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Identification of Mycobactins by Nuclear-Magnetic-Resonance Spectroscopy

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Details are given of the n.m.r. spectra of mycobactins F, H, M, N, P, S and T, and resonances are ascribed to all the protons in these molecules. A simplified system is described for identifying known mycobactins by the n.m.r. spectrum alone. This method will not distinguish mycobactins S and T, whose nuclei differ only in the configuration at an asymmetric centre.

The structures of mycobactins produced by various species of mycobacteria have already been published (Snow, 1965*a*,*b*; White & Snow, 1969; Snow & White, 1969). In these papers, n.m.r. spectroscopy was used to confirm the stereochemistry of the hydroxy acid fragment of mycobactin P (Snow, 1965*a*), and to compare the structures of the cobactins S and T derived from the corresponding mycobactins (White & Snow, 1969). The n.m.r. spectra of seven mycobactins are reported here and general correlations are shown between the spectra and structure. The use of n.m.r. measurements for the identification of novel mycobactins is discussed.

METHODS

The n.m.r. spectra of the metal-free mycobactins were measured in C^2HCl_3 solution at 2–10% (w/v) concentrations, according to the amounts of material available, with a Varian HA100 spectrometer. Where signal enhancement was necessary the spectrometer was linked to a Varian C1024 Time Averaging Computer. Double-resonance experiments were performed with a Muirhead Wigan Decade Oscillator. The n.m.r. spectra of cobactins were similarly measured in [U-^{2}H]acetone.

RESULTS AND DISCUSSION

The features of the n.m.r. spectra of the mycobactins are summarized in Table 1. The spectra could be divided into five general regions: 6·5–7·7, 4·0–6·0, 3·0–4·0, 2·0–3·0 and 0·5–2·0δ. The lowest-field region was assigned to the aromatic protons and the 4·0–6·0δ region contained olefinic protons and all protons located on carbon bearing one strong electronegative grouping or two less electronegative groupings, i.e. O or C:O and N respectively. Protons on carbon adjacent to nitrogen absorbed in the 3·0–4·0δ region, and the area 2·0–3·0δ included protons on carbon adjacent to double bonds. The highest-field region contained all the aliphatic protons.

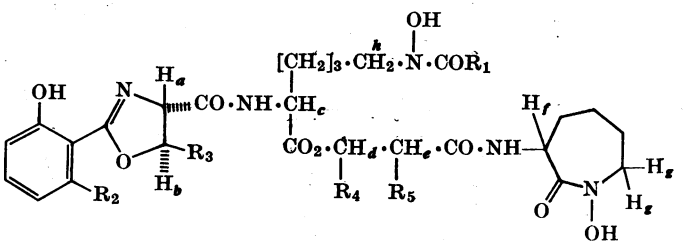
A doublet of doublets ($J = 8$ and 2Hz) at 7·62δ was assigned to the aromatic proton ($R_2 = H$), which was deshielded by the oxazoline ring. When $R_2 = CH_3$, this 7·62δ resonance was absent and was replaced by a three-proton singlet at 2·5δ assignable to the aromatic methyl group. The mycobactin from *Mycobacterium fortuitum* was a mixture of mycobactin H with mycobactin F, the components differing only in the presence or absence of the aromatic methyl group. The ratio of the compound where $R_2 = H$ to the compound where $R_2 = CH_3$ was calculated by measuring the integral of the 7·62δ peak and comparing it with the integral of the rest of the aromatic protons.

The olefinic protons of the unsaturated aliphatic chain ($R_1 = CH{:}CH{\cdot}[CH_2]_n{\cdot}CH_3$) appeared as a broad multiplet centred at 5·9δ. A multiplet at 2·5δ was assigned to the methylene group adjacent to the double bond, and the remaining protons of the aliphatic chain appeared as a strong single peak at 1·2δ with a triplet for the terminal methyl group at 0·85δ; these latter two assignments also applied when $R_4 = [CH_2]_n{\cdot}CH_3$.

When $R_3 = CH_3$, H_a was a doublet ($J = 8$Hz) at 4·3δ and H_b was a complex multiplet at 4·8δ. On using the technique of double irradiation at this position the methyl doublet at 1·55δ collapsed to a single peak, and conversely irradiation of the methyl doublet produced a further doublet ($J = 8$Hz) at 4·8δ. A degenerate AB_2 pattern was produced when $R_3 = H$, i.e. H_b appeared as four lines at 4·88δ and H_a and $R_3 = H$ had identical chemical shifts at about 4·56δ. The fact that the chemical shift of H_b stayed constant on changing R_3 from H

Table 1. *Identification of peaks in the n.m.r. spectra of mycobactins*

The following abbreviations are used: (s), singlet; (d), doublet; (t), triplet; (se), sextet; (b), broadened singlet. The n.m.r. spectrum of mycobactin T was identical with that of mycobactin S. The n.m.r. spectrum of a mixture of mycobactins F and H resembled that of mycobactin H except that resonances for $R_2 = H$ and $R_2 = CH_3$ were both present; the estimated F/H ratio was 2:5. Other resonances common to all mycobactins were: protons in benzene ring, 6·6–7·3; protons in aliphatic chains, 1·2; protons on terminal methyl, 0·85; protons in seven-membered ring, 1·0–2·0; OH and NH protons, variable between 12·0 and 6·0. Resonances in mycobactins H, P, S, and T: CH = CH, 5·9; = CH·CH_2, 2·5.



Mycobactin	R_1 Group	R_1 δ	R_2 Group	R_2 δ	R_3 Group	R_3 δ	R_4 Group	R_4 δ	R_5 Group	R_5 δ	H_a	H_b	H_c	H_d	H_e	H_f	H_g	H_h
S	Unsatd. paraffin	See others	H	7·62	H	4·56	CH_3	1·3(d)	H	See H_e	4·56	4·88	4·4	5·25 (se)	2·5 (d)	4·4	3·65	3·5
M	CH_3	2·0(b)	H	7·62	CH_3	1·55 (d)	Satd. paraffin	See others	CH_3	1·15 (d)	4·36 (d) $J = 8$Hz	4·8	4·6	4·9	2·6	4·6	3·65	3·5
N	CH_3CH_2	CH_2 2·3 CH_3 1·1 (t)	H	7·68	CH_3	1·58 (d)	Satd. paraffin	See others	CH_3	1·16 (d)	4·38 (d) $J = 8$Hz	4·8	4·6	4·9	2·62	4·6	3·66	3·5
H	Unsatd. paraffin	See others	CH_3	2·5 (s)	CH_3	1·54 (d)	CH_3	1·3 (d)	H	See H_e	4·3 (d) $J = 8$Hz	4·86	4·4	5·25 (se)	2·5	4·4	3·65	3·54
P	Unsatd. paraffin	See others	CH_3	2·5 (s)	H	4·6	$CH_2 \cdot CH_3$	CH_2? CH_3 0·9 (t)	CH_3	1·14 (d)	4·6	4·86	4·4	5·0	2·5	4·4	3·7	3·55

to CH_3 whereas H_a moved upfield from 4·56δ to about 4·36δ could be explained by the *cis* relationship of H_a and R_3.

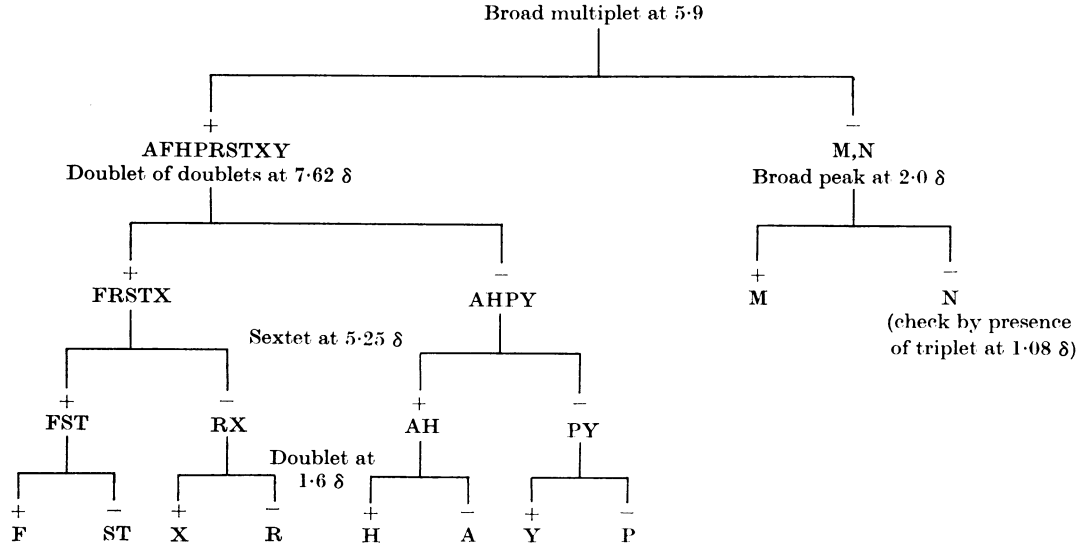
When $R_5 = H$, H_d appeared as a sextet at 5·25δ. Double irradiation of this sextet caused collapse of the methyl doublet at 1·3δ and also of a doublet at 2·5δ assigned to R_5 and H_e. Changing R_5 to methyl caused H_d to shift upfield to 5·0δ when $R_4 = CH_2CH_3$ and to 4·9δ when $R_4 = [CH_2]_n \cdot CH_3$. An explanation could be that the additional methyl group produced a change in the ratio of the rotamers at H_d and H_e and hence a different shielding of H_d.

White & Snow (1969) described the pattern for the H_e protons in cobactin T as more complex than in cobactin S and explained the difference in terms of different optical centres at H_d. This distinction was not seen in the mycobactins S and T as the pattern was obscured by other resonances. In mycobactin M, however, H_e appeared in the spectrum as a doublet of quartets at 2·6δ, agreeing with the pattern observed by Snow (1968) for the *erythro* hydroxy acid fragment derived from mycobactin P. This pattern was also observed when the n.m.r. spectra of the cobactin fragments of mycobactins P, R and M were measured in [U-^{2}H]acetone as solvent.

In mycobactin M, $R_1 = CH_3$ and this group was observed as a broad peak at 2·0δ. The broadness was attributed to hindered rotation in the amidic C–N linkage. In mycobactin N where $R_1 = CH_2 \cdot CH_3$ the methylene group gave a broad multiplet at 2·3δ and the methyl group gave a triplet at 1·1δ.

Other identifiable signals were H_f at 4·4–4·6δ and H_g and H_h at about 3·68δ and 3·5δ respectively. The methylene 'hump' at 1·0–2·0δ contained all the protons not already discussed excluding the NH and OH groupings, which were not assigned because of concentration variables but appeared between 12·0δ and 6·0δ.

The n.m.r. spectra of pure metal-free mycobactins can be used to identify known compounds and to predict the structures of new mycobactins that may yet be found. A simple scheme for identification of mycobactins is shown in Scheme 1. It relies on the recognition of a number of well-defined peaks that are unlikely to be obscured by other resonances. The first step separates mycobactins with Δ^2-acyl groups in the mycobactic acid moiety from those with saturated acyl groups. Next, the presence of a resonance at 7·62δ shows that $R_2 = H$. The sextet at 5·25δ is characteristic of 3-hydroxybutyric acid as the hydroxy acid fragment whereas the doublet at 1·6δ shows the presence of a methyl group in the oxazoline ring. So far, saturated acyl groups in the mycobactic acid moiety have been found only in mycobactins M and N. The protons of the acetyl group of mycobactin M are shown as a peak at 2·0δ, whereas mycobactin N is characterized by the



Scheme 1. Distinctions between mycobactins by n.m.r. resonances. The expected peaks for mycobactins A and R are included although their n.m.r. spectra have not been measured. The mycobactin F spectrum is deduced from the spectrum of its mixture with mycobactin H. X and Y show the expected peaks for two unknown mycobactins having R_2, R_3, R_4, R_5 = H, Me, Et, Me and Me, Me, Et, Me respectively. +, Resonance present; − resonance absent.

resonance due to the protons of the methylene carbon in its propionyl group. The scheme distinguishes between known mycobactins carrying different substituents (spectra for mycobactins A and R are deduced, but have not been measured); it also predicts the n.m.r. characteristics of two unknown mycobactins. Other possible mycobactins of the M or N type with variants at R_2 and R_3 could also be distinguished.

Distinction cannot be made between mycobactins S and T, where the sole difference in the nuclei lies in the configuration of the asymmetric centres.

REFERENCES

Snow, G. A. (1965*a*). *Biochem. J.* **94**, 160.
Snow, G. A. (1965*b*). *Biochem. J.* **97**, 166.
Snow, G. A. & White, A. J. (1969). *Biochem. J.* **115**, 1031.
White, A. J. & Snow, G. A. (1969). *Biochem. J.* **111**, 785.