The Structure of Erythromycin A in [$^2H_6$]DMSO and Buffered D_2O: Full Assignments of the ^1H and ^{13}C NMR Spectra

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The ^1H and ^{13}C NMR spectra of erythromycin A in [$^2H_6$]-DMSO and buffered D_2O have been assigned using a range of one and two dimensional NMR techniques. Assignments have been made for both the major (keto) and minor forms of the drug, and the minor form has been shown to be the 12,9-cyclic hemiacetal.

Erythromycin A **1** is a clinically important member of the macrolide group of antibiotics. It consists of a 14-membered polyfunctionalized lactone ring bearing desosamine and cladinose residues. The molecule has been extensively studied; the stereochemistry of each chiral centre has been deduced from X-ray crystallographic analysis of the hydroiodide dihydrate,[1] and the solution conformation of erythromycin A and its relatives have been studied by a variety of methods[2–8] including a detailed NMR spectroscopic investigation using nuclear Overhauser effects.[9] A full and unambiguous assignment of the ^1H and ^{13}C NMR spectra of erythromycin A in $CDCl_3$ based on two-dimensional shift correlation experiments was published in 1985.[10]

The antibacterial action of erythromycin A is mediated by the binding of the drug to the bacterial ribosome, so inhibiting protein biosynthesis. The ribosomal proteins L15 and L16 are known to be important in this interaction.[11] The interactions of erythromycin A with ribosomes and with ribosomal components are amenable to study by NMR spectroscopy, and as a prerequisite to such studies the ^1H and ^{13}C NMR spectra of the drug in aqueous solution at physiological pH must be assigned. In $CDCl_3$ the drug exists almost entirely in the 9-keto form, but in aqueous solution each signal is doubled, indicating the presence of a second compound in equilibrium with the ketone. At ambient temperature the ratio of ketone to this second compound is *ca.* 5:2. In preparation for biochemical NMR spectroscopic studies we set out to assign the NMR spectra of both forms of erythromycin A, and to deduce the structure of the minor form of the drug. Preliminary conclusions from the work described below were communicated in ref. 12.

Initial 1 and 2D spectra were measured in aqueous solution. The characteristic carbonyl resonance at δ 225 in the ^{13}C NMR spectrum was a key feature in determining that the major form of the drug was the 9-ketone observed in the crystal and in $CDCl_3$ solution. This resonance was replaced by a signal at δ 109 in the minor form, suggesting the cyclization of the ketone with the 6-OH or 12-OH had occurred, giving a five-membered ring hemiacetal. In order to distinguish between these possibilities it would be necessary to assign the quaternary carbons C-6 and C-12 using long range coupling to, say, 13-H and 5-H, and then to determine which carbon had participated in ring formation and which still bore a hydroxy group. The poor solubility of erythromycin in water made long range experiments such as COLOC and HMBC impractical, and it would be particularly difficult to obtain information about the position of an OH group in this solvent. Chemical exchange experiments were also ruled out because the two compounds are in very slow exchange on the NMR timescale (no hint of line broadening was obtained on heating to 90 °C) and because the quaternary signals for the major isomer had not been assigned. An aprotic solvent in which both isomers were present was therefore sought. In most aprotic solvents only the ketone is formed, but in [$^2H_6$]-DMSO both isomers are present in a ratio of about 5:1 ketone:hemiacetal. The drug is also very soluble in this solvent. Our strategy therefore was to assign the spectra of the drug in [$^2H_6$]-DMSO solution and to determine the structure of the minor isomer. Mixed solvent experiments would then be used to complete the assignments in aqueous solution and to confirm that the structure of the minor isomer was the same as in [$^2H_6$]-DMSO.

Results

DMSO Solution.—All spectra were measured at 300 MHz proton frequency. The ^1H and ^{13}C 1D spectra are shown in Fig. 1. Initial assignments were made using the double quantum filtered COSY spectrum in conjunction with the 1D proton spectrum. Signals for protons 13, 15, 7′/8′ and the 8″ were clearly identifiable in the 1D spectrum. The spin systems 13–14–14–15, 5–4(17)–3–2–16, 7–7–8–19, 20–10–11–11-OH, 1′–2′–3′–4′–4′–5′–6′, 1″–2″–2″ and 4″(4″-OH)–5″–6″ of the major isomer could be identified from the COSY spectrum by their characteristic shift and coupling patterns. Interestingly, weak cross-peaks between 12-OH and 21 and between 6-OH and 18 (assigned using HMBC results, see below) were visible in the COSY spectrum despite the small magnitude of the four bond couplings involved and the intermediate exchange rate of the OH protons. In the 1D spectrum, even extreme resolution enhancement failed to reveal any residual splitting of the methyl signals or any narrowing of the methyl resonances on irradiation of the OH protons. In the proton spectra of the minor isomer, the same spin systems were identified, with the exceptions that cross-peaks linking protons 7 and proton 8, and between OH and methyl signals, were too weak to detect.

The remaining assignments required evidence from heteronuclear experiments, notably the HMBC technique. Apart from carbons 1 and 9, which show characteristic shifts, initial ^{13}C assignments were made using the heteronuclear shift correlation spectrum in conjunction with the proton shifts already identified. This allowed assignment of carbons 2–5, 7, 8, 10, 11, 13–15, 1′–8′, 1″, 2″, 4″, 5″ and 8″ in the major isomer, and carbons 2–5, 11, 13, 1′–5′, 7′/8′, 1″, 2″, 4″, 5″ and 8″ in the minor isomer. Evidence of long-range ^1H–^{13}C couplings from the HMBC spectrum (data were processed with a number of

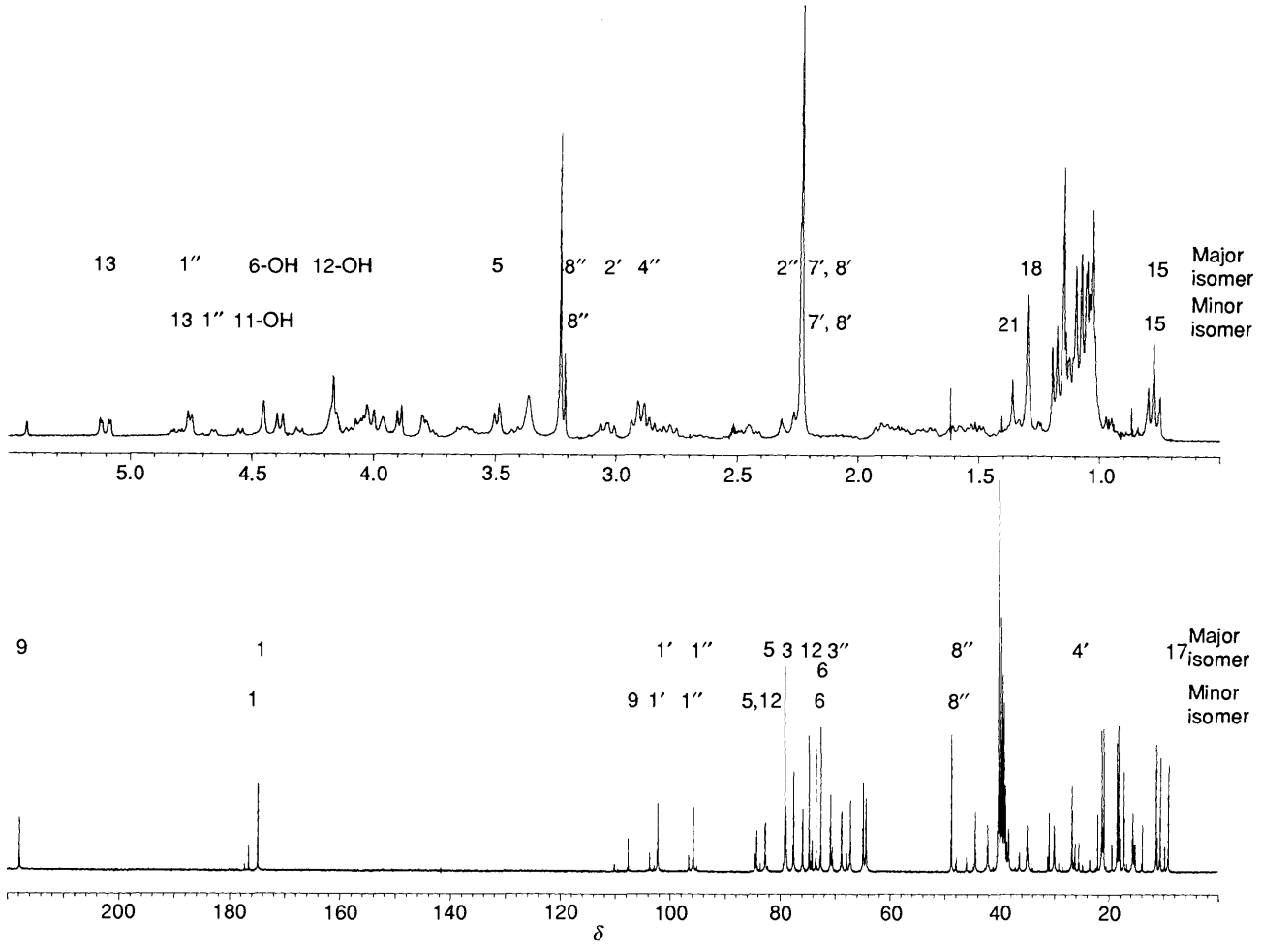


Fig. 1 300 MHz proton (top) and 75 MHz ^{13}C (bottom) spectra of a saturated solution of erythromycin A in [$^2H_6$]-dimethyl sulphoxide. Selected signals are identified.

different weighting functions depending on the magnitude of the coupling expected and the linewidths of the ^1H multiplets concerned) was then used to identify carbons 6, 12, 16–21 and 3″ in the major isomer and 6, 7, 9, 10, 12, 14 and 19–21 in the minor. Having assigned the ^1H signal of methyl-19 in this way, the assignment of proton- and carbon-8 in the minor isomer followed from the COSY data. The HMBC results also allowed the assignment of the proton signals 18, 21, 6-OH and 12-OH in the major isomer and 18, 21 and 9-OH in the minor.

At this stage the only remaining uncertainties in the shifts of the major component were carbons 6″ and 7″, and protons 7″ and 2′-OH. The assignment of the rest of the methyl signals by HMBC resolved the ambiguity over the position of carbon 6″ since only one CH correlation peak in the shift correlation spectrum remained at this proton shift, and hence by process of elimination the only remaining CH_3 cross-peak must be that of 7″. The key to assigning the remaining OH signals lay in the NOESY spectrum. Although numerous (negative) NOEs were seen in the NOESY spectrum, these were not needed for assignment purposes. More useful were the cross-peaks attributable to exchange amongst the OH proton and residual water signals. These allowed the immediate identification of the missing 2′-OH signal from the major isomer proton spectrum, and by analogy also that from the minor isomer spectrum (see Table 1).

In the minor isomer, the assignments lacking at this point were carbons 16–18, 6′, 3′, 6″ and 7″, and protons 6″, 7″ and 6-OH. The missing OH was identified as that on carbon 6, and the nature of the hemiacetal linkage 9–12 confirmed, by measuring a decoupled ^{13}C spectrum on a sample of erythromycin in a [$^2H_6$]-DMSO solution containing a small amount of a mixture of H_2O and D_2O (the SIMPLE experiment[13,14]). This showed doubling of the signals of all carbons bearing OH groups, because of the secondary isotope effect on chemical shift, establishing that in the minor isomer C-6 carries a hydroxy group but C-12 does not; the shift of the proton 6-OH was determined from the last remaining minor component OH peak in the NOESY spectrum. The H_2O/D_2O exchanged ^{13}C NMR spectrum also identified 3″ in the minor isomer by its chemical shift and lack of doublet structure. Comparison of the remaining minor isomer CH_3 peaks in the heteronuclear shift correlation spectrum with the ^1H assignments and the combined ^1H and ^{13}C NMR data for the methyls 6′, 6″ and 7″ in the major form gave the assignments of 17, 6′, 6″ and 7″. These assignments removed the uncertainty over methyl 16, identified by COSY cross-peak with proton 2, leaving methyl 18 as the sole signal remaining unaccounted for, and finally completing the assignment of the minor isomer of erythromycin (see Table 2).

Aqueous Solution.—Initial assignments were made using phase-sensitive COSY and HOHAHA (Homonuclear Hartmann Hahn) spectra in conjunction with the 1D proton spectrum. Erythromycin A is poorly soluble in aqueous media (20 mmol dm^{-3} at pH 7.4) so these spectra were run at 500 MHz, and a COSY spectrum was also run at 600 MHz. Signals due to protons 13, 15, 1′, 7′/8′, 1″, 5″ and 8″ were clearly distinguishable.

For the major isomer, the spin systems 13–14–14–15, 11–10–20, 7–7–8–19, 1′–2′–3′–4′–4′–5′–6, 1″–2″–2″ and 4″–5″–6″ were identifiable from their characteristic chemical shifts a[1] cross-

Table 1 1H and ^{13}C spectra of erythromycin A ketone

Site	δ_C(DMSO)	$\delta_C(D_2O)$	δ_{OH}(DMSO)	δ_H(DMSO)	$\delta_H(D_2O)$		J_{HH}/Hz (500 MHz D_2O spectrum)
1	174.8	178.0					
2	44.4	45.4		2.77	3.07	dq	8.5, 7.2
3	78.9	81.0		4.01	3.83	dd	8.5, 2.0
4	39.1	38.0		1.90	2.03	d quint	7.2, 2.0
5	82.6	86.4		3.49	3.57	d	7.2
6	73.4	75.1 [a]	4.45				
7	38.3	37.7		1.71	1.94	dd	14.5, 9.2
				1.31	1.61	dd	14.5, 4.0
8	39.8	44.7		2.85	2.79	m	
9	217.8	225.0					
10	42.2	*d*		2.88	3.30	dq	6.9, 2.0
11	68.8	70.0	3.89	3.79	3.82	d	2.0
12	74.7	76.6 [a]	4.16				
13	75.8	78.4		5.10	5.07	dd	10.75, 2.5
14	21.2	21.7		1.82	1.82	ddd	14.3, 7.5, 2.5
				1.38	1.55	m	
15	10.6	10.9		0.78	0.84	t	7.5
16	15.6	15.6		1.10	1.22	d	7.2
17	9.1	9.8		1.02	1.08	d	7.4
18	26.7	26.5		1.30	1.37	s	
19	18.2	18.5		1.04	1.19	d	7.3
20	11.3	12.1		1.06	1.12	d	6.9
21	17.2	17.4 [b]		1.03	1.21 [c]	s	
1′	102.2	102.6		4.38	4.61	d	7.2
2′	70.8	70.1	3.95	3.03	3.52	dd	10.7, 7.2
3′	64.4	65.5		2.45	3.48	ddd p.obs.	10.7, 4.5
4′	30.0	30.4		1.59	2.12	ddd	16.5, 4.5, 2.5
				1.06	1.54	m p.obs.	
5′	67.1	68.7		3.62	3.88	dq	6.5, 2.5
6′	21.3	20.9		1.08	1.29	d	6.5
7′8′	40.2	39.8		2.22	2.85	s	
1″	95.8	97.5		4.75	4.93	d	4.8
2″	34.9	35.0		2.28	2.51	dd	15.3, 1.4
				1.51	1.68	dd	15.3, 4.8
3″	72.5	74.0 [a]					
4″	77.5	77.8	4.16	2.90	3.23	d	9.45
5″	64.8	66.5		4.06	4.15	dq	9.45, 6.5
6″	18.5	18.6		1.18	1.31	d	6.5
7″	20.9	21.1		1.14	1.24 [c]	s	
8″	48.8	49.9 [b]		3.21	3.31	s	

[a,b,c] May be exchanged. [d] Assignment uncertain, see text. p.obs. = partially obscured.

peaks in the COSY and HOHAHA spectra. The spin system 5–4(17)–3–2–16 could also be assigned although no cross-peak between 3-H and 4-H was detected. For the minor isomer, the same spin systems could be identified, but, in this case, the 3-H/4-H cross-peak was quite distinct. A cross-peak between 7-H and 8-H could be detected only in the 600 MHz COSY spectrum, in which it was quite weak. Thus, only the methyl singlets 21-H_3, 18-H_3 and 7″-H_3 remained to be assigned at this stage.

In the ^{13}C NMR spectrum, signals due to C-9 and C-1 were assigned on the basis of chemical shift. DEPT spectra and a ^1H–^{13}C correlation spectrum allowed the assignment of much of the rest of the spectrum, but some of the cross-peaks in this spectrum were weak or undetectable and the following ambiguities and uncertainties remained.

(i) C-11 appeared to have very different shifts in the two isomers (δ 70.0 and 85.3).

(ii) Only a single cross-peak for 5-H was observed in the heteronuclear correlation spectrum, although there were two peaks (one of which was rather broad) in the ^{13}C NMR spectrum.

(iii) No peak was observed in the heteronuclear correlation spectrum for either 8-H (minor) or 10-H (major).

(iv) Analogy with the DMSO spectrum suggests that the small CH_2 peak at δ 25.1 should be assigned to C-14 (minor) and that at δ 39.4 to C-7 (minor). The high field signal, however, gives a single weak cross-peak (which could, perhaps, be an artefact) to δ 1.3 which corresponds to 7-H (minor). The lower field signal has no cross-peaks.

(v) The isolated methyl groups 18-CH_3, 21-CH_3 and 7″-CH_3 show cross-peaks in the heteronuclear correlation spectrum such that the three pairs of peaks corresponding to the methyl groups in the major isomer are at δ 17.40/1.26, δ 21.28/1.20 and δ 26.48/1.37 but cannot be distinguished. Similarly for the minor isomer the peaks are at δ 21.40/1.20, δ 24.41/1.22 and δ 26.6/1.42.

(vi) The three quaternary signals for each isomer C-3″, C-6 and C-12 appeared at δ 74.0, 76.6 and 75.1 (major) and δ 74.3, 85.4 and 75.5 (minor) but could not be distinguished.

Points (i) and (ii) were resolved using an HMQC experiment on a partially ^{13}C enriched sample of erythromycin (a gift from Professor D. E. Cane). This sample was prepared biosynthetically and was enriched in carbons 1, 3, 5, 7, 9, 11 and 13. Carbons 1 and 9 are quaternary, so in the HMQC spectrum ^1H–^{13}C cross-peaks were seen only for carbons 3, 5, 7, 11 and 13. Eight ^{13}C signals, as expected, appeared in the methine region. The cross-peaks were used to confirm the assignments of C-11 and C-5, for which two distinct peaks could be identified. It should be noted that the NMR spectra of erythromycin A (in particular the ^{13}C spectra) are very sensitive to slight changes in sample preparation and running conditions. Changes in chemical shifts of up to 0.1 ppm were observed from one spectrum to another even when there were no discernible difference in composition, pH or temperature of the sample. All

Table 2 1H and ^{13}C spectra of erythromycin A 12,9-hemiacetal

Site	δ_C(DMSO)	$\delta_C(D_2O)$	δ_{OH}(DMSO)	δ_H(DMSO)	$\delta_H(D_2O)$		J_{HH}/Hz (500 MHz D_2O spectrum)
1	176.4	180.4					
2	46.0	48.3		2.64	2.90	m obs.	
3	78.7	82.0		4.13	3.94	dd	8.6, 5.25
4	39.7	41.8		2.16	2.15	m obs.	
5	84.2	85.0		3.42	3.54	d obs.	
6	74.1	75.5[a]	4.35				
7	36.37	39.4		1.91	1.89	dd obs.	
				1.06	1.32	dd obs.	
8	36.35	*d*		2.10	2.14	dd obs.	
9	107.6	108.8					
10	47.9	47.4		2.04	2.25	quint.	7.2
11	84.5	85.3	4.54	3.75	4.03	d	7.2
12	84.2	84.2					
13	78.9	83.0		4.80	4.85	dd	11.0, 5.0
14	23.5	25.1		1.84	1.89	m obs.	
				1.68	1.78	m obs.	
15	11.1	12.0		0.78	0.88	t	7.5
16	15.3	16.3		1.07	1.28	d obs.	
17	9.7	11.5		1.01	1.08	d obs.	
18	25.5	24.4[b]		1.14	1.22[c]	s	
19	19.4	18.2		0.95	1.11	d obs.	
20	15.1	14.3		1.05	1.13	d	7.2
21	26.1	26.6		1.35	1.42	s	
1′	103.7	103.5		4.30	4.55	d	7.3
2′	70.5	69.7	4.01	3.07	3.52	dd	11.5, 7.3
3′	64.2	65.8		2.50	3.49	ddd p.obs.	11.5, 4.25
4′	30.0	30.4		1.61	2.16	m p.obs.	
				1.12	1.57	m p.obs.	
5′	67.8	69.5		3.52	3.83	m obs.	
6′	21.0	20.9		1.11	1.33	d	6.2
7′8′	40.2	40.3		2.22	2.84	s	
1″	96.6	99.0		4.65	4.87	d	4.25
2″	34.8	35.7		2.28	2.48	d	16.0
				1.49	1.69	dd	16.0, 4.25
3″	72.5	74.3[a]					
4″	77.5	78.0	4.16	2.89	3.24	d	9.5
5″	64.8	66.7		4.08	4.20	dq	9.5, 6.5
6″	18.0	18.1		1.16	1.28	d p.obs.	6.5
7″	20.9	21.4[b]		1.14	1.24[c]	s	
8″	48.8	50.2		3.19	3.29	s	

[a,b,c] May be exchanged. [d] Assignment uncertain, see text. obs. = obscured. p.obs. = partially obscured.

the ^{13}C chemical shifts, especially those derived from the HMQC experiment, are therefore of limited precision.

In the methylene region there was only one large broad ^{13}C peak, with an extended series of cross-peaks to δ 1.3–2.5 in the ^1H spectrum. This suggests strongly that the ^{13}C signals for C-7 of the two isomers lie close together leaving the signal at δ 21.8 to be assigned to C-14 (minor).

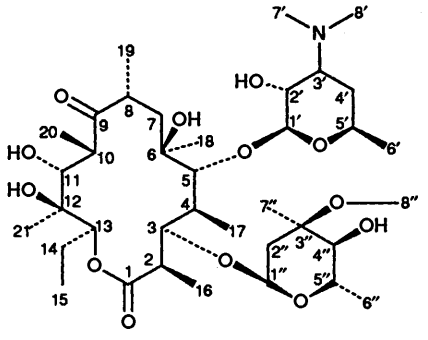
HMBC experiments on the dilute aqueous samples were unsuccessful. Attempts were made to assign the remaining signals using mixed solvent experiments. Mixed solvent ^{13}C experiments were also unsuccessful; the solubility of the drug in DMSO–D_2O mixture was poor and the DMSO signal led to dynamic range problems. Mixed solvent ^1H experiments were successful, however, and no large changes in chemical shift were observed on going from DMSO through 1:4, 2:3, 1:1, 3:2, 4:1 DMSO–D_2O and finally 100% D_2O. This result confirmed that both isomers had the same structures in aqueous as in DMSO solution. C-12 and 21-H_3 of the minor isomer and 18-H_3 of the major isomer could therefore be assigned with reasonable certainty on the basis of chemical shift.

It seems likely that C-10 (major) lies under the C-7′, 8′ signal and that C-8 (minor) is coincident with C-8 (major) but these tentative assignments could not be confirmed. Several ambiguities also remained in the assignments of the quaternary carbons and the isolated CH_3 peaks. The major objective, the determination of the structures of both isomers in aqueous solution had, however, been achieved; these structures are the 9-ketone **1a** and the 12,9-hemiacetal, **1b**.

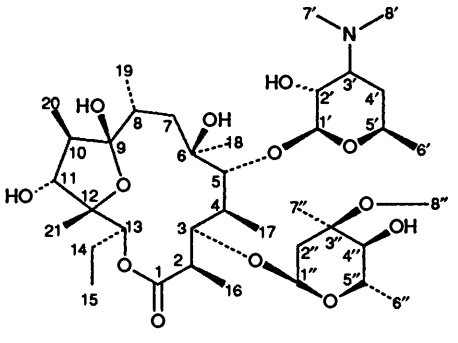
Effects of Temperature.—As mentioned in the introduction, the mixture of isomers is in slow exchange on the NMR timescale. When the sample was heated no line broadening appeared in the ^1H spectrum, but the relative proportions of the isomers changed. In aqueous solution at 50 °C the ketone:hemiacetal ratio is 5:1; at 4 °C it is about 1:1 (see Fig. 2). This probably reflects the more constrained structure of the hemiacetal which is unfavoured at higher temperature. Above 50 °C a third compound, a very minor component at room temperature, becomes more abundant. We have not attempted to identify this compound but the identification as a very minor component of erythromycin A in DMSO solution as the 6,9-hemiacetal is described in the adjacent paper.[15]

Molecular Modelling.—Molecular modelling was used in an attempt to determine the likely stereochemistry at C-9 in the hemiacetal. When the two possible structures were minimized the *S* stereochemistry for the ring junction was preferred (by 37.5 kcal mol^{-1})* over *R* stereochemistry, suggesting that the structure of the minor isomer is that shown in **1b**. Experimental data also suggesting *S* stereochemistry has been obtained using

* 1 cal = 4.184 J.



1a



1b

an erythromycin derivative and this is presented by Everett and co-workers in the adjacent paper.[15]

Discussion

Surprisingly, the aqueous solution structure of erythromycin A has not previously been discussed in the literature, although the crystal structure has been known for 25 years. We have now demonstrated that in aqueous solution, and therefore, presumably, in the cell, the drug exists in at least two forms, the ketone and the 12,9-hemiacetal. (We have observed trace amounts of a third compound in spectra of erythromycin A but have not identified it.) The two isomers are in slow exchange, and may have different biological properties. In particular we have shown that *E. coli* ribosomes are able to bind the major isomer only in a fast exchange process.[16] Cyclized derivatives of erythromycin A have been reported to be instrumental in inducing resistance to the drug.[17] The elucidation of the structures of both isomers is of potential importance in the design of erythromycin analogues. It may be possible to develop improved drugs by designing structures in which hemiacetal formation is prevented.

Experimental

Erythromycin A (Boehringer) was recrystallised twice from chloroform–hexane, and dissolved in [$^2H_6$]-DMSO or buffered D_2O (60 mmol dm^{-3} sodium phosphate, 200 mmol dm^{-3} potassium chloride, apparent pH 7.4). All spectra were run using saturated solutions (200 mg cm^{-3} in DMSO, 15 mg cm^{-3} in aqueous buffer) except for 1D ^{1}H experiments in D_2O for which solutions of 3 mg cm^{-3} were used).

DMSO Solution.—Spectra were measured at 30 °C, using the 5 mm proton and 10 mm broadband probes of a Varian XL300 spectrometer. Standard methods were used to acquire proton and carbon-13 1D, ^{13}C DEPT, non-quaternary suppressed ^{13}C 1D, double quantum filtered COSY, NOESY, relayed coherence transfer, ^{1}H–^{13}C heteronuclear shift correlation and HMBC spectra.

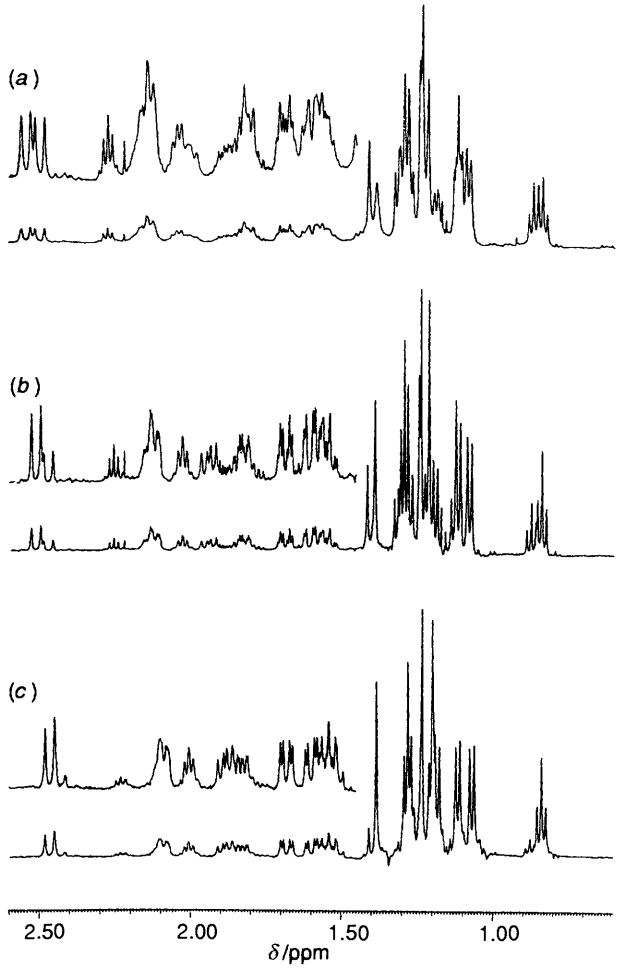


Fig. 2 500 MHz proton spectra (upfield region) of (*a*) 4 mmol dm^{-3} erythromycin A in deuteriated phosphate buffer at 4 °C; (*b*) the same sample at ambient temperature; (*c*) the same sample at 50 °C

Aqueous Solution.—Samples were made up in 50 mmol dm^{-3} sodium phosphate, 200 mmol dm^{-3} KCl in D_2O, apparent pH 7.4. Saturated solutions were used, except for 1D ^{1}H spectra, when the sample contained 4 mmol dm^{-3} erythromycin A. Spectra were measured at 27 °C using the 5 mm proton and 10 mm broadband probes of a Bruker AM-500 spectrometer, except for a ^{1}H–H COSY spectrum which was run using the 5 mm proton–carbon dual probe of a Varian VXR 600S spectrometer. Standard methods were used to acquire proton and carbon-13 1D, ^{13}C DEPT, non-quaternary suppressed ^{13}C 1D, and phase-sensitive COSY spectra.

The HOHAHA spectra were acquired in 5 h using the MLEV17 pulse sequence as suggested by Bax and Davis.[18] The spin-lock field was achieved by using a decoupler setting of 3 H, 90° pulse = 14 μs giving a spin-lock field of 17 kHz. Optimum mixing time was found to be 54 ms.

The ^{13}C spectrum with proton detection was obtained using the method suggested by Bax and Subramanian.[19] A BIRD sequence was introduced at the beginning of the polarisation transfer scheme to achieve better suppression of protons bound to ^{12}C. The experiment was optimised for a J_{H-C} of 135 Hz. The 2D spectrum took 16 h to accumulate.

Molecular Modelling.—Molecular modelling was carried out on a Silicon Graphics IRIS 4D/240 GTX hardware system using the Polygen QUANTA and CHARMm software.

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