An Analysis of the ^1H and ^{13}C N.m.r. Spectra of Erythromycin A using Two-dimensional Methods

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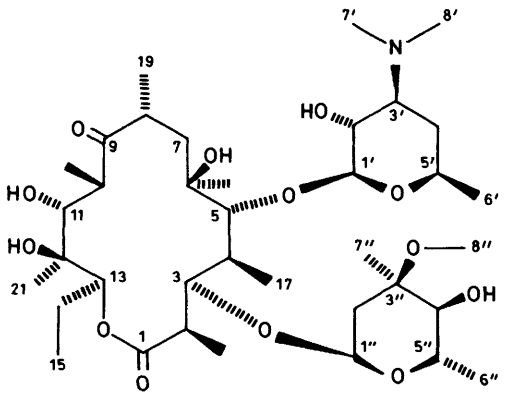
An unambiguous and almost complete assignment of the carbon-13 and proton n.m.r. spectra of erythromycin A (**1**) in deuteriochloroform has been made using two-dimensional (2D) chemical-shift correlation methods.

Erythromycin A (**1**) is a commercially important, naturally occurring member of the macrolide class of antibiotics. The compound is composed of a poly-functionalised 14-membered lactone ring substituted with desosamine and cladinose sugar units. The stereochemistry at each asymmetric centre is known from an *X*-ray crystallographic analysis[1] of the hydroiodide dihydrate of (**1**). In view of the medical importance of the erythromycin antibiotics the solution conformations of these molecules and their aglycones have been studied intensively[2-8] by many techniques including n.m.r. spectroscopy. The usefulness of proton and ^{13}C n.m.r. spectroscopy in this area is unquestioned. However, difficulties have arisen in the interpretation of the spectra. The proton n.m.r. spectrum of (**1**) is complex and overlapped. No complete assignment has been published although incomplete data (23 out of 41 ^1H resonances assigned) is available.[2,9] Four complete or nearly complete interpretations of the ^{13}C n.m.r. spectrum of (**1**) have been published.[7,8,10,11] All four assignments are different and there is no indication as to which is the most reliable.[12,13] We therefore



(**1**)

embarked on a program of one-dimensional (1D) and 2D n.m.r. experiments in order to obtain complete and unambiguous assignments of the ^1H and ^{13}C n.m.r. spectra of (**1**).

Results

Table 1 lists all the proton and carbon-13 n.m.r. chemical shifts and all the proton-proton coupling constants for (**1**) in $CDCl_3$.

Discussion

Method of Analysis.—The ^1H n.m.r. spectrum of (**1**) in $CDCl_3$ was too complex and overlapped even at 400 MHz for proton-to-proton *J*-connectivities to be established by selective decoupling experiments. These *J*-connectivities were, therefore, determined by 2D COSY-45 ^1H n.m.r. experiments[14] at 400 MHz. Having established these connectivities, the assignment of the ^1H n.m.r. spectrum was unambiguous and complete except for two hydroxy protons.

The ^{13}C n.m.r. spectrum of (**1**) in $CDCl_3$ was then assigned by the use of a 2D ^{13}C, ^1H COSY experiment[14-16] which linked the ^1H and ^{13}C n.m.r. resonances of directly bonded (one-bond) protons and ^{13}C nuclei. Nearly all the protonated carbon resonances were assigned from one 2D spectrum. In order to resolve ambiguities in the assignment of two pairs of carbon resonances a 2D relayed coherence transfer experiment[17] (2D ^{13}C, ^1H, ^1H RELAY) was performed. This RELAY experiment establishes connectivities between ^{13}C nuclei and directly bonded ^1H nuclei (*via* $^1J_{CH}$) as well as more remote ^1H nuclei linked to the directly bonded ^1H nuclei *via* homonuclear coupling. The RELAY experiment thus contains elements of both the 2D ^1H COSY and the 2D ^{13}C, ^1H COSY experiments. In addition to resolving the aforementioned ambiguities, the RELAY experiment also served as a check on the results of the other two experiments.

The values of all the homonuclear proton coupling constants were measured from resolution-enhanced 400 MHz and 500 MHz 1D ^1H n.m.r. spectra.

Choice of Solvent.—Deuteriochloroform is normally the solvent of choice for n.m.r. studies of (**1**) and related compounds[1,3,5,6,8,10-13] and was, therefore, used in this work. Erythromycin A is not very soluble in $CDCl_3$ but it does reside almost exclusively in the C-9 ketone form in this solvent.[18]

2D ^1H COSY-45 Spectra.—The 400 MHz COSY-45 2D ^1H n.m.r. spectrum of (**1**) in $CDCl_3$ is shown in Figure 1 as a contour plot beneath the 1D ^1H n.m.r. spectrum. The experiment was run with the pulse sequence 90°-t_1-D2-45°-D2-FID, where t_1 was the incremented evolution time and D2 was set to zero. The 'normal' spectrum runs along the diagonal (F1 = F2) from lower left to upper right. The multitude of off-diagonal cross peaks established the connectivities between all pairs of mutually coupled protons. An expansion of the upper right hand quadrant of the spectrum is shown in Figure 2, together with the analysis of connectivities in the 19-H, 8-H, 7eq-H, 7ax-H spin-system (bold lines). This isolated CH_3–CH–CH_2 spin-system is unique in the molecule and could be assigned unambiguously. The rest of the COSY spectrum was analysed in the same way and an unambiguous assignment of all the protons of (**1**) was achieved with the exception of the 6-OH and 2′-OH hydroxy protons. Figure 3 shows the proton connectivities found in (**1**). 18-H Resonated as an isolated singlet and was assigned by default. A considerable number of long-range connectivities over 4 and 5 bonds were found in the COSY-45 ^1H n.m.r. spectrum. These connectivities

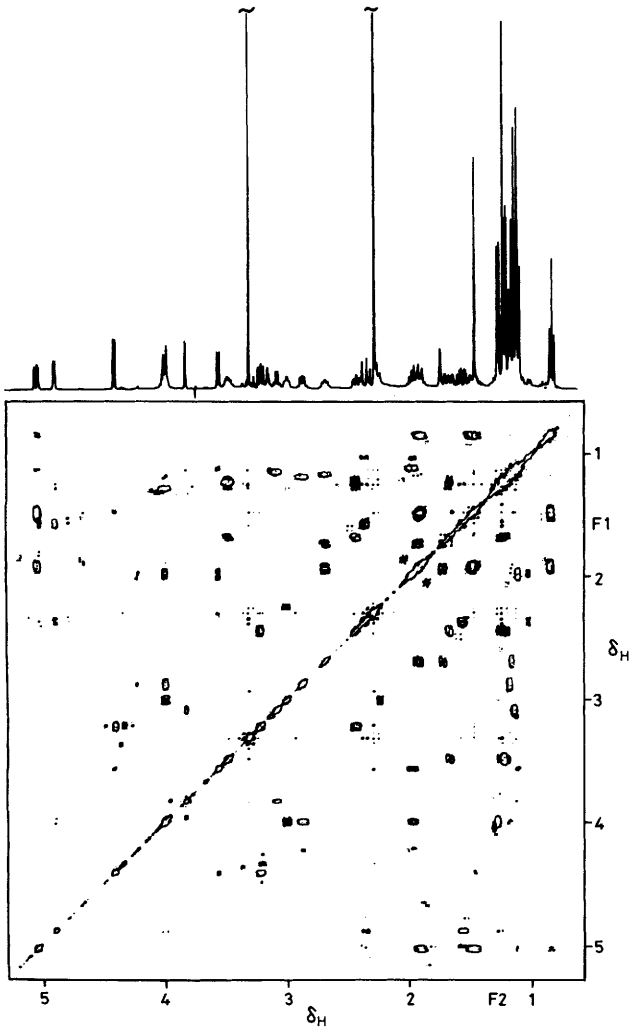


Figure 1. The 400 MHz 2D ^{1}H COSY-45 n.m.r. spectrum (D2 = 0) of (**1**) in $CDCl_3$, as a contour plot beneath the corresponding 1D ^{1}H n.m.r. spectrum.

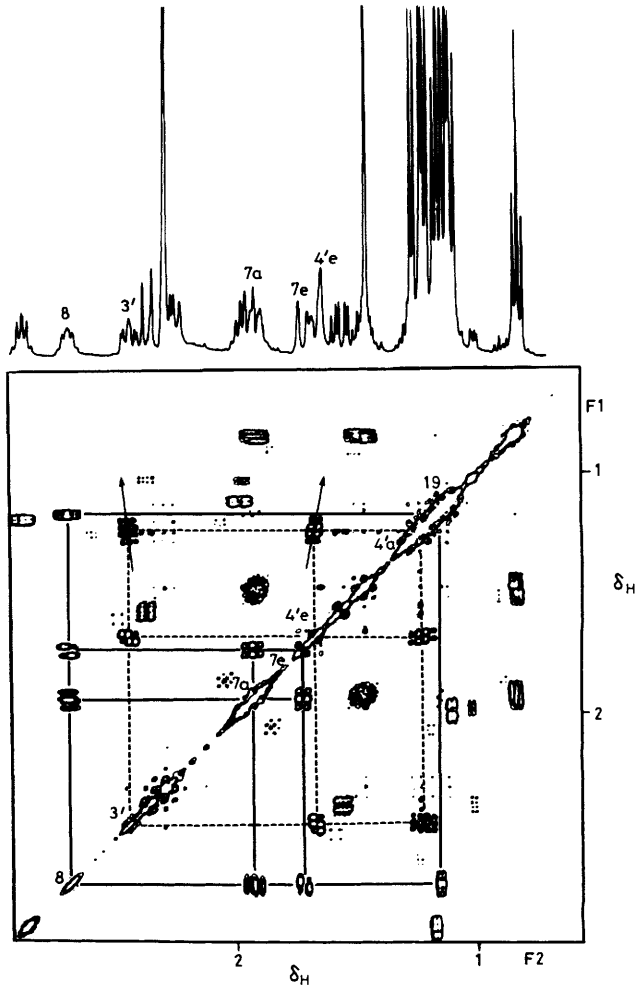


Figure 2. An expansion of the high field quadrant of the 2D ^{1}H COSY-45 n.m.r. spectrum (D2=0) of (**1**) in $CDCl_3$, as a contour plot beneath the corresponding 1D spectrum. The solid lines (—) and broken lines (– – –) trace out the cross peaks due to *J*-connectivities in the 19-CH_3, 8-H, 7ax-H, 7eq-H and the 4'ax-H, 4'eq-H, 3'-H spin systems respectively. The arrows on two of the cross peaks serve to indicate their opposite slope.

were verified by repeating the experiment with D2 = 0.25s, thus enhancing effects due to small couplings. Some of the weak cross peaks seen in Figures 1 and 2 are due to these long-range connectivities but many of them are due to the presence of small amounts of another species in solution. This species could not be removed by repetitive re-crystallisation. A useful feature of the COSY-45 experiment is that the relative signs of coupling constants in certain types of spin-systems can be obtained by inspection of the slopes of the cross-peaks.[19] This is clearly seen for the 3'-H, 4'eq-H, 4'ax-H spin-system in Figure 2 (dashed lines) where the cross-peaks due to geminal coupling ($^2J < 0$) have the opposite slope to the cross-peaks due to vicinal coupling ($^3J > 0$). Finally, it will be noted that the digital resolution and processing functions chosen for the COSY spectra allowed the resolution of multiplet structure in most of the cross peaks. This was a most valuable aid to the assignment of the cross peaks, especially in crowded regions of the spectrum where resonances were overlapped.

2D ^{13}C, ^{1}H *COSY N.m.r. Spectrum.*—The 63, 250 MHz ^{13}C, ^{1}H COSY spectrum of (**1**) in $CDCl_3$ is shown in Figure 4 as a contour plot. Cross-peaks occur in this spectrum at location δ_{Hi}, δ_{Ci} where H_i and C_i are directly bonded proton and carbon-13 nuclei. Since the assignment of the proton n.m.r. spectrum was complete, this 2D spectrum gave an almost complete analysis

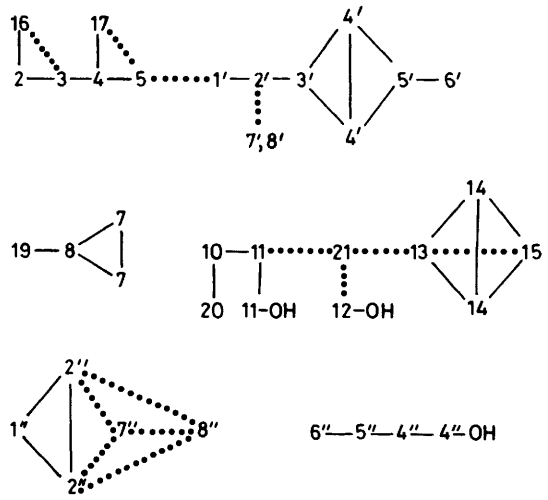


Figure 3. A diagram of the proton connectivities found in the 2D ^{1}H COSY-45 n.m.r. spectra of (**1**) in $CDCl_3$. The bold lines indicate connectivities *via* ^2J and ^3J whilst the dotted lines indicate connectivities via ^4J and ^5J.

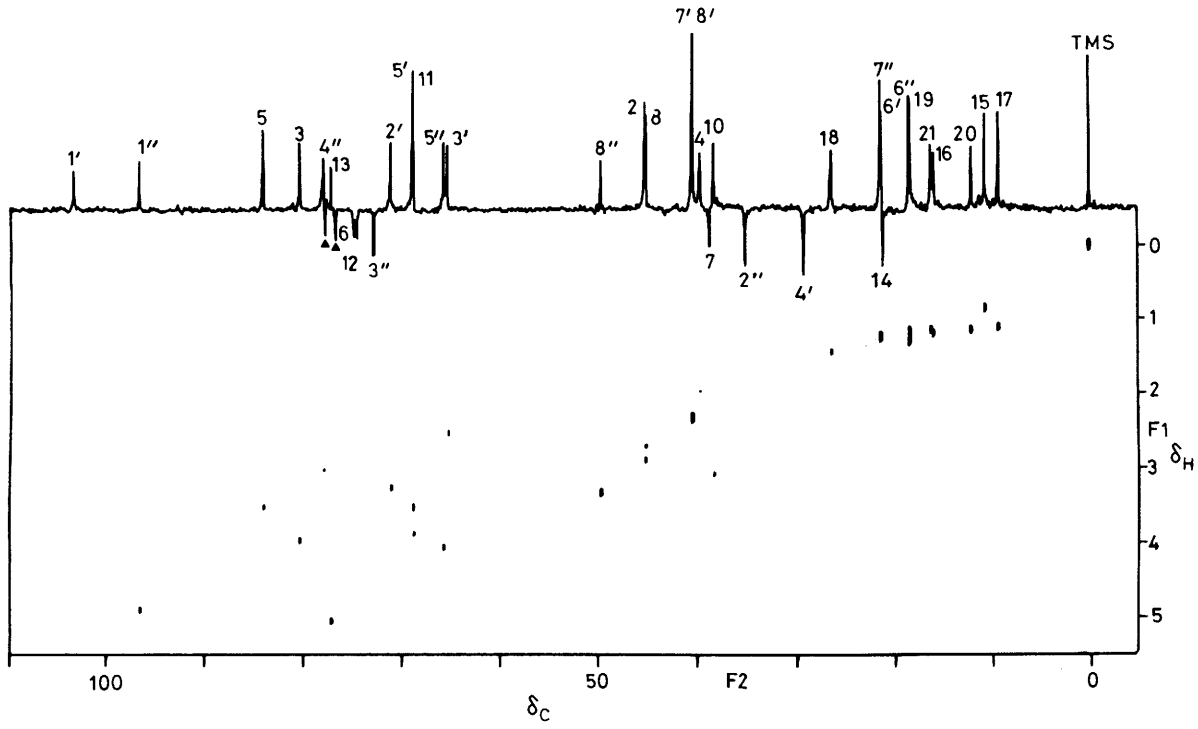


Figure 4. The 2D ^{13}C, ^{1}H COSY n.m.r. spectrum of (**1**) in $CDCl_3$ as a contour plot beneath the corresponding 1D ^{13}C spin-echo n.m.r. spectrum. The spin-echo spectrum (pulse sequence: 90°-τ-180°-τ-ACQUIRE) was acquired with broadband proton decoupling during the second τ period and data accumulation. With $\tau = 8$ ms CH_2 and C resonances are inverted relative to CH_3 and CH resonances. The small triangles (▲) indicate the two outer lines of the solvent triplet. The contour plot levels are higher than the cross peaks due to methylene moieties.

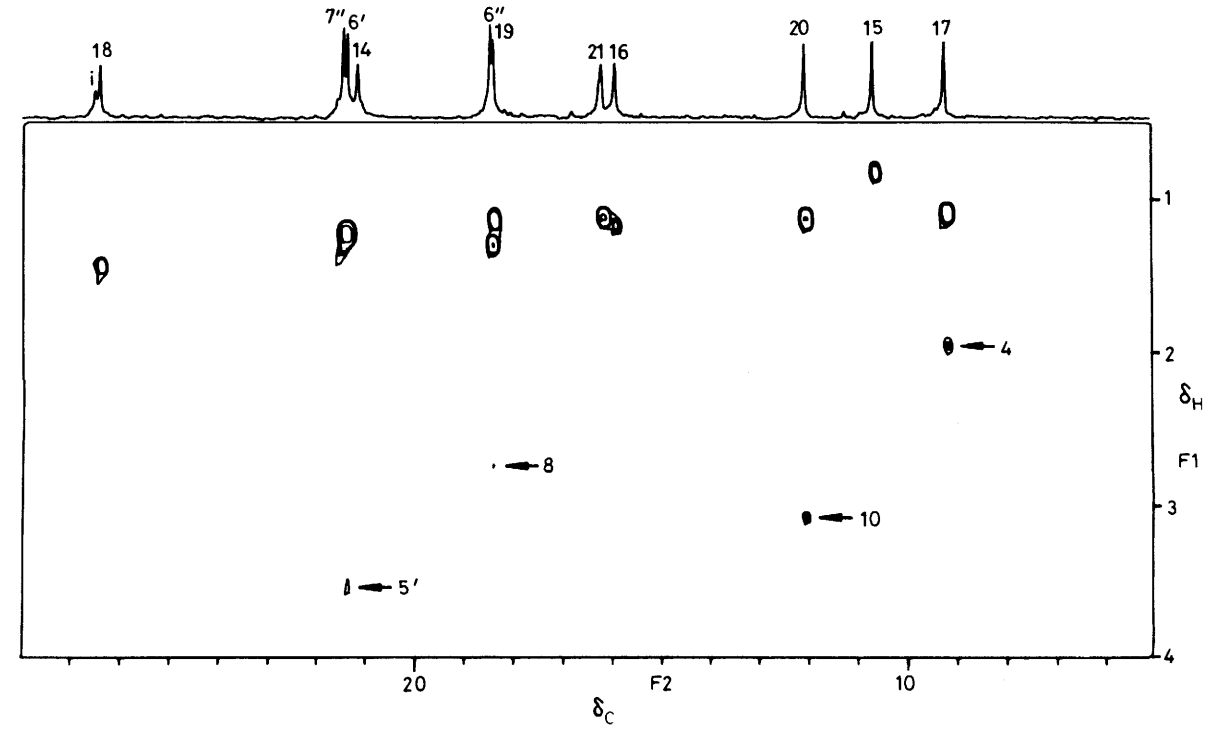


Figure 5. The highfield portion of the 2D ^{13}C, ^{1}H, ^{1}H RELAY n.m.r. spectrum of (**1**) in $CDCl_3$ as a contour plot beneath the corresponding 1D DEPT-45 ^{13}C n.m.r. spectrum. Peaks due to relayed coherence transfer are indicated with horizontal arrows. The peak marked 'i' is due to an 'impurity'.

A Table of ^1H and ^{13}C n.m.r. chemical shifts and ^1H, ^1H coupling constants for erythromycin A in $CDCl_3$

Position	δ_H in (p.p.m.)	M[a]	$^nJ_{HH}$ (Hz)				δ_C (p.p.m.)	
1″	4.88	d,br			$^3J \sim$ b	$^3J \sim 4.5$	96.5	
2″eq	2.35	d,d	$^2J \sim 15.2$		$^3J \sim 0.8$		35.0	
2″ax	1.56	d,d	$^2J \sim 15.1$		$^3J \sim 5.0$			
3″	—	—					72.7[d]	
4″-OH	2.23	d			$^3J \sim 10.1$		—	Cladinose
4″	3.00	d,d			$^3J \sim 9.7$	$^3J \sim 9.7$	77.9	
5″	3.99	d,q			$^3J \sim 6.2$	$^3J \sim 9.4$	65.7	
6″	1.27	d			$^3J \sim 6.2$		18.46	
7″	1.23	s					21.43	
8″	3.31	s					49.5	
1′	4.40	d		$^3J \sim 7.2$			103.3	
2′	3.21	d,d		$^3J \sim 7.3$	$^3J \sim 10.3$		71.1	
3′	2.43	d,d,d		$^3J \sim 12.3$	$^3J \sim 10.2$	$^3J \sim 3.9$	65.3	
4′eq	1.665	d,d,d	$^2J \sim 12.5$		$^3J \sim 2.2$	$^3J \sim 3.9$	29.2	Desosamine
4′ax	1.22	d,d,d	$\Sigma J \sim 37$					
5′	3.48	d,d,q		$^3J \sim 6.1$	$^3J \sim 2.1$	$^3J \sim 10.8$	68.81	
6′	1.22	d		$^3J \sim 6.1$			21.35	
7′, 8′	2.29	s					40.3	
1	—	—					176.3	
2	2.87	d,q		$^3J \sim 9.5$	$^3J \sim 7.1$		45.02	
3	3.99	d,d		$^3J \sim 9.4$	$^3J \sim 1.4$		80.3	
4	1.97	d,d,q		$^3J \sim 7.5$	$^3J \sim 1.5$	$^3J \sim 7.5$	39.5	
5	3.56	d		$^3J \sim 7.8$			84.0	
6	—	—					74.78[d]	
7ax	1.93	d,d	$^2J \sim 14.9$	$^3J \sim 11.9$			38.54	
7eq	1.74	d,d,d	$^2J \sim 14.9$		$^3J \sim 2.4$	$^4J \sim 1.0$[c]		
8	2.68	d,d,q		$^3J \sim 11.4$	$^3J \sim 2.3$	$^3J \sim 7.0$	44.93	
9	—	—					221.9	
10	3.08	d,q		$^3J \sim 1.4$	$^3J \sim 6.9$		38.1	
11	3.82	d,br		$^3J \sim 1.2$			68.76	
11-OH	3.95	s,v,br					—	Lactone
12	—	—					74.45[d]	ring
12-OH	3.13	s,br					—	
13	5.03	d,d			$^3J \sim 11.0$	$^3J \sim 2.3$	77.1	
14eq	1.91	d,d,q	$^2J \sim 14.2$	$^3J \sim 7.5$		$^3J \sim 2.4$	21.16	
14ax	1.475	d,d,q	$^2J \sim 14.3$	$^3J \sim 7.3$	$^3J \sim 11.0$			
15	0.84	t		$^3J \sim 7.4$			10.7	
16	1.175	d,br			$^3J \sim 7.8$		15.9	
17	1.10	d				$^3J \sim 7.4$	9.2	
18	1.46	s,br					26.4	
19	1.155	d				$^3J \sim 7.1$	18.40	
20	1.135	d			$^3J \sim 6.9$		12.0	
21	1.12	s,br					16.2	

[a] Multiplicity of ^1H resonance. [b] Unresolved, < 1 Hz. [c] $^4J_{7eq6-OH}$. [d] Ref. 11.

of the ^{13}C n.m.r. spectrum of (**1**). However, unambiguous assignments could not be achieved for C-7″, C-6′, C-21, and C-20 since the proton chemical shifts of the pairs 7″-H, 6′-H and 20-H, 21-H were very close. Naturally, no non-protonated carbon-13 nuclei may be observed in this type of experiment. These non-protonated ^{13}C nuclei were assigned on the basis of chemical shifts.[7,8,10,11]

2D ^{13}C, ^1H, ^1H *RELAY Spectrum.*—The highfield region of the 63 250, 250 MHz relayed coherence transfer spectrum of (**1**) in $CDCl_3$ is shown in Figure 5. Relayed coherence transfers from 6′-CH_3 to 5′-H and from 20-CH_3 to 10-H are indicated. These served to resolve the ambiguities remaining from the simple ^{13}C, ^1H COSY spectrum. Other transfers (not shown) served to corroborate previous assignments for many other resonances. The ^{13}C assignments given in the Table are different in some respects from all previous reports[7,8,10,11] but are close to those given by Egan *et al.*[11]

Conclusions.—An unambiguous and almost complete analysis of the proton and carbon-13 n.m.r. spectra of erythromycin A in deuteriochloroform has been made for the first time. Two-dimensional chemical shift correlation experiments were invaluable in unravelling connectivities in the complex and crowded n.m.r. spectra.

Experimental

The erythromycin A base was obtained from K & K—Greef Chemicals Ltd. and was recrystallised twice from $CHCl_3$–hexane before use. The material was homogeneous as judged by t.l.c. and h.p.l.c.

Proton n.m.r. data at 250, 400 and 500 MHz were acquired at ambient temperature (21—23 °C) for 20—60 mM $CDCl_3$ solutions in 5 mm o.d. n.m.r. tubes on Brüker WM 250, WH 400, and AM 500 n.m.r. spectrometers. The 2D ^1H, ^1H COSY n.m.r. spectra were acquired at 400 MHz with sweep widths of 2 000

Hz into 2 048 data points in F2. The 90° pulse was 9.8 μs, the relaxation delay was 1.5 s and each F.I.D. was acquired with 32 scans and 4 dummy scans. 512 Values of the evolution time were sampled but the data was zero-filled to 1 024 points in F1 prior to double Fourier transformation with unshifted sine-bell squared window functions in both dimensions. Carbon-13 n.m.r. data were acquired at 63 MHz and ambient temperature for a 0.14M-solution in a 5mm o.d. n.m.r. tube on a Bruker WM 250 n.m.r. spectrometer. The 2D ^{13}C, ^{1}H COSY and 2D ^{13}C, ^{1}H, ^{1}H RELAY n.m.r. data was acquired with sweep widths and data points of 8 064 Hz, 2 048 pts. and 2 016 Hz, 128 pts. in F2 and F1 respectively. The 90° pulses for ^{1}H and ^{13}C were 15.0 and 9.5 μs respectively. Each FID was acquired with 160 scans and a relaxation delay of 1.5 s. Both experiments were acquired using standard Bruker microprograms which gave ^{1}H decoupling in F2. However, the COSY experiment was acquired with ^{1}H decoupling in F1 as well. Both experiments were tuned for optimum polarisation transfer with $^{1}J_{CH}$ values of $\simeq$ 140 Hz. The RELAY experiment was tuned to give optimum relayed coherence with $^{n}J_{HH}$ values of 7 Hz. The data matrix of each experiment was zero-filled to 4 096 × 512 points prior to double Fourier transformation with unshifted sine-bell window functions in both dimensions. Increasing the solution concentration to 0.14M caused small shifts in some proton resonances ($|\Delta\delta_{av.}| = 24 \pm 24$ ppb) but these were too small to cause problems. The ^{13}C n.m.r. sample was dissolved in CD_3OD and then evaporated to dryness prior to dissolution in $CDCl_3$.

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