (see Scheme 1); in particular, 8-aza-D-homo-oestradiol 3-methyl ether (II) was demethylated by *Cunninghamella blakesleeana*. The product was shown to be 8-aza-D-homo-oestradiol (IV) by analysis, by the shift of the u.v.-absorption maximum on adding alkali and by methylation with diazomethane to the 3-methyl ether. The addition of Zn^{2+} to the medium led to the formation of more strongly acidic compounds instead of the phenol. 8-Aza-D-homo-oestrone 3-methyl ether (I) was demethylated by *Aspergillus flavus* to the phenol (III), which was identical with a product obtained by oxidizing 8-aza-D-homo-oestradiol (IV) with chromium trioxide.

EXPERIMENTAL

Melting points were determined on a Kofler block and are corrected. Aq. 5% (w/v) Na_2CO_3 was used for adjusting the pH of aqueous solutions. The pH 4 buffer used was 7.5 mM- Na_2HPO_4 -22 mM-citric acid. Aluminium oxide G (from E. Merck, Darmstadt, Germany) was used for thin-layer chromatography, and fluorescent spots were observed in ultraviolet light (Hanovia lamp; maximum emission 366 m μ) after heating the chromatoplates with a hair-dryer for 10 min. The numbers given with the names of fungi refer to the I.C.I. (Akers) Culture Collection; where possible the number in the Commonwealth Mycological Institute Collection (I.M.I.) or the American Type Culture Collection (A.T.C.C.) is also given.

Fungus culture. The *Aspergillus* cultures were grown on media containing 5% of Dextrolact (Corn Products Ltd.), 1% of KNO_3 , 0.2% of KH_2PO_4 , 0.05% of $MgSO_4 \cdot 7H_2O$, 0.1% of yeast extract (Oxoid) and 1 ml. of minor element concentrate/l. (Brian, Curtis & Hemming, 1947). *Cunninghamella blakesleeana* was grown on media containing 1% of Dextrolact, 0.15% of KNO_3 , 0.2% of KH_2PO_4 , 0.05% of $MgSO_4 \cdot 7H_2O$, 0.1% of yeast extract and 1 ml. of minor element concentrate (from which the $ZnSO_4$ had been omitted)/l.

Portions (20 ml.) of medium were inoculated in 100 ml. conical flasks and incubated at 25° . After 2 days a solution of the aza steroid (8 mg./ml. of ethanol) was added to give a concentration of 200 mg./l. in the medium. Incubation was continued for 3 more days in shake culture or 5 days in surface culture.

Isolation and characterization of products. (a) 8-Aza-D-homo-oestrone 3-methyl ether as precursor. Each culture filtrate (400 ml.) was adjusted to pH 8.5-9.0 and extracted with benzene (3×40 ml.). The combined benzene extracts were extracted with pH 4 buffer (2×100 ml.). The combined buffer extracts were adjusted to pH 8.5-9.0 and extracted with benzene (2×20 ml.). These benzene extracts were dried with Na_2SO_4 , concentrated and chromatographed on neutral alumina columns, which were developed with benzene and increasing proportions of ethyl acetate in benzene.

From shake-culture filtrate of *Aspergillus ochraceus* (1357 = I.M.I. 35038) a mixture of two 17A epimeric alcohols were eluted from the column (38 g. of Al_2O_3 ; Woelm grade III) with 5% and 10% (v/v) ethyl acetate in benzene. The minor component of this mixture, though not isolated, was found to be identical with the $NaBH_4$ reduction product by thin-layer chromatography with three solvent systems. The major component was crystallized from light petroleum (b.p. $80-100^\circ$) in 22% yield of needles, m.p. 100° and $[\alpha]_D^{25} - 90^\circ$, and was distinguished by different R_F values on thin-layer chromatograms and by slight differences of infrared spectrum. Residual precursor was isolated in 31% yield from the benzene eluates of the chromatogram, and by further extraction of the benzene extract of culture filtrate with 0.1 N-citric acid. After recrystallization from light petroleum (b.p. $60-80^\circ$) and from ethanol, the 8-aza-D-homo-oestrone 3-methyl ether had m.p. $154-157^\circ$ and $[\alpha]_D^{25} + 94^\circ$, and its infrared spectrum in bromoform was identical with that of the racemic form.

From surface-culture filtrate of *Aspergillus flavus* (1076), by elution of the column (25 g. of Al_2O_3 ; Woelm grade V) with 10% (v/v) ethyl acetate in benzene, a solid was isolated in 3% yield, λ_{max} (in $CHBr_3$) 3280 (OH) and 1684 cm^{-1} (CO). It was shown to be 8-aza-D-homo-oestrone (see below).

(b) 8-Aza-D-homo-oestradiol 3-methyl ether as precursor. Shake-culture filtrate (440 ml.) of *Cunninghamella blakesleeana* (1346 = A.T.C.C. 8688b) was adjusted to pH 8.5-9.0 and extracted with ethyl acetate (2×90 ml.). The combined extracts were extracted with pH 4 buffer (2×130 ml.). The ethyl acetate was removed from the buffer extracts in a stream of N_2 . The buffer extracts, combined and adjusted to pH 8, were extracted with benzene (3×12 ml.) and each benzene extract was successively back-washed with two 250 ml. portions of 70 mM- Na_2HPO_4 . The combined aqueous solutions were extracted with ethyl acetate (2×150 ml.). Prisms of 8-aza-D-homo-oestradiol (16% yield) separated on concentrating the ethyl acetate extracts. The optically inactive solid was recrystallized from acetone and had m.p. 216° (sublimes) (Found: C, 75.1; H, 8.9; N, 5.0; methoxyl, none. $C_{18}H_{25}O_2N$ requires C, 75.2; H, 8.8; N, 4.9%). In ethanol (50 $\mu g./ml.$) it had λ_{max} 281 m μ ; after adding 2 drops of N-NaOH it had λ_{max} 300 m μ .

If Zn^{2+} is present in the medium, the crystalline phenol was replaced by a yellow acidic gum.

Oxidation of (-)-8-aza-D-homo-oestradiol 3-methyl ether. (-)-8-Aza-D-homo-oestradiol 3-methyl ether (40 mg.), dissolved in 5 ml. of acetone at 0° was treated with 0.04 ml. of 8N- CrO_3 in H_2SO_4 (Curtis, Heilbron, Jones & Woods, 1953). After 5-10 min. the reaction mixture was diluted with 24 ml. of distilled water, adjusted to pH 8.7 and extracted with benzene (2×8 ml.). The dry concentrated extracts were chromatographed on 18 g. of Al_2O_3 (Woelm grade III). (-)-8-Aza-D-homo-oestrone 3-methyl ether (15 mg.) was eluted with benzene, and unoxidized 8-aza-D-homo-oestradiol 3-methyl ether (25 mg.) with 10% (v/v) ethyl acetate in benzene. The ketone, after recrystallization from ethanol, had m.p. $155-157^\circ$ and $[\alpha]_D^{18} = -86^\circ$, and was identified with (+)-8-aza-D-homo-oestrone 3-methyl ether by its infrared spectrum.

Oxidation of 8-aza-D-homo-oestradiol. 8-Aza-D-homo-oestradiol (1 mg.), dissolved in 0.4 ml. of acetone at 0° , was treated with 0.004 ml. of 8N- CrO_3 in H_2SO_4 . After 35 min. when no further green precipitate was forming, the reaction

mixture was diluted with 9ml. of distilled water, made alkaline and extracted with benzene (2×2 ml.).

The 8-aza-D-homo-oestrone in these extracts was found to have the same R_F values on thin-layer chromatograms, with two solvent systems, as the product isolated from the culture filtrates of *Aspergillus flavus*.

Methylation of 8-aza-D-homo-oestradiol. About 1 mg. of 8-aza-D-homo-oestradiol was dissolved in 0.5 ml. of methanol; on adding diazomethane solution immediate decolorization occurred; further diazomethane was added drop by drop until the yellow colour persisted. The residue from the reaction mixture was dissolved in ethanol, and thin-layer

chromatography with three solvent systems showed the presence of 8-aza-D-homo-oestradiol 3-methyl ether.

The author gratefully acknowledges the advice of Dr B. E. Cross and the skilled assistance of Mr M. S. O. Bongilli.

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