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THE PROTON MAGNETIC RESONANCE SPECTRUM OF AMPHOTERICIN B

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Abstract—The high resolution proton NMR spectrum of Amphotericin B in dimethyl sulphoxide has been analysed by a combination of spin decoupling. NOE difference and spin decoupling difference techniques. The parameters are compared with those obtained for the related macrolide antibiotic pimaricin, and the methyl ester and acetamide of Amphotericin B.

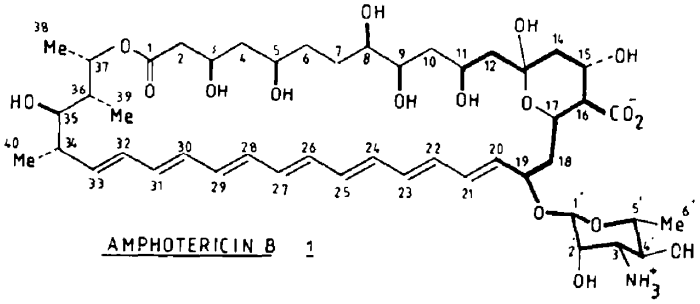
The polyene macrolides produced by a range of *Streptomyces* organisms are toxic to fungi but without effect on bacteria.[1] This is ascribed to selective complexation of the macrolide with yeast sterols leading to coaggregation in the cell membranes. For larger-ring macrolides (e.g. Amphotericin B) at low concentrations (*ca* 6.5 mM) this results in an increase in membrane porosity manifested by leakage of potassium ions. At higher concentrations (*ca* 65 mM) complete disruption of the membrane occurs causing cell death.[2] With smaller-ring polyene macrolides (e.g. Pimaricin) only the latter effect is observed.[2] There is recent interest[3] in the potentiation of carcinostatic properties by polyene macrolides, which is again presumed to be related to increased membrane permeability.

Several groups of workers have studied the interaction between macrolides and sterols, either *in vitro* or *in vivo*. Cholesterol and ergosterol have been most commonly employed and the techniques used include, *inter alia*, membrane conductance[4] freeze-etch electron microscopy,[5] monolayer reflectance spectra,[6] fluorescence spectra[7] and resonance Raman spectra.[8] The UV absorption spectrum of large polyenes such as Amphotericin B in water is complicated by aggregation but the addition of cholesterol produces changes in relative band intensity at low concentrations. Similar effects are observed when the components are incorporated in lecithin liposomes.[9] In all cases it is thought that a 1:1 complex, either monomeric or co-aggregated is responsible for the changes in spectral properties.[10] There is no firm evidence to support a particular structure for the association complex although it is recognised that hydrophobic forces and the apparent fit between polyene and sterol aligned with their long axes parallel[11] must play a role. For this reason we have studied the proton NMR spectrum of Amphotericin B and will report later on the changes induced by sterol complexation.

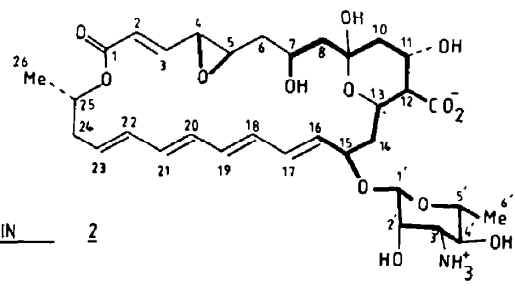
RESULTS AND DISCUSSION

Amphotericin B, which is clinically the most widely used antifungal polyene, is the only one for which an X-ray structure is available.[12] The conformation adopted in the solid state by the N-iodoacetamide is as depicted in 1 with both the polyene and hydroxylated chains extended. Only one report of the NMR spectrum of Amphotericin B has appeared and this is very poorly resolved in d_6-dimethylsulphoxide solution, even at 220 MHz.[13]

Our initial NMR studies were carried out employing Amphotericin B methyl ester since this has similar



AMPHOTERICIN B 1



PIMARICIN 2

biological activity to the parent compound and increased solubility in D_2O at pH 2–3.[14] It was quickly found that realistic concentrations of macrolide produced broad featureless spectra, and thus subsequent work was carried out with Amphotericin B in d_6-dimethylsulphoxide, at a concentration of 0.02 M. Even in the aprotic medium the natural line-width was 7–8 Hz and spectra were processed with sufficient resolution enhancement (see Experimental section) to reduce this to 1.5 Hz. The antibiotic was pre-exchanged with D_2O to remove signals due to hydroxyl protons. In some cases HOD was then removed *in vacuo*; otherwise the spectrum was accumulated with concurrent irradiation at 3.5 ppm. This produces the spectrum displayed in Fig. 1 in which the resonances of two single C–H protons at 3.46 ppm are lost due to their proximity to the irradiating frequency.

Systematic spin-decoupling was then applied. Irradiation of the high-field methyl group at 0.88 ppm led to collapse of the broad quartet at 1.73 ppm, whereas irradiation of the Me group at 1.07 ppm led to collapse of the quartet at 5.19 ppm. Irradiation of the Me-group at 1.00 ppm collapses the multiplet at 2.25 ppm. In each case the reciprocal spin-decoupling was carried out and in the case of the multiplet at 2.25 ppm there was a further collapse of the resonance at 5.41 ppm to a doublet. Since this latter signal, with 14 Hz and 10 Hz couplings must be an olefinic proton it is identified as H 33 and signal at 2.25 ppm is therefore H 34. The same experiment permitted the location of H 35 at 3.06 ppm, overlapping with a second signal. The existence of a mutual coupling (<2 Hz) between the signal at 5.19 ppm and one at 1.6 ppm enables them to be identified as H 37 and H 36 respectively. The high degree of deshielding of H 37 is interesting and suggests a conformation in which it is *syn*-coplanar with the CO-group; for comparison the methine hydrogen of iso-propyl acetate is at 5.02 ppm in $CDCl_3$.[15]

The remaining Me-group may be assigned to H 6′ of the mycosamine by elimination. Irradiation of this doublet shows that the Me-group is coupled to a proton at 3.24 ppm and hence this is H 5′. It was observed that chemical shifts in this region of the spectrum were very sensitive to the precise amount of water added (0–5%). Thus, a sufficiently large chemical shift difference exists in the presence of a judicious quantity of D_2O to permit irradiation of H 5′ and thereby demonstrate that it is coupled to the adjacent signal to lower field which is thus H 4′. The single proton at 2.96 ppm (here, but not in other spectra part-obscured by a spike) was suspected to be H 3′ by analogy with simple primary ammonium salts.[15] Irradiation revealed a strong coupling to H 4′ and another weak coupling to a proton at 3.79 ppm thus assigned as H 2′. The broadened signal at 4.5 ppm is assigned to the anomeric proton H 1′ which is at higher field in β-pyanosides than in the α-isomer.[16] This set of experiments identified the resonances due to the sugar residue.

At this stage the NMR spectrum of pimaricin 2 was recorded under similar conditions. The highlighted region of this latter is identical to the region of Amphotericin B between C 11 and C 19 and their sugars have identical structure and stereochemistry. The NMR spectrum of N-acetylpimaricin has been recorded in d_5-pyridine,[17] and that of pimaricin in d_3-trifluoroethanol;[18] in both cases the chemical shifts prove to be widely divergent from those observed for the parent compound in d_6-dimethylsulphoxide (Fig. 2). It was thus necessary to carry out a full set of double resonance experiments in order to obtain the assignments listed in Table 1 for pimaricin. The line-width observed was about 2 Hz so that much less deconvolution is required and for a given

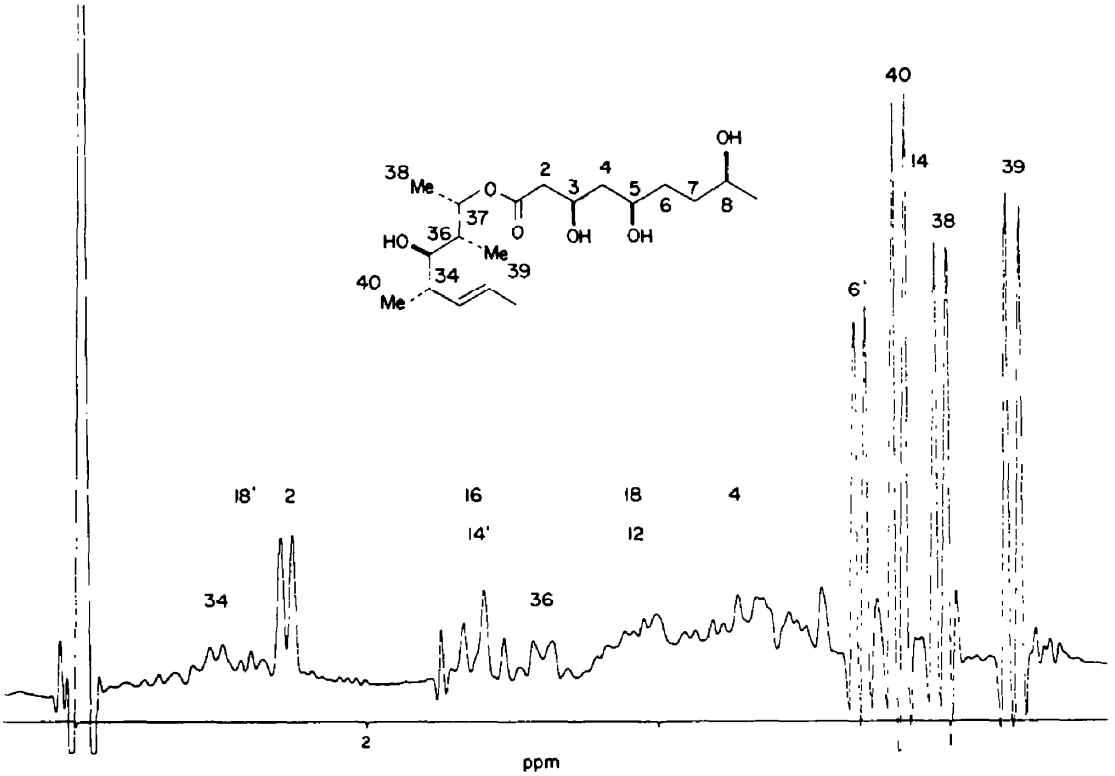


Fig. 1(a).

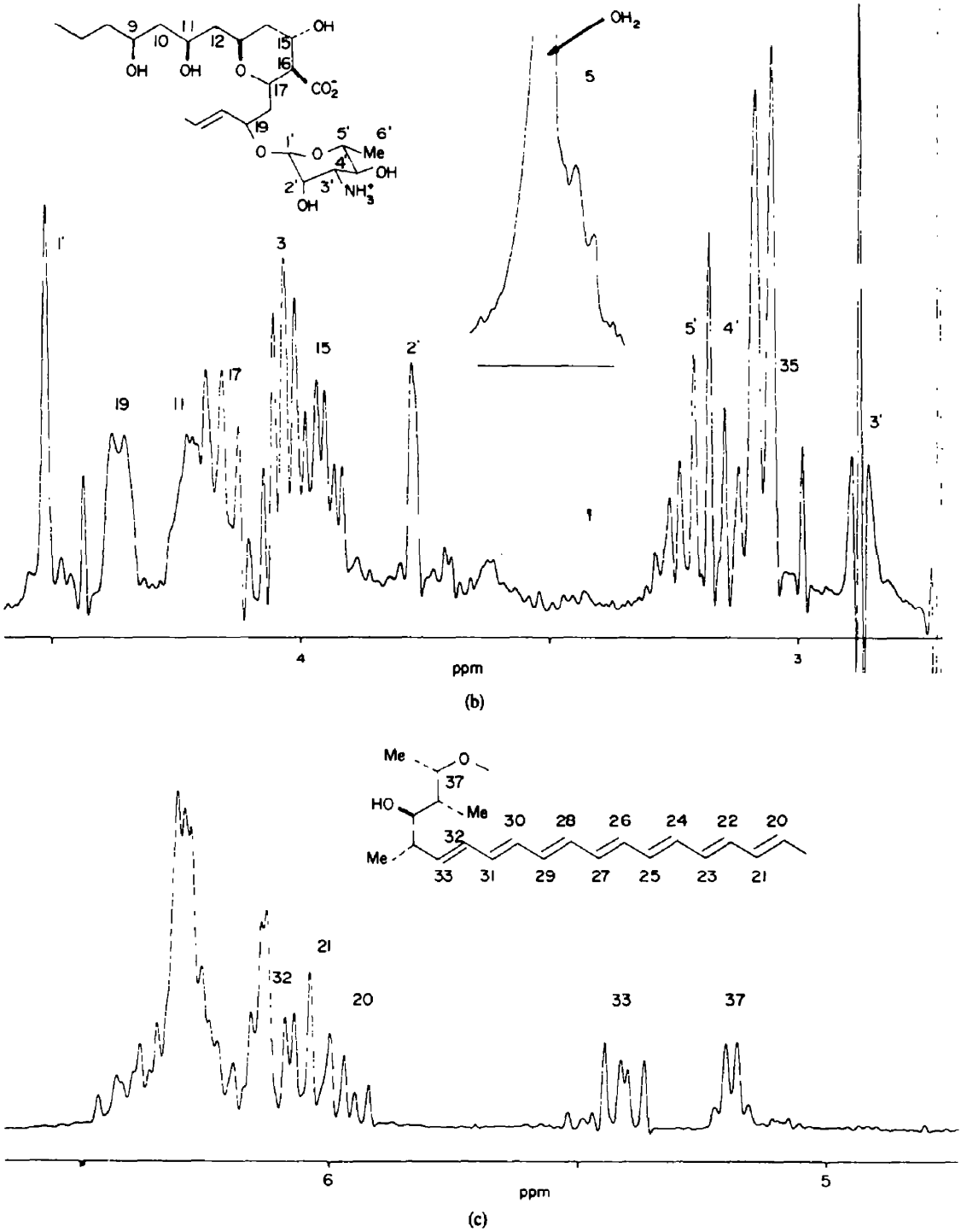


Fig. 1. Proton NMR spectrum of Amphotericin B in d_6 DMSO recorded as described in the Experimental. For clarity, expanded regions of the spectrum are shown: (a) 0.5–2.5 ppm, (b) 2.7–4.7 ppm, with inset of spectrum lacking solvent suppression, and (c) 5.0–6.5 ppm.

line-width and signal-to-noise ratio spectra may be accumulated in one-third of the time taken for accumulation of Amphotericin B spectra. In addition, the simpler spectrum obtained for the smaller polyene removed many of the potential ambiguities.

With the assigned pimaricin spectrum in hand the identity of protons in the region of Amphotericin B between C 11 and C 20 was established by spin-decoupling experiments. This was complicated by overlap of multiplets, particularly between 1 and 2 ppm. Resonance H 19 was readily established through its coupling to one of the olefinic protons, namely H 20. The identification of H 15, H 16 and H 17 followed directly by comparison with pimaricin and was confirmed by double resonance. One of the diastereotopic protons at C 14 is buried under the H 16 triplet and the other resonates at high field,

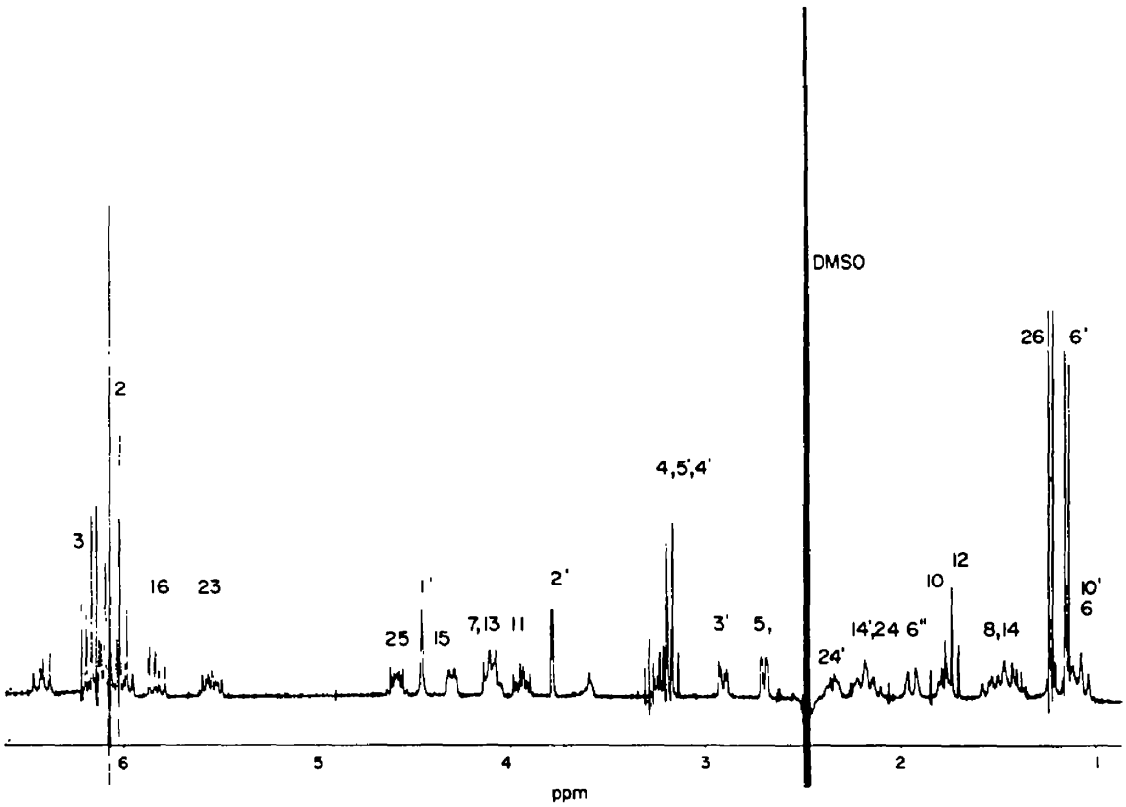


Fig. 2. Proton NMR spectrum of pimaricin in d_6-DMSO recorded using 90° pulse, 16 K data points, and an acquisition time of 2.72 sec. Processed using GB = 0.2; LB = −4.0.

1.10 ppm. One of the diastereotopic protons at C 18 is obscured by H 2 but was tentatively identified by sharpening of H 19 when this site was irradiated. The other was also obscured and in order to assist identification, a nuclear Overhauser experiment[19] was carried out. Irradiation at a particular site perturbs the intensities of protons which participate in its spin-lattice relaxation. Since the magnitude of the effect is inversely proportional to the sixth power of the inter-proton distance, this is a powerful technique for locating signals close to the site of irradiation. For small molecules which have short correlation times the effect is positive, but for larger molecules having sufficiently long correlation times negative NOE's are observed. The phenomenon is frequency dependent, favouring negative NOE's at higher fields.[19] Thus the observation of negative NOE's in our experiments is in accord with results obtained on molecules of a similar size under related conditions.[20] Observation is made particularly simple by a pulse program which accumulates the difference between normal and irradiated spectra which has been put to recent effective use. Low-power irradiation at H 19 and accumulation of NOE difference spectra is recorded in Fig. 3. Because of the proximity of other resonances it proved necessary to adjust parameters very carefully and these optimised conditions demonstrate spin saturation transferred to neighbouring sites so that the r^{-6} dependence does not hold. This very effectively locates the two C 18 protons, as shown. Experiments of this type hold much promise in the elucidation of complex organic structures.

Much of the polyol chain remained unassigned, although H 2 is readily recognised by its conspicuous two-proton doublet at 2.12 ppm. The adjacent signal H 3 is located by double resonance but no further information could be obtained under normal conditions. The technique of spin decoupling difference spectra has been effectively employed in a recent full assignment of the proton NMR spectra of some steroids.[21] By this means it proved possible to identify H 4 at 1.36 ppm on irradiation of H 3 at 4.03 ppm and thence H 5 at 3.46 ppm on irradiation of H 4 (Fig. 4). Two protons on OH-bearing carbons at C 8 and C 9 are not securely identified; one is isochronous with H 5 and the other overlaps with H 35. Methylene groups at C 6, C 7, C 10 and C 12 form part of the broad unresolved envelope in the region of 1.3–1.6 ppm.

Amphotericin B methyl ester and acetamide. Loss of the zwitterionic structure of Amphotericin B leads to considerable changes in chemical shift in that region of the molecule. Sites which are very remote from the carboxylate and ammonium group are unaffected, so that it is reasonable to suppose that the molecular conformation is independent of charge-type, and that association phenomena are unimportant in dimethyl sulphoxide. Changes which occur are recorded in Table 2 and are as expected at the sites of substitution and in their immediate vicinity. One of the C 18 protons is strongly deshielded in the parent compound relative to both methyl ester and amide. This must reflect a spatial proximity of H 18' to the ammonium carboxylate, which might be inferred from the X-ray structure of its derivative.[12]

Summary and extensions. The identification of 28 separate proton resonances in the NMR spectrum of Amphotericin B is the first step towards identifying the

Table 1. Chemical shifts of assigned protons in Amphotericin B (1) and Pimaricin (2) in d_6-dimethyl sulphoxide. (a) Asterisked resonances may have crossed assignments. (b) Chemical shifts of N-acetyl pimaricin in pyridine. Observed coupling constants (±0.5 Hz): $J_{2,3} = 6.0$; $J_{3,4} = 6.0$; $J_{14,15} = 4.75$; $J_{14,16} = 10.5$; $J_{16,17} = 10.5$; $J_{17,18} = 10.5$; $J_{19,20} = 8.75$; $J_{20,21} = 15.0$; $J_{32,33} = 14.0$; $J_{33,34} = 10.0$; $J_{34,35} = 10.25$; $J_{34,40} = 6.25$; $J_{36,39} = 7.0$; $J_{37,38} = 6.0$; $J_{2',3'} = 2.75$; $J_{3',4'} = 9.25$; $J_{4',5'} = 9.25$; $J_{5',6'} = 5.75$.

	2	3	4	5	6	7	8	9	10	11	12	12'	14	14'	15
1	*2.12*	*4.03*	*1.36*	*3.46*	--	—	*3.46*[a]	*3.05*[a]	—	*4.22*	—	--	*1.10*	*1.80*	*3.96*
				2	3	4	5	6	6''	7	8	8'	10	10'	11
2				*6.10*	*6.25*	*3.22*	*2.72*	*1.10*	*1.98*	*4.15*	*1.5*	*1.5*	*1.10*	*1.80*	*3.98*
2[b]				*6.08*	*6.47*	*3.21*	*2.97*	*1.27*	*2.12*	*4.50*	*1.83*	*1.73*	*1.40*	*2.13*	*4.25*
	16	17	18	18'	19	20	1'	2'	3'	4'	5'	6'	33		
1	*1.86*	*4.17*	*1.47*	*2.18*	*4.28*	*5.94*	*4.46*	*3.79*	*2.96*	*3.18*	*3.24*	*1.15*	*5.41*		
	12	13	14	14'	15	16	1'	2'	3'	4'	5'	6'	23		
2	*1.76*	*4.15*	*1.50*	*2.20*	*4.38*	*5.90*	*4.50*	*3.73*	*2.92*	*3.21*	*3.24*	*1.16*	*5.60*		
2[b]	*2.13*	*4.25*	*1.83*	*2.13*	*4.36*	*5.93*	*4.57*	*3.92*	*2.65*	*3.21*	*3.22*	*1.37*	*5.67*		
	34	35	36	37	38	39	40								
1	*2.25*	*3.06*	*1.73*	*5.19*	*1.07*	*0.88*	*1.00*								
	24	24'		25	26										
2	*2.22*	*2.38*		*4.64*	*1.26*										
2[b]	*2.26*	*2.40*		*4.75*	*1.37*										

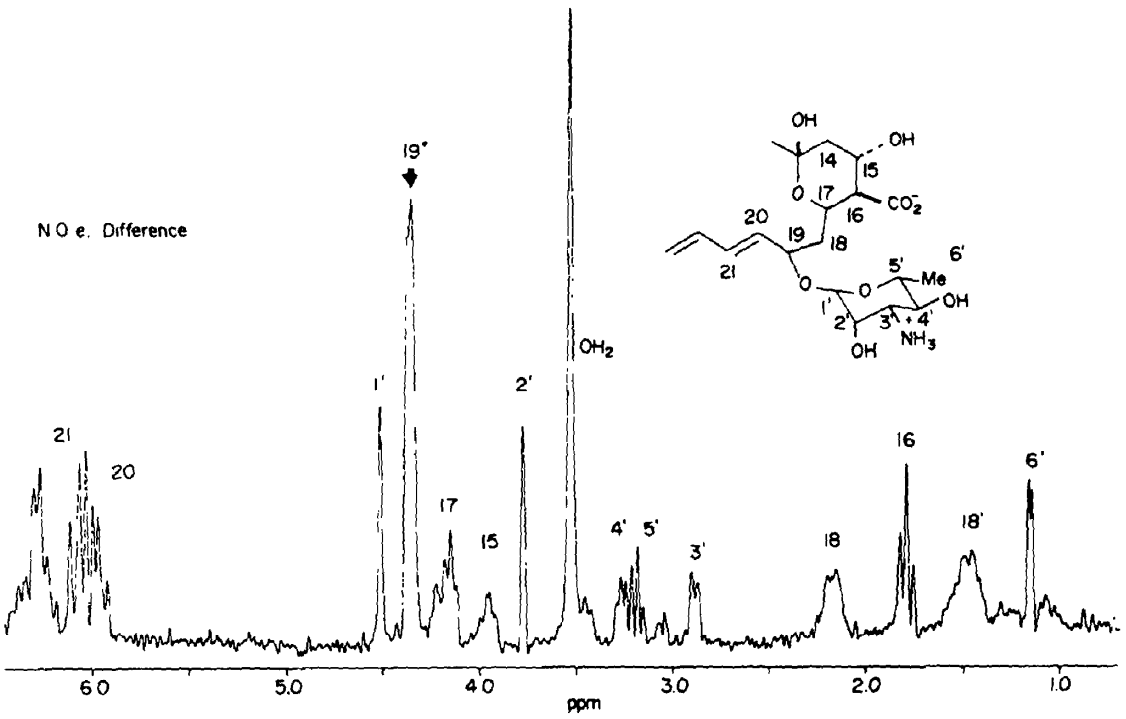


Fig. 3. Nuclear Overhauser effect on irradiation of H 19, in Amphotericin B, recorded by difference from the normal spectrum. Conditions: see experimental.

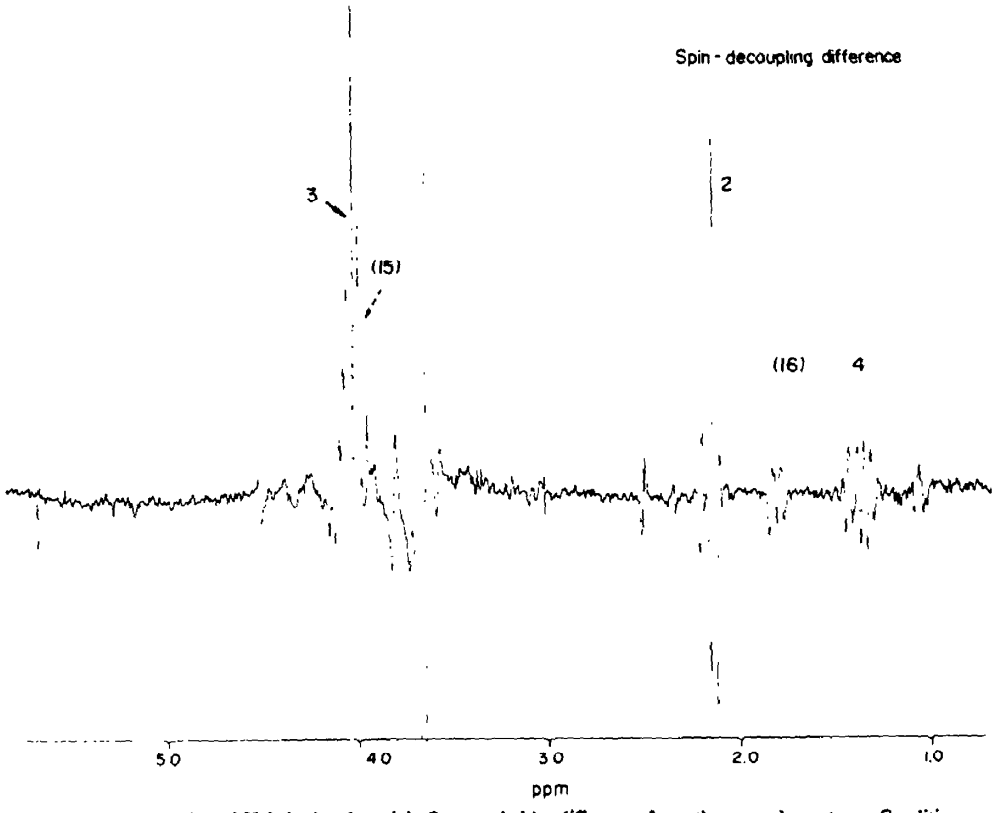


Fig. 4. Spin decoupling of H 3, in Amphotericin B, recorded by difference from the normal spectrum. Conditions: see experimental; 0.5 Hz line broadening applied.

Table 2. Comparison of ^{1}H NMR spectrum of Amphotericin B (1) with that of its methyl ester (3) and acetamide (4). Unspecified resonances lie within 0.05 ppm

	14	14'	15	16	17	18	18'	19	20	1'	2'	3'	4'	5'	6'
1	1.10	1.80	3.96	1.86	4.17	1.47	2.18	4.38	5.94	4.46	3.79	2.96	3.18	3.24	1.15
3	1.11	1.88	4.00	2.06	4.20	1.53	1.85	4.32	5.88	4.2	3.5	2.28	2.85	3.09	1.13
4	1.10	1.88	3.98	1.96	4.18	1.55	1.95	4.35	5.88	4.34	3.52	3.62	3.09	3.14	1.14

structure of macrolide–sterol complexes in solution. In d_6-dimethylsulphoxide, no complexation occurs (more strictly speaking none is detected by ^{1}H NMR or UV) and we plan to extend the investigation to perdeuterated surfactant micelles. The synthesis of their precursors is in progress.

EXPERIMENTAL

Amphotericin B was obtained from Sigma, Ltd., and pimaricin was a gift from I.C.I. Pharmaceuticals through the courtesy of Dr. N. F. Elmore. Both were stored at −30° with exclusion of light. Amphotericin B methyl ester and acetamide were prepared by published methods[14] and each gave a single spot by tlc on silica (5:2:1:3 butan-1-ol:ethanol:acetone:30% aqueous ammonia).

NMR spectra were recorded at 303 K on a Bruker WA 300 spectrometer fitted with an Aspect 2000 computer. Samples were prepared by dissolving the macrolide in d_6-DMSO (0.5 mls) and adding D_2O (2 drops). The soln was either used directly or evaporated to dryness at room temp redissolved in d_6-DmSO (0.5 ml) and D_2O (1 drop) added. Amphotericin spectra were normally collected using a 90° pulse angle, 8 K data points and an acquisition time of 1.395 sec. Resolution enhancement was achieved by using the Gaussian multiplication function available in the control program FTQNMR. In the routine the F.I.D. is multiplied by $e^{(-at-bt^2)}$ where $a = \pi LB$ and $6 = -a/(2*GB*AQ)$, AQ being the acquisition time and LB and GB being operator-entered variables. For Amphotericin B spectra values of LB = −8 and GB = 0.15 were employed.

NOE and decoupling difference spectra were measured using automated sequences which enable alternate batches of 8 pulses to be accumulated with the decoupler firstly off-resonance and then on-resonance; the former data is substracted from memory. The NOE spectra were obtained using a decoupler power of 30 L applied for 2 s. just prior to the data acquisition.† Difference decoupling spectra were recorded using a power level of 22 L applied only during acquisition, a 2 s. delay being inserted before the succeeding pulse in order to allow the NOE to decay.

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†Using shorter decoupling times down to 0.5 s($< T_1$) while increasing the time required for data collection (more scans then required for adequate signal-to-noise) had little effect on the degree of spin diffusion.

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