Uropygiols: confirmation of structure by proton magnetic resonance

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Abstract Lipids were extracted from excised uropygial glands of domestic chickens and the wax diesters were isolated by preparative thin-layer chromatography (TLC). The diesters were hydrolyzed and the liberated diols were resolved by boric acid TLC into two fractions. These were investigated by proton magnetic resonance at 360 MHz of the free diols and of their acetonide derivatives. The results showed that the *cis* and *trans* acetonides, formed from the *erythro* and *threo* isomers of the diols, respectively, could be distinguished by the degree of magnetic nonequivalence of the two acetonide methyl groups in each molecule. On the presumption that the *cis* isomer should show the greater nonequivalence of the methyl groups, this configuration was assigned to the acetonides of these diols which had the lesser TLC mobility on boric acid/silica gel. This agrees with the assignment of configuration made by earlier workers on the basis of the relative TLC mobility of the diol isomers on boric acid/silica gel, but was contrary to a previous assignment based on gas-liquid chromatographic (GLC) retention times. We conclude that the *erythro* isomers of the diols are characterized by lower mobility on boric acid TLC, as well as on silica gel TLC, and form acetonides that have longer retention times on GLC, and greater nonequivalence of the acetonide methyl groups in the NMR spectrum, than do the acetonides of the *threo* isomers. —**Wertz, P. W., W. Abraham, P. M. Stover, and D. T. Downing.** Uropygiols: confirmation of structure by proton magnetic resonance. *J. Lipid Res.* 1985. **26:** 1333–1337.

Supplementary key words chicken • preen gland • 2,3-diols • acetonides

The uropygial gland of birds produces a clear oil that differs in composition among species (1). In the domestic chicken (*Gallus domesticus*), the oil gland secretion consists predominantly of a mixture of wax diesters. These are formed from long chain 2,3-diols, termed uropygiols (2, 3), esterified with fatty acids ranging in chain length from C_{10} to C_{20}. The diols and the fatty acids are exclusively straight chained and saturated (2, 3). The chicken uropygiols were reported to consist of a mixture of about equal proportions of the *threo* and *erythro* stereoisomers, the existence of which was revealed by differences in the gas-liquid chromatographic mobilities of the acetonides of these isomers. Haahti and Fales (2) proposed that the acetonides that were more mobile on a nonpolar gas-liquid chromatographic column were the derivatives of the *erythro* isomers. However, on the basis of the relative TLC mobilities of the isomers on boric acid-impregnated silica gel, Hansen, Tang, and Edkins (3) proposed the reverse assignment. In the present study we have separated the two series of isomers and investigated their structures by chemical procedures as well as by high field proton magnetic resonance. Some recent publications illustrate the use of this technique in the structure determination of lipids (4, 5).

METHODS

Collection of uropygial gland secretion

Several mature laying hens were killed and the uropygial glands were excised and freed as completely as possible of adhering tissues. The glands were then coarsely chopped and placed in chloroform–methanol 2:1. After 24 hr the tissue was discarded and the solution was evaporated in a rotary evaporator, leaving a clear yellow oil. The yield was approximately 450 mg per 2.3 g gland.

Isolation of the wax diesters

Analytical TLC on silica gel revealed a major component that migrated in the wax diester region when chromatograms were developed with chloroform. This was isolated by preparative TLC in which 50-mg aliquots of the gland lipids were chromatographed on 0.5-mm-thick layers of silica gel H, using chloroform as the developing solvent. The lipid bands were visualized by spraying the chromatograms with 8-hydroxy-1,3,6-pyrene-trisulfonic acid trisodium salt and viewing under UV light. The band containing the wax diesters was scraped from the plate and the lipid was recovered by elution with chloroform–methanol 2:1.

Abbreviations: TLC, thin-layer chromatography; GLC, gas-liquid chromatography; RT, retention time.

Preparation of the uropygiol isomers

The isolated wax diester fraction was saponified in 1 N KOH in methanol–water 95:5 at 60°C for 2 hr. The reaction mixture was then acidified and the hydrolysis products were recovered by extraction into chloroform. After evaporation of the solvent, the lipid residue was treated with BCl_3–methanol to esterify the fatty acids. The mixture of fatty acid methyl esters and wax diols was then separated by preparative TLC on silica gel H, using toluene as developing solvent.

The isolated wax diols were then chromatographed on thin-layer plates coated with silica gel H containing 5% boric acid, using diethylether as developing solvent. Two major bands were revealed under UV fluorescence, having R_f values of 0.44 and 0.59. These were scraped from the plate and the two diol fractions were eluted with chloroform–methanol 2:1.

Preparation of the uropygiol acetonides

Each of the recovered diol isomers was dissolved in acetone and treated with a trace of HCl to produce the acetonides. After 1 hr the mixture was treated with excess aqueous K_2CO_3 and the acetonides were extracted into chloroform. The solvent was evaporated and the residual lipid mixture was examined by GLC at 280°C on a 50 m × 0.2 mm quartz capillary column wall-coated with cross-linked methyl silicone (BP1, Scientific Glass Engineering, Austin, TX).

Proton magnetic resonance spectrometry

Proton NMR spectra were obtained with a Bruker Model WM-360 spectrometer operating in the Fourier transform mode with signal averaging of multiple scans. The lipid samples were dissolved in deuterochloroform and the solutions were dehydrated by addition of two beads of 3A Davison Molecular Sieve. The residual $CHCl_3$ peak at 7.20 was used as reference for chemical shift measurements. Nuclear Overhauser Effect (NOE) difference spectra were obtained using the Bruker NOE pulse program. The decoupler was set at the downfield end of the spectral window for the control spectrum.

Confirmation of chain lengths

After GLC analysis, each acetonide fraction was reconverted to diols by treatment with BCl_3–MeOH for 1 hr. After evaporation of the solvent with a stream of nitrogen, the diol residues were dissolved in *t*-butanol and treated with an equal volume of periodate-permanganate reagent (6) to cleave the molecules between the vicinal hydroxyl groups and produce series of fatty acids each having two carbon atoms less than the diols from which they were derived. The free fatty acids were recovered in chloroform and converted to methyl esters with BCl_3–MeOH. The methyl esters were analyzed by GLC, using a series of C_{14} to C_{24} fatty acid methyl esters (Applied Science Kit KF) as standards.

RESULTS

Fig. 1A shows the proton NMR spectrum of the acetonides of the diol fraction that was less mobile on boric acid TLC. The spectrum shows a triplet at 0.82 ppm and a tall broad peak at 1.20 ppm corresponding to the methyl and the methylenes, respectively, of the alkyl chain. The doublet at 1.07 ppm is assigned to the 1-methyl group protons. There are two singlets at 1.27 and 1.39 ppm assigned to the two methyl groups of the acetonide moiety. This large chemical shift difference between the two methyl groups attached to the same carbon suggests that they experience quite different molecular environments. The methine protons on the ring appear at 3.97 and 4.16 ppm. These were assigned by inspection (as they show a simple first-order coupling pattern) and later confirmed by decoupling experiments. Thus, the lowfield multiplet at 4.16 ppm is a doublet of quartets and is assigned to H1, while the upfield multiplet at 3.97 ppm is a doublet of triplets and is assigned to H2 (Fig. 1A).

Fig. 1B shows the spectrum of the acetonide derivatives of the diols which were more mobile on boric acid TLC. The spectrum shows a triplet at 0.82 ppm and a broad peak at 1.20 ppm which were assigned to the end methyl and the methylenes of the alkyl chain, respectively, as in the other isomer. There is a shoulder on the upfield limb of the strong methylene peak at 1.18 ppm which was assigned to the 1-methyl group protons by decoupling experiments. There are two singlets around 1.33 ppm and separated by ~0.02 ppm, equivalent to three protons each. These were assigned to the two methyl groups of the acetonide moiety. These two methyl groups are almost equivalent in chemical shift in this isomer, while there is a much greater difference between them in the other isomer. This is the principal evidence upon which the isomeric structures are assigned. Thus, in the *trans* isomer, each of the acetonide methyl groups has a proton and an alkyl substituent on the same side of the acetal ring, and hence the two methyl groups can be expected to be almost chemical-shift equivalent (homotopic). In the *cis* isomer, on the other hand, one acetonide methyl group "sees" both substituents—the methyl group and the long chain—while the other "sees" neither. Therefore, the *cis* isomer clearly should produce the greater difference in chemical shift between the two acetonide methyl groups.

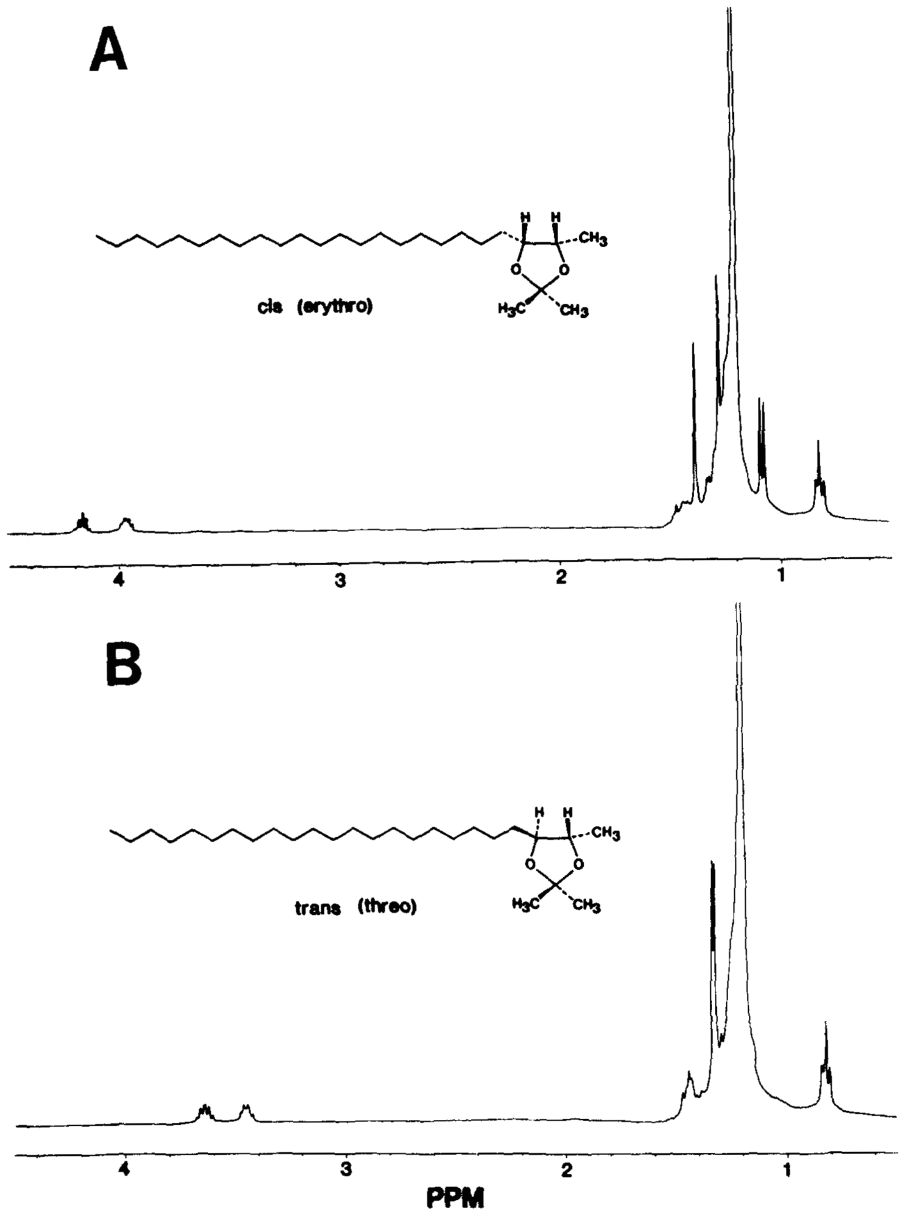

Fig. 1. Structures and proton NMR spectra of the *cis* (A) and *trans* (B) isomers of acetonides derived from the *erythro* and *threo* 2,3-diols, respectively, that were isolated from chicken preen gland wax diesters.

In an attempt to distinguish between the two acetonide methyl groups, an experiment was conducted to determine whether a homonuclear Overhauser effect could be detected between the 1-methyl group protons and one of the acetonide methyls. In both of the isomers, the acetonide methyl resonance having the smaller chemical shift (1.33 ppm in the *trans* isomer and 1.27 ppm in the *cis* isomer) was found to gain in intensity on saturating the 1-methyl group protons. Thus, the upfield acetonide methyl was shown to be on the same side as the 1-methyl group in both isomers.

In the *trans* isomer, the methine protons also appeared downfield—H1 at 3.64 ppm and H2 at 3.45 ppm. An interesting feature of the spectra of the two isomers is the relative positions of the two methine protons and the 1-methyl group protons. For the *cis* isomer, the methine protons are further downfield (by about 0.5 ppm) and the 1-methyl group protons are further upfield (by about 0.1

ppm) compared to the *trans* isomer. This suggests a preferential conformation of the nonplanar five-membered acetal ring for the *cis* isomer wherein there is higher electron density on one side of the ring than the other. Thus, the methine protons that lie on the electron-deficient side of the ring are deshielded and hence move downfield, while the 1-methyl group protons move upfield due to additional shielding. This also causes an upfield shift of the acetonide methyl group on the same side of the ring as the 1-methyl group and a downfield shift of the other acetonide methyl group protons in the case of the *cis* isomer as compared to those in the *trans* isomer.

Gas chromatograms of the two series of diol acetonides revealed that each consisted of five principal components forming homologous series (**Fig. 2**). The peaks for each *trans* isomer emerged somewhat earlier than the corresponding peak for the *cis* isomer having the corresponding chain length. Consequently, a gas chromatogram of the acetonides of unfractionated diols showed ten significant peaks (Fig. 2).

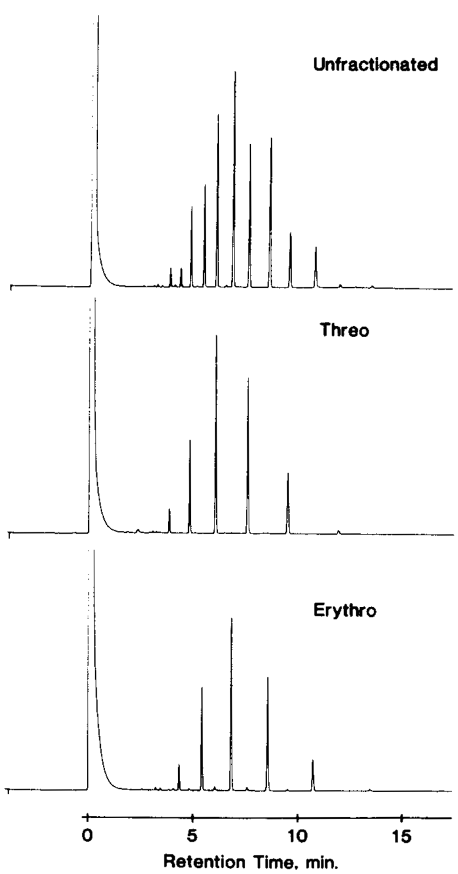

Fig. 2. Gas chromatograms of the acetonide derivatives of unfractionated 2,3-diols and of the *threo* and *erythro* isomers that were resolved by boric acid TLC.

TABLE 1. Composition (wt%) of the mixture of 2,3-diols from chicken preen gland wax diesters

Chain Length	*Threo*		*Erythro*	
	RT[a]	%	RT[a]	%
C_{20}	3.88	1.80	4.37	2.35
C_{21}	4.87	6.87	5.50	11.25
C_{22}	6.11	15.04	6.91	22.77
C_{23}	7.67	12.79	8.66	16.84
C_{24}	9.61	5.42	10.85	4.87
Total		41.92		58.08

[a]RT, gas-liquid chromatographic retention time (min) on a 50-m quartz capillary column at 260°C.

The fatty acids produced by oxidation of each series of diol isomers, when analyzed by GLC of their methyl esters, were each found to consist of a series of five components, matching the retention times of the C_{18} to C_{22} reference methyl esters. This implies that each set of diol isomers consists of the C_{20} to C_{24} homologues, the quantitative composition of which is shown in **Table 1**.

DISCUSSION

The present study is in agreement with both earlier investigations (2, 3) in concluding that the uropygial gland secretion of the chicken consists predominantly of the diesters of several long-chain 2,3-diols, and that both the *threo* and *erythro* isomers of the diols are present in about equal proportions. The isomers may be distinguished by differences in several physical properties, including differences in GLC mobilities of the respective acetonides and in the TLC mobility of the free diols on silica gel and on boric acid/silica gel. The disparity between the earlier studies lies only in which properties are associated with which isomers. The relationships observed in the present study are shown in **Table 2**.

The assumption of Hansen et al. (3) that the *erythro* isomer of the diols would have the lower mobility on boric acid TLC appears to be correct. Although this assumption is intuitively reasonable, inspection of structural models does not indicate any clear reason why this should be so. As shown in **Fig. 3**, both isomers are free to orient the vicinal hydroxyl groups in similar spatial arrangements relative to each other and thus to the chromatographic substrate.

The assignment of chain lengths for the uropygiols was made initially by Haahti and Fales (2) on the basis of mass spectra of the unfractionated mixture of their acetonides. The assignments were confirmed by Hansen et al. (3) for both chicken and turkey diols by oxidation of the diol

TABLE 2. Relative physical properties of the *threo* and *erythro* isomers of uropygiols and their respective acetonides

Physical Property	*Threo*	*Erythro*
GLC retention time of acetonides (nonpolar phase)	Shorter RT	Longer RT
TLC mobility of free diols on silica gel	More mobile	Less mobile
TLC mobility of free diols on boric acid/silica gel	More mobile	Less mobile
NMR equivalence of acetonide methyl groups	More equivalent	Less equivalent

mixture to aldehydes followed by gas-liquid chromatography. These assignments were confirmed by the present oxidation of the diols to fatty acids which were then analyzed by GLC as their methyl esters and compared with authentic methyl esters. However, in the mixture obtained in the present study, five significant homologues (C_{20} to C_{24}) were observed for each isomer of the chicken preen gland diols, rather than the three homologues (C_{22} to C_{24}) in each isomer fraction reported by both previous groups for chicken preen gland. ■

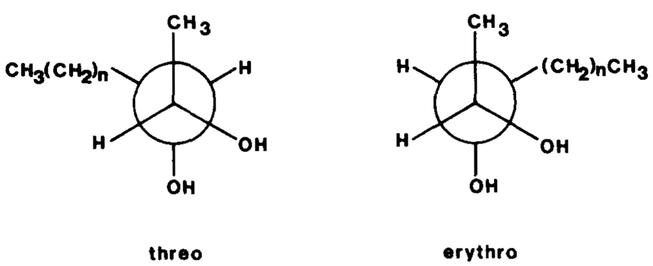

Fig. 3. Newman projections of the *threo* and *erythro* isomers of the 2,3-diols of chicken preen gland, showing the similar spatial relationships that are possible for the hydroxyl functions.

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REFERENCES

1. Jacob, J. 1976. Bird waxes. *In* Chemistry and Biochemistry of Natural Waxes. P. E. Kolattukudy, editor. Elsevier, New York. 93-146.
2. Haahti, E. O. A., and H. M. Fales. 1967. The uropygiols: identification of the unsaponifiable constituent of a diester wax from chicken preen glands. *J. Lipid Res.* **8:** 131-137.
3. Hansen, I. A., B. K. Tang, and E. Edkins. 1969. *erythro*-Diols of wax from the uropygial gland of the turkey. *J. Lipid Res.* **10:** 267-270.
4. Dabrowski, J., H. Egge, and P. Hanfland. 1980. High resolution nuclear magnetic resonance spectroscopy of glycosphingolipids. I. 360 MHz ^1H and 90.5 MHz ^{13}C NMR analysis of galactosylceramides. *Chem. Phys. Lipids* **26:** 187-196.
5. Abraham, W., P. W. Wertz, and D. T. Downing. 1985. Linoleate-rich acylglucosylceramides of pig epidermis: structure determination by proton magnetic resonance. *J. Lipid Res.* **26:** 761-766.
6. Downing, D. T., and R. S. Greene. 1968. Rapid determination of double bond positions in monoenoic fatty acids by periodate-permanganate oxidation. *Lipids.* **3:** 96-100.