APPENDIX

Nuclear-Magnetic-Resonance and Mass-Spectral Study of Myxinol Tetra-acetate

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Myxinol tetra-acetate was examined by both nuclear-magnetic-resonance and mass-spectral techniques. The results are compared with corresponding data for 5 α -ranol tetra-acetate and 5 α -cyprinol tetra-acetate. The structure deduced for myxinol tetra-acetate, 3 β ,7 α ,16 α ,27-tetrahydroxy-5 β -cholestane, is in agreement with that simultaneously arrived at by Haslewood (1966).

EXPERIMENTAL

Nuclear-magnetic-resonance (n.m.r.) spectra were recorded on a Varian A-60 spectrometer with the assistance of Mr J. W. Murphy. Solutions of concentration 5–8% were employed containing tetramethylsilane as an internal reference. Chemical shifts are quoted in τ units and coupling constants, J , as cyc./sec. Accuracies are of the order of $\pm 0.02\tau$ and ± 0.5 cyc./sec. respectively.

Mass spectra were kindly recorded by Dr Ryhage, Karolinska Institute, Stockholm, Sweden, and by Dr Nickels, Atlas Co., Bremen, Germany, both using Atlas mass spectrometers.

RESULTS AND DISCUSSION

The n.m.r. spectrum of myxinol tetra-acetate in deuteriochloroform showed acetate methyl resonances equivalent to four acetate groups. One acetate methyl showed resonance at 2.04 τ , upfield by 0.04 p.p.m. from any other acetate proton resonance previously encountered in our studies of bile alcohols and related compounds. This suggested that a novel site of substitution might be involved. Other methyl proton resonances totalled 12 protons, distributed as two 3-proton singlets at 9.30 τ and 9.04 τ and a broad unresolved 6-proton singlet at 9.16 τ . No free hydroxyl groups could be detected by the addition of deuterium oxide to the deuteriochloroform solution. A multiplet at 5.87–6.37 τ corresponded by area measurement to only one $\text{CH}\cdot\text{CH}_2\cdot\text{O}\cdot\text{CO}\cdot\text{CH}_3$ methylene group and argued strongly in favour of the terminal unit $-\text{CH}(\text{CH}_3)\cdot\text{CH}_2\cdot\text{OH}$ in myxinol. An unresolved complex multiplet at 4.83–5.55 τ was assigned to three protons in the environment $\text{CH}\cdot\text{O}\cdot\text{CO}\cdot\text{CH}_3$. From the overall shape of the resonance 'envelope' it was clear that this multiplet was composed of an equatorially oriented proton ($\text{CH}\cdot\text{O}\cdot\text{CO}\cdot\text{CH}_3$

multiplet, half-band width approx. 6 cyc./sec). overlying broader multiplets for axial or pseudo-axial protons (i.e. equatorial acetate groups). Similar superposed multiplets featured in the n.m.r. spectra of ranol tetra-acetate and cyprinol tetra-acetate (Cross, 1964*a,b*). All known bile alcohols contain 3- and 7-hydroxyl groups but a 12 α -hydroxyl function was readily excluded since myxinol readily undergoes complete acetylation. No low-field resonance equivalent to allylic $\text{CH}\cdot\text{O}\cdot\text{CO}\cdot\text{CH}_3$ protons was detectable nor did the n.m.r. spectrum of myxinol tetra-acetate show broad resonance for protons allylic to any tetra-substituted double bond. Since no vinyl proton resonance was visible the myxinol molecule is therefore free of unsaturation. Angular methyl proton chemical-shift values (see below) were, moreover, incompatible with the presence of Δ^8 - or $\Delta^{8,14}$ -double bonds, both of which are known to cause pronounced shifts of these resonances.

Previously it was found that the mass spectra of 5 α -ranol tetra-acetate and 5 α -cyprinol tetra-acetate (III and IV respectively) are dominated by m/e peaks corresponding to overall loss of acetic acid and water from acetate and unesterified 12 α -hydroxyl functions (Cross, 1964*a,b*). Thus $M-60$, $M-(60+18)$, $M-120$ and $M-(120+18)$ peaks were intense whereas the molecular ion was difficult to retain. A similar situation was obtained with myxinol tetra-acetate. However, by using a direct inlet system, Dr Nickels (Atlas Co., Bremen) was able to record the molecular ion (m/e 604). Other peaks were recorded corresponding to $M-120$ and $M-180$, equivalent to the overall elimination of two and three molecules of acetic acid respectively. Three more similarities noted in the mass spectra of all three bile alcohol tetra-acetates consisted in intense m/e peaks at 253 and 313, and a weak peak

(I) $R = H$ (II) $R = CO \cdot CH_3$ (III) $R = CH(O \cdot CO \cdot CH_3) \cdot CH_2 \cdot CH_2 \cdot O \cdot CO \cdot CH_3$ (IV) $R = CH_2 \cdot CH(CH_2 \cdot O \cdot CO \cdot CH_3)_2$

at 373. The first of these corresponds to $C_{19}H_{25}^+$, the basic tetracyclic skeleton with three double bonds, resulting from loss of the 17β -side chain and thermally induced overall elimination of nuclear hydroxyl and acetate groups as water and acetic acid. Loss of less acetate leads to the units $C_{19}H_{26}(O \cdot CO \cdot CH_3)^+$ containing two double bonds (m/e 313) and $C_{19}H_{27}(O \cdot CO \cdot CH_3)_2^+$ with one double bond (m/e 373). The occurrence of these three peaks therefore indicated that myxinol contains three hydroxyl groups on the tetracyclic nucleus and only one as a side-chain substituent.

For 5α -ranol tetra-acetate and 5α -cyprinol tetra-acetate the strong peaks corresponding to losses of acetate and hydroxyl groups are accompanied by much weaker peaks, lower by 15 mass units, as expected for smaller fragmentations involving loss of methyl groups. In striking contrast the mass spectrum of myxinol tetra-acetate showed $M-120$ (m/e 484) and $M-180$ (m/e 424) peaks of much lower intensity than the peaks resulting from additional loss of methyl groups (m/e 469 and 409). Indeed, the m/e 409 peak was the strongest in the spectrum. It was clear that the extra nuclear acetate in myxinol tetra-acetate is located so that elimination furnishes a double bond allylic to the C-10, C-13 or C-20 positions and capable of stabilizing the radical generated by loss of methyl. Two further diagnostic peaks, absent from the mass spectra of the reference compounds (Cross, 1964a), were also observed. These were assigned to the triply unsaturated tetracyclic nucleus bearing a 17β -CH $\cdot$ CH $_3$ side chain (m/e 281) and the analogous double unsaturated tetracycle $C_{21}H_{30}(O \cdot CO \cdot CH_3)^+$ retaining one acetate group (m/e 341). This observation, combined with that described above on methyl lability, indicated a 16-hydroxyl group in myxinol. Deacetylation of the tetra-acetate leads to a Δ^{16} -steroid with the double bond allylic to C-13 and C-20 and therefore capable of stabilizing radicals that result from $C_{(13)}-C_{(13)}$, $C_{(20)}-C_{(21)}$ or $C_{(20)}-C_{(22)}$ bond fission. Precedent for the increased loss of methyl from 16-oxygenated steroids is provided by the work of

Djerassi and his colleagues, who also have discussed mechanisms of methyl loss (Beard, Wilson, Budzikiewicz & Djerassi, 1964).

Consideration of the strongly negative contribution to molecular rotation made by the 16-hydroxyl group required the 16α -configuration (Haslewood, 1966). The ensuing discussion on n.m.r. data established a 5β -configuration at the A/B ring junction, and formation of a digitonide from myxinol was indicative of the 3β -hydroxyl- 5β -stereochemistry. Structure (I) for myxinol followed.

By analogy with previous work on bile alcohols (Cross, 1960, 1961, 1964a,b; Cross, Landis & Murphy, 1965), one of the three methyl proton resonances for myxinol tetra-acetate (II) was readily assigned, $\delta_{18-H} = 9.30\tau$. The remaining two resonances at 9.16τ (6H, broad singlet, half-band width 5cyc./sec.) and 9.04τ (3H) could be interpreted in two ways. Either $\delta_{19-H} = 9.04\tau$, and the broad singlet corresponds to two ill-resolved overlapping secondary methyl resonances, or $\delta_{19-H} = \text{approx. } 9.16\tau$, and the secondary methyl groups give rise to clearly resolved doublets, J , approx. 7cyc./sec., with the two sets of protons having accidentally equivalent chemical shifts. The latter interpretation infers that the 9.16τ resonance is a composite of a 19-H, 3-proton singlet and the two higher-field arms of two secondary methyl doublets, and that the 9.04τ resonance composes two superposed lower-field arms of the two doublets. Theoretical considerations eliminate the second interpretation and strongly favour the first. It is known (Anet, 1961) that for the structural unit:

the methyl proton resonance cannot be treated as an independent AB_3 system if $H_{(a)}$ and $H_{(b)}$ couple strongly and J_{ab} is of the same order of magnitude

as the chemical shift δ_a between them. Under such circumstances the methyl proton resonance loses its clearly resolved doublet character and appears as an ill-resolved doublet with a small apparent coupling constant or even a broad singlet. This situation obtains in the side chain of myxinol for both C-20- and C-25-methyl groups. Well-resolved doublets for these methyl groups are consequently not expected and therefore the C-19 protons resonate at 9.04τ . Similar resonances for side-chain methyl groups were observed previously for 26-deoxyscymnol and related compounds (Cross, 1960, 1961).

The side-chain secondary methyl proton resonances of myxinol tetra-acetate were further studied in switching to aromatic solvents. Bhacca & Williams (1964) have discussed the use of benzene solvent for n.m.r. studies of steroid ketones. The recorded methyl proton resonances (Fig. 1) illus-

trate that this solvent is also of utility with polar polyacetates for redistributing the methyl proton resonance pattern. The value of pyridine solvent for this purpose was disclosed by Slomp & MacKellar (1960). With these aromatic solvents the angular methyl proton resonances experienced upfield shift, thereby exposing the secondary methyl proton resonances with the expected band profiles.

The stereochemistry at C-5 in myxinol tetra-acetate (II) was readily determined by comparison of calculated and observed angular methyl proton chemical shifts (Table 1). Additive shift values for 3- and 7α -acetate substituents and base frequencies for the tetracyclic nucleus were taken from the literature (Zürcher, 1963). (A positive shift increment indicates an upfield shift towards the tetramethylsilane reference. Negative values correspond to downfield shifts.) Shifts of 0.04τ and

Fig. 1. Methyl proton resonance for myxinol tetra-acetate in: (a) CDCl_3 ; (b) benzene; (c) pyridine.

Table 1. *Calculated and observed 18- and 19-proton chemical shifts for myxinol tetra-acetate (calculations for 5α - and 5β -stereoisomers)*

Chemical unit	δ_{18-H}		δ_{19-H}	
	5α	5β	5α	5β
Unsubstituted tetracycle	9.31τ	9.31τ	9.21τ	9.08τ
$3\beta\text{-O}\cdot\text{CO}\cdot\text{CH}_3$	-0.01	-0.01	-0.05	-0.06
$7\alpha\text{-O}\cdot\text{CO}\cdot\text{CH}_3$	0.00	0.00	-0.01	-0.01
$16\alpha\text{-O}\cdot\text{CO}\cdot\text{CH}_3$	-0.03	-0.03	0.00	0.00
17β -Side chain	0.04	0.04	0.01	0.01
Calculated frequency	9.31τ	9.31τ	9.16τ	9.02τ
Observed frequency	9.30τ		9.04τ	

0.01 τ for δ_{18-H} and δ_{19-H} respectively were used, in accordance with recent practice, for the 17 β -side-chain contribution in angular methyl proton frequency calculations (Cross *et al.* 1965). Although ring D has been shown capable of conformational distortion owing to steric interactions of adjacent substituents, thereby invalidating frequency-shift calculations in certain cases (Cross & Beard, 1964), the 16 α -orientation of the ring D acetate was not expected to invoke problems of this nature. Comparison of published n.m.r. curves for 16 α -acetoxy steroids (Neeman & Hashimoto, 1962) with data for the corresponding 16-unsubstituted compounds suggested $\Delta\delta_{18-H}=0.03\tau$ and $\Delta\delta_{19-H}=0.0\tau$ as reasonable additive shift values for a 16 α -acetoxy substituent (unpublished observations from the Syntex Research n.m.r. data collection).

The assembled data clearly establish the 5 β -rather than the 5 α -formulation. Differentiation between 3 α - and 3 β -orientation of the ring A acetate was not possible from the n.m.r.-spectral study.

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