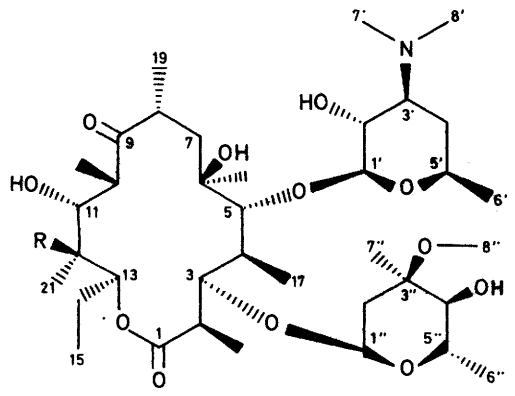
The Conformational Analysis of Erythromycin A

Jeremy R. Everett * and John W. Tyler
Beecham Pharmaceuticals, Brockham Park, Betchworth, Surrey, RH3 7AJ

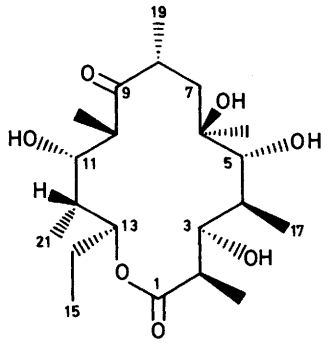
^1H and ^{13}C n.m.r. spectroscopy combined with molecular modelling techniques were used to show that the major conformation of erythromycin A (**1**) in $CDCl_3$ solution is very similar to the crystalline state conformation (**A**) of erythromycin A hydroiodide dihydrate. However, ^1H nuclear Overhauser enhancement (n.O.e.), variable-temperature and variable-solvent n.m.r. experiments showed that, in contrast to previous reports, the major conformation of (**1**) is in fast equilibrium with a second, minor conformation. This minor conformation is related to the major conformation *via* a reorganisation of the macrocyclic lactone ring in the C-2 to C-9 region.

^{13}C N.m.r. relaxation measurements showed that the desosamine sugar possesses more conformational freedom than the cladinose sugar, in agreement both with previous results and with energy calculations based on the crystal structure. In addition, the relaxation experiments indicated that some methyl groups in (**1**) are sterically hindered whereas others possess motional freedom. A good agreement was found between the n.m.r. relaxation results and calculations of methyl group rotational energy barriers in the crystal structure. This good agreement provided further evidence of the similarity between the major solution-state conformation of (**1**) and the crystalline-state conformation **A**.

Erythromycin A (**1**) is an important member of the macrolide class of antibiotics. The compound is composed of a polyfunctionalised 14-membered lactone ring substituted with desosamine and cladinose sugar units. In view of their medical importance, the solution conformations of the erythromycin antibiotics and their aglycones have been studied by many techniques including n.m.r. spectroscopy. The crystalline-state conformation (**A**) of (**1**) has been known since 1965 from an *X*-ray analysis of the hydroiodide dihydrate.[1] The first ^1H n.m.r. data on the solution conformation of (**1**) were published in 1969.[2] An analysis of ^1H n.m.r. ^3J values was used to establish that erythronolide B (**2**), (**1**), and erythromycin B (**3**) all adopted the Celmer-Dale, diamond-lattice conformation in solution. However, further ^1H n.m.r. and circular dichroism studies from the same group concluded that the lactone rings of (**1**) and (**3**) as well as several aglycones were in the 'Perun' conformation [derived from an alternate diamond-lattice and very similar to the crystalline-state conformation (**A**)] and that this conformation was stable.[3–5] These results were contradicted when Egan *et al.* showed that (**2**) and some of its derivatives were conformationally flexible in the C-6 to C-9 region of the lactone ring, as well as exhibiting some differences between their C-4–C-5 torsion angles.[6] The earlier thesis of Egan[7] had already concluded that whilst the solution-state and crystalline-state conformations of the lactone ring of (**1**) were the same, it was not possible to rule out a conformational equilibrium in the C-6 to C-9 portion of the molecule in solution. Nourse and Roberts applied ^{13}C n.m.r. spectroscopy to the problem of the solution conformation of (**1**) but with limited success because of incorrect assignments.[8] Ōmura *et al.*[9] used tenuous ^{13}C n.m.r. evidence to conclude that the aglycones of (**1**) and (**3**) adopted the 'Perun' conformation in solution. ^{13}C N.m.r. relaxation time measurements were used to show that the desosamine sugars in the cyclic 11,12-carbonate of (**1**)[10] and (**1**) itself[11] had more motional freedom than the cladinose sugars. The entire literature was reviewed in 1984.[12] In summary, the solution-state conformation of the lactone ring of (**1**) is expected to be the same as the crystalline-state conformation (**A**) although there is the possibility of a conformational equilibrium in the C-6 to C-9 portion of the molecule. However, all the previous n.m.r. work was severely hampered by two main factors; firstly, incomplete and/or incorrect n.m.r. spectral assignments and secondly, the use of vicinal coupling constants, $^3J_{HH}$, to probe the solution conformations, thus obtaining no information on the overall orientations of the sugar rings and no information on proton-proton spatial proximity. In this



(**1**) R = OH
(**3**) R = H



(**2**)

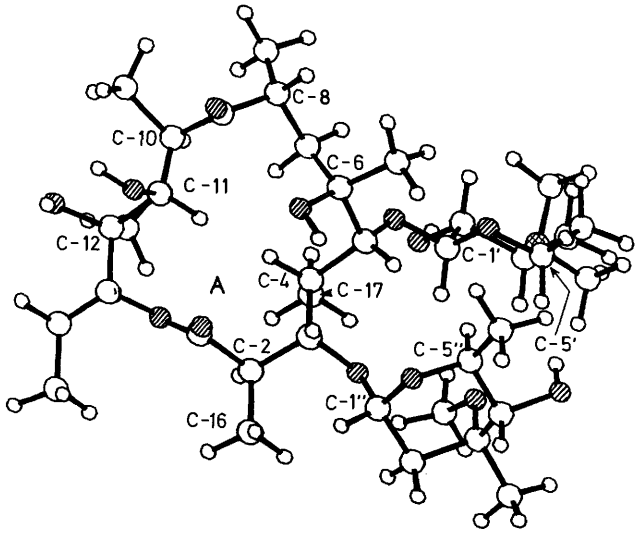


Figure 1. A view of the crystal structure of erythromycin A hydroiodide dihydrate. Oxygen atoms are shaded and the nitrogen atom is cross-hatched

work these problems were overcome. First, the preliminary 2D n.m.r. studies of (**1**) had established unambiguous assignments of the ^1H and ^{13}C n.m.r. spectra.[13] Secondly, in this work, ^1H n.O.e. difference spectroscopy was used as the main tool for conformational analysis. The n.O.e. between two protons H_A and H_B is proportional to the inverse sixth power of the internuclear distance r_{AB}.[14] The n.O.e. thus drops off rapidly as r_{AB} increases but is an excellent monitor of spatial proximity ($r_{AB} \leqslant \sim 4$ Å) and is independent of the number of chemical bonds separating H_A and H_B. Hence close spatial connectivities between protons in structurally remote regions may be monitored. Using the ^1H n.O.e.s, information was obtained for the first time on proton-to-proton spatial proximity and on the orientations of the sugar rings of (**1**) in the solution state. The orientation and mobilities of the sugar rings are important in considerations of biological activity. In particular, the flexibility of the desosamine ring is thought[15] to help initiate complexation between the antibiotic and its site of action — the bacterial 50S ribosomal subunit. In this work, the mobilities of the sugar rings were probed using ^{13}C n.m.r. relaxation time experiments. A preliminary report of this work has already appeared.[16]

Results and Discussion

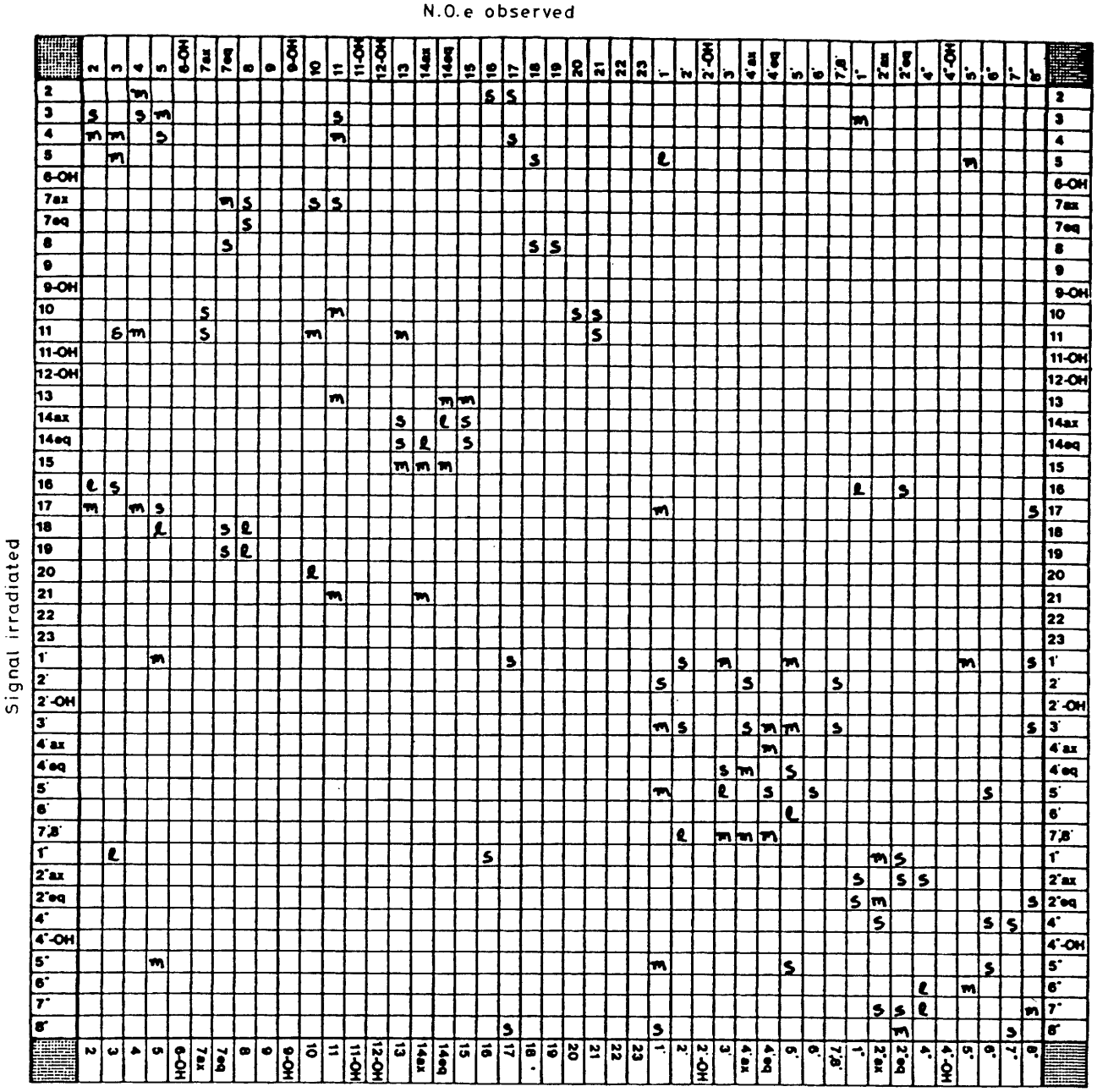
Crystal Structure of Erythromycin A Hydroiodide Dihydrate.—A view of the crystal structure[1] of erythromycin A hydroiodide dihydrate is shown in Figure 1. Several points are of interest. First, as has been previously noted[15] the sugar rings are orientated approximately perpendicularly to the macrocyclic lactone ring with Me-6″ and Me-6′ pointing up. However, as can be seen, the orientation of the cladinose sugar is flatter than the desosamine sugar. This orientation of the sugars may be designated 'up up'. Owing to steric crowding the bulky sugar rings are not free to rotate and therefore three other rotational isomers of (**1**) are possible, corresponding to 'up down', 'down up', and 'down down' orientations of the sugars. It has been assumed[7,15] that owing to their bulky nature the sugars of (**1**) will be in the same 'up up' orientation in solution. There is no experimental evidence to support this assumption except for some very tenuous ^{13}C n.m.r. data.[8] Secondly, the two sugar rings are themselves each in chair conformations with the maximum number of substituents in the more stable equatorial positions on the rings. Thirdly, the macrocyclic lactone ring is in a conformation such that 11-H is close in space to 4-H ($r_{4,11} \sim 2.2$ Å) even though they are separated by nine bonds in the ring.

Solution Conformations of (**1**).—(i) *Conformation of the sugar rings.* The sugar rings are known to adopt the same chair conformations observed in the crystal structure (Figure 1) on the basis of the observed vicinal proton-proton coupling constants.[7] These former results have been confirmed.[13] The large values of the axial couplings $^3J_{2',3'}$, $^3J_{3'4'ax}$, $^3J_{4'ax,5'}$, and $^3J_{4'',5''}$ (9.7—12.3 Hz)[13] indicated little or no population of the ring-inverted chair conformations of the two sugar rings.

Several other points are of note. First, the large value of $^3J_{4'',4''\text{-OH}}$ indicated that the 4″-OH proton was *trans*-diaxial to 4″-H. Secondly, the observation of n.O.e.s from Me-8″ to Me-7″ and 2″eq-H *i.e.* n.O.e. [8″]7″ and n.O.e. [8″]2″eq but not n.O.e. [8″]5″ [Figure 2(b)] showed that the solution conformation of the methoxy group on cladinose was the same as in the crystal structure with Me-8″ close in space to Me-7″ and 2″eq-H but pointing away from 5″-H (Figure 1). Thirdly, the observation of n.O.e.s from Me-7′,8′ to 2′-H, 3′-H, 4′eq-H, and 4′ax-H was consistent with the 3′-NMe_2 group on desosamine occupying the same conformation in solution as in the crystal structure (Figure 1).

(ii) *Conformation of the lactone ring and the orientation of the sugar rings.* (a) *Conformation of the lactone ring.* A comparison was made between the vicinal proton-proton coupling constants found for (**1**) in $CDCl_3$ solution[13] and the corresponding dihedral angles in the crystal structure (Table 4). Large dihedral angles ($|\varphi| > 150°$) in the crystal were associated with large $^3J_{HH}$ values in solution whereas dihedral angles close to $\pm 90°$ were associated with small $^3J_{HH}$ values (Table 4). This was the result expected on the basis of a crude Karplus-type ^3J analysis if the solution-state and crystalline conformation (**A**) were similar. Furthermore the extreme values of some of the vicinal couplings *e.g.* $^3J_{2,3}$, $^3J_{3,4}$, $^3J_{7ax,8}$, $^3J_{10,11}$, and $^3J_{13,14ax}$ showed that any motional averaging in the macrocyclic lactone ring was limited or did not involve the single bonds which could be monitored by $^3J_{HH}$ values. These results agreed with previous n.m.r. analyses.[7] However, it was obvious that much more information was required in order to define adequately the solution conformation of (**1**). Vicinal proton-proton coupling constants could only monitor the torsion angle relationships between protons on adjacent carbon atoms. No longer-range information was obtained. In order to overcome this problem ^1H n.O.e. experiments were used to probe the molecule and determine which pairs of protons were spatially proximate in solution. Figure 2(a) shows the n.O.e. difference ^1H n.m.r. spectrum[17] for (**1**) upon irradiation of 11-H. The n.O.e.s observed at 10-H, 13-H, and Me-21 indicate the spatial proximity of 11-H to these protons in solution as in the crystal structure. More interestingly, the n.O.e.[11]4 shows that the close, cross-ring approach of 11-H and 4-H observed in the crystal structure (Figure 1) is also present in solution. Similarly, n.O.e.[11]7ax indicates that these two protons are spatially proximate in solution. The very small n.O.e.[11]3 which would not be predicted on the basis of the crystal structure will be returned to later. Many more n.O.e. experiments were performed in order to obtain as complete a picture of the solution conformation of (**1**) as possible. The results are shown in Table 1 in the form of a matrix. These one-dimensional (1D) experiments were quite time-consuming. In order to investigate whether or not alternative two-dimensional (2D) experiments might have been more time efficient, both normal and phase-sensitive 2D ^1H NOESY experiments were performed on (**1**) in $CDCl_3$ solution. The normal 2D NOESY experiment was not

Table 1. ^{1}H N.O.e.s for (**1**) in $CDCl_3$[a]



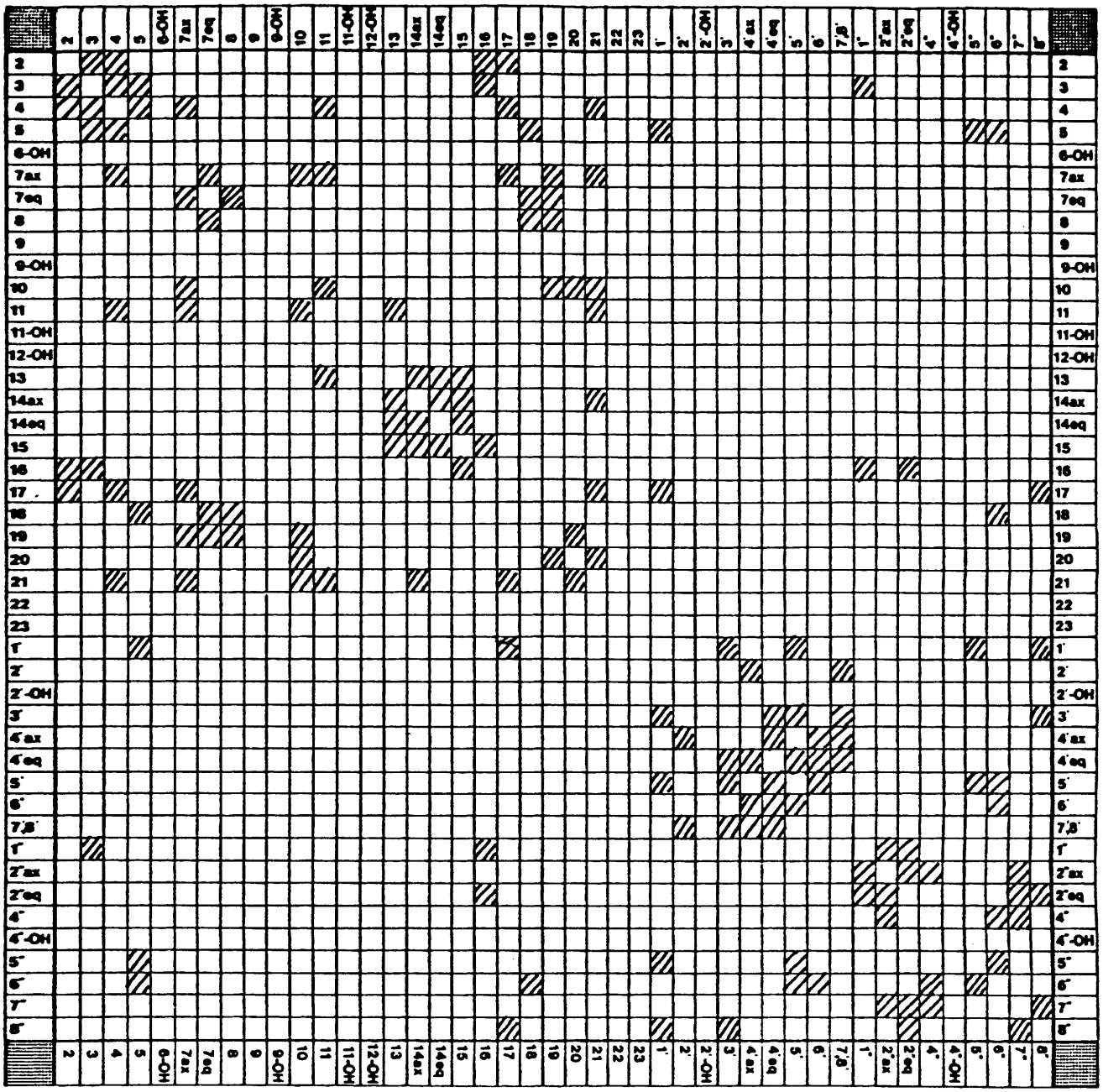
[a] S = small (0—2%), m = medium (2—5%), l = large (>5%) effect.

useful. However, the phase-sensitive 2D ^{1}H NOESY experiment[18] (Figure 4) was useful for confirming the corresponding 1D results (especially where resonance overlap was a problem) but the sensitivity of the 2D method was lower and artefact suppression more problematical than in the 1D experiments.

(b) *Orientation of the sugar rings.* The n.O.e. difference experiments proved to be even more powerful in solving the problem of the orientation of the sugar rings and revealed sugar–lactone, intra-sugar, and inter-sugar interactions. Figure 3 shows a typical n.O.e. difference spectrum obtained by the irradiation of 1′-H. As expected, on the basis of the desosamine sugar ring conformation, n.O.e.s are observed for 3′-H and 5′-H which are both 1,3-diaxial to 1′-H (Figure 1). More interestingly two inter-sugar n.O.e.s were observed — n.O.e. [1′]5″ and n.O.e. [1′]8″ as well as two sugar–lactone n.O.e.s — n.O.e.[1′]5 and n.O.e.[1′]17. These results showing the spatial proximity of 1′-H to 5-H, Me-17, 5″-H, and Me-8″ are exactly those expected if the two sugars had the same 'up up' orientation as in the crystal structure (Figure 1). These data represent the first unambiguous determination of the orientations of the sugar rings in an erythromycin molecule.

Comparison of the Solution-state and Crystalline-state Conformations of (**1**).—The matrix of n.O.e. results obtained on (**1**) in $CDCl_3$ solution (Table 1) represents a 'spatial proximity map' for pairs of protons in the solution-state conformation. In order to compare the solution-state conformation with the crystalline-state conformation (**A**) a similar spatial proximity map was constructed for pairs of protons in the crystal structure

Table 2. Pairs of protons less than 3 Å apart (shaded squares) in the crystalline-state conformation of erythromycin A hydriodide dihydrate [conformation (**A**)]



(Table 2), using distance calculation algorithms in the molecular modelling program suite 'MODEL'.[19] An arbitrary cut-off distance of 3.0 Å was used in the calculations. Thus Table 2 represents a map of all those pairs of protons less than 3.0 Å apart in the crystal structure. If the solution-state conformation was exactly the same as the crystalline-state conformation (**A**) and if all the necessary n.O.e. experiments could be performed then the two matrices should be exactly superimposable (Tables 1 and 2). However, comparison of the two matrices showed that this was not the case. The crystal structure matrix showed 162 contacts (81 proton pairs, times 2) whereas the solution-state matrix contained only 122 n.O.e.s. Of these 122 n.O.e.s, 114 corresponded to interactions between protons less than 3 Å apart in the crystal structure, a remarkable level of agreement. However, eight n.O.e.s were observed for which no corresponding crystal structure contact existed and 48 crystal structure contacts were found with no corresponding n.O.e. contact. Of the eight outstanding n.O.e.s five were small *trans*-diaxial interactions and one was a small n.O.e.[17]5 all of which were compatible with the crystal structure ($r_{HH} < 3.3$ Å) and were excluded merely because of the arbitrary nature of the cut-off distance (3.0 Å) applied. Of the 48 missing n.O.e.s, 30 were 'missing' due to technical difficulties (selective irradiation or selective observation impossible). A further 15 missing n.O.e.s

Table 3. Vicinal coupling constants $^3J_{HH}$ (in Hz) for (**1**) in various solvents

	Solvent							
^3J	$CDCl_3$ 292 K[a]	$CDCl_3$ 323 K[a]	CD_2Cl_2	CD_3OD	$[^2H_6]$DMSO 340 K	$[^2H_7]$DMF	$[^2H_5]$Pyridine	$[^2H_6]$Acetone
$^3J_{2,3}$	9.3	9.0	9.2	9.1	8.5	9.0	9.1	9.2
$^3J_{3,4}$	1.4	1.7	1.5	1.2	*b*	~1—2	*b*	1.7
$^3J_{4,5}$	7.7	7.4	7.7	7.8	7.3	7.5	7.8	7.3
$^3J_{7e,8}$	2.3	2.7	2.3	*b*	*b*	~6	*b*	3.3
$^3J_{7a,8}$	11.3	11.0	11.3	8.4	5.9	~7	8.4	10.2
$^3J_{10,11}$	1.4	1.5	1.3	1.3	*b*	1.8	1.3	1.8
$^3J_{13,14e}$	2.3	2.6	2.4	2.4	2.7	2.2	2.4	2.5
$^3J_{13,14a}$	11.0	10.6	11.1	10.9	10.3	10.8	10.9	11.0

[a] D_2O Shake solution. [b] Not clearly resolved.

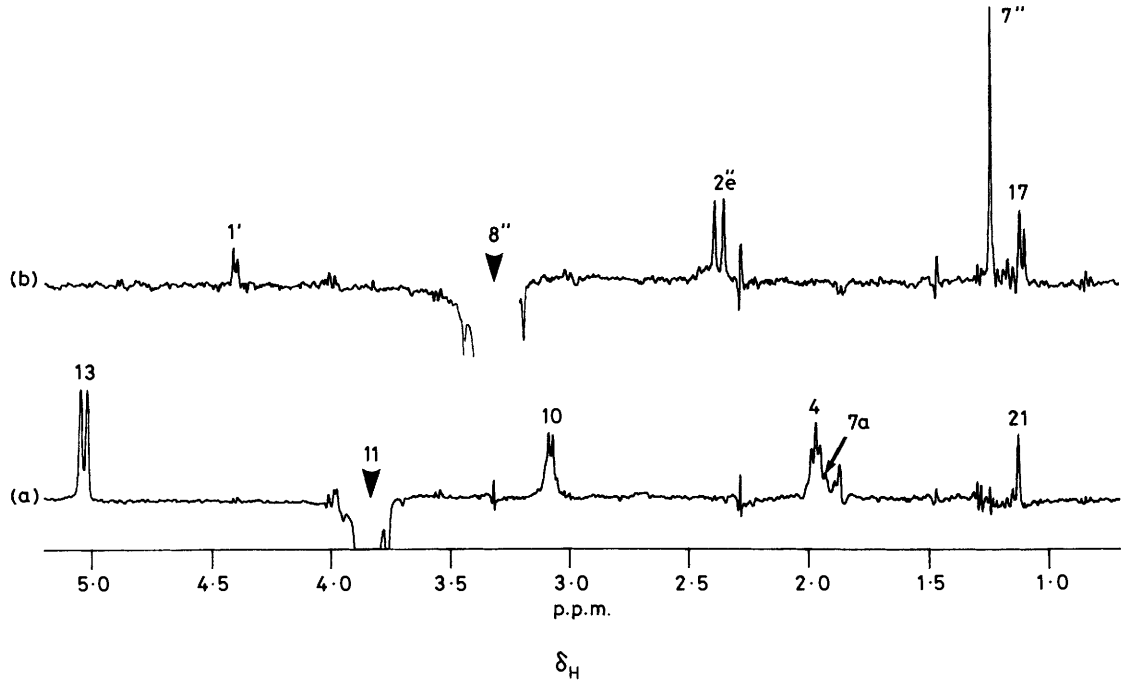


Figure 2. 400 MHz ^1H N.O.e. difference spectra of (**1**) in $CDCl_3$–TMS produced by irradiation of (a) 11-H and (b) Me-8″

were n.O.e.s to methyl groups or methylene protons. These latter n.O.e.s are inherently weak[17] and are frequently not observed. This left only three n.O.e.s not observed but expected on the basis of the crystal structure—n.O.e.[8″]3′, n.O.e.[6″]5, n.O.e.[5′]5″, and two n.O.e.s observed but unexpected—n.O.e.[11]3 and its partner n.O.e.[3]11. These discrepancies will be discussed in turn as they are of importance in the determination of the solution conformation of (**1**).

(i) *N.O.e.*[8″]3′. In conformation (**A**), $r_{8'',3'}$ is ~ 2.3 Å minimum* but n.O.e.[8″]3′ was not observed and n.O.e.[3′]8″ was very small (<0.5%). In view of the fact that a medium-sized n.O.e.[1′]3′ was observed [Figure 3(b), $r_{1,3'}$ ~ 2.5 Å] the lack of n.O.e.[8″]3′ [Figure 2(b)] indicated that in solution $r_{8'',3'} > 3$ Å.

(ii) *N.O.e.*[6″]5. In the crystal structure $r_{6'',5}$ 2.97 Å minimum, thus, on average $r_{6'',5} > 3$ Å due to methyl group rotation. The non-observation of n.O.e.[6″]5 may thus simply have been due to the relatively large distance involved or may indicate that Me-6″ and 5-H are further apart in the solution state than in the crystalline state.

(iii) *N.O.e.*[5′]5″. In the crystal structure $r_{5'',5'}$ ~ 2.1 Å, $r_{5'',5}$ ~ 2.3 Å, and $r_{1',5'}$ ~ 2.5 Å. Medium-sized n.O.e.[5]5″ and n.O.e.[1′]5′ and a small n.O.e.[5″]5′ were observed. Since n.O.e.[5′]5″ was not observed it was concluded that in solution $r_{5'',5'} > r_{1',5'}$ and $r_{5'',5'} > r_{5'',5}$ *i.e.* $r_{5',5''} > 2.5$ Å minimum.

(iv) *N.O.e.*[3]11 *and n.O.e.*[11]3. In the crystal structure $r_{3,11}$ ~ 3.7 Å and $r_{4,11}$ ~ 2.2 Å whilst in solution, n.O.e.[11]3 0.2—0.5%, n.O.e. [11]4 ~ 4%, n.O.e. [3]11 ~ 0.1%, and n.O.e.[4]11 ~ 3.3%. The small n.O.e.s between 3-H and 11-H could have been due to the slow build-up of long-range n.O.e. with $r_{3,11}$ ~ 3.7 Å. This possibility was eliminated by an n.O.e. kinetics experiment which showed that the rate of build-up of n.O.e.[11]3 was no slower than the rate of build-up of n.O.e.[11]4. Three more explanations were considered. First, the small n.O.e.[11]3 could have been due to the presence of a small amount of another compound in solution. Previous work

* Here and later in the text 'minimum' refers to the minimum distance found by rotation of the methyl group.

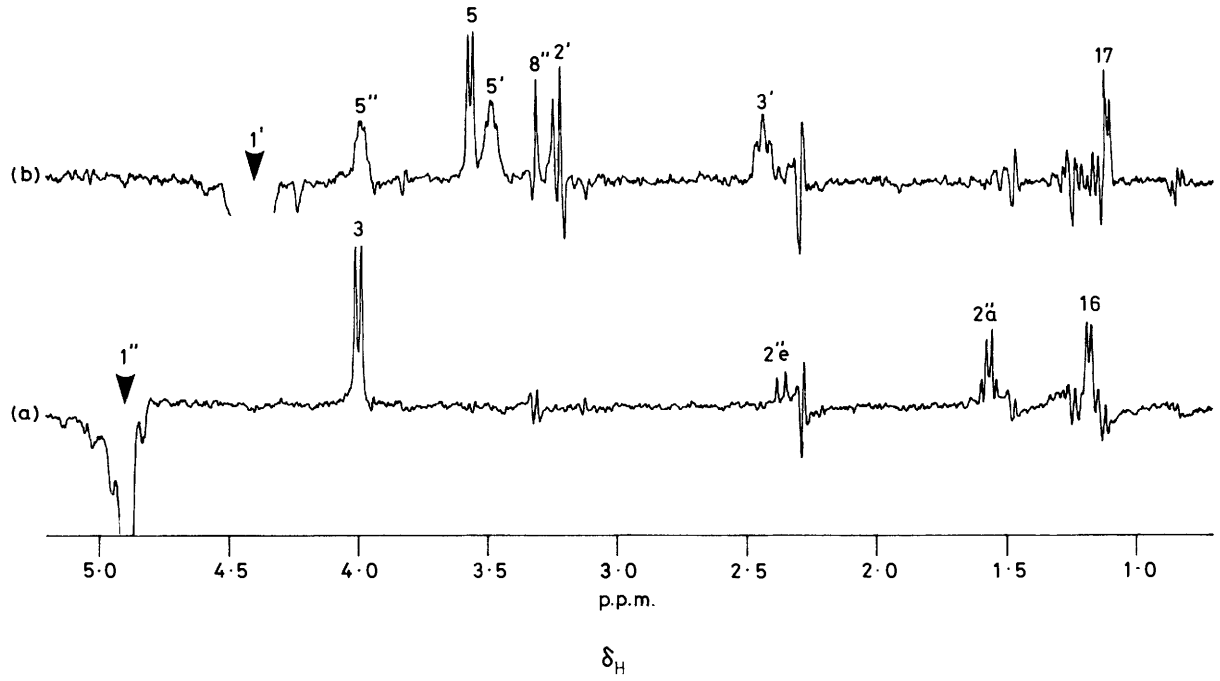


Figure 3. 400 MHz ^{1}H N.O.e. difference spectra of (**1**) in $CDCl_3$–TMS produced by irradiation of (a) 1″-H and (b) 1′-H

Table 4. Crystalline-state dihedral angles (φ in °) and corresponding solution-state coupling constants (^3J in Hz) for vicinal proton pairs in erythromycin A

Vicinal proton pair	φ	^3J
2-H, 3-H	174	9.5
3-H, 4-H	−70	1.5
4-H, 5-H	125	7.5
7ax-H, 8-H	164	11.7
7eq-H, 8-H	−76	2.4
10-H, 11-H	70	1.3
13-H, 14ax-H	175	11.0
13-H, 14eq-H	31	2.4
2′-H, 3′-H	172	10.3
4″-H, 5″-H	−177	9.7

had shown that small amounts of another species * were present in deuteriochloroform solutions of (**1**) and that this contamination could not be removed by repetitive recrystallisation.[13] However, although this explanation could not be eliminated, it was considered unlikely as the degree of 'contamination' was low (< ~8%). Furthermore the contaminant would have to have the same δs for 3-H and 11-H as (**1**) whilst possessing a much shorter $r_{3,11}$. The second explanation of the existence of n.O.e.[11]3 *etc.*, would invoke a solution-state structure different to the crystal structure such that (**1**) existed in a single, stable conformation with $r_{3,11}$ < 3.7 Å. One characteristic of a molecule which exists in a single, stable conformation is that the vicinal coupling constants remain invariant with respect to solvent and temperature changes. The proton n.m.r. spectrum of (**1**) was rerun in various different solvents and at different temperatures (Table 3). Raising the temperature of a $CDCl_3$ solution from ~292 to ~323 K caused all the $^3J_{H,H}$ values associated with the lactone ring protons to become more averaged *i.e.* large ^3J values became smaller and *vice versa*. Changing solvents caused even more dramatic effects. In CD_3OD, [$^2H_6$]dimethyl sulphoxide (DMSO), [$^2H_7$]dimethylformamide (DMF), and [$^2H_5$]pyridine, the C-6 to C-9 region of the molecule became destabilised and the ^3J values were indicative of the fast averaging of different conformations. Formation of the 6,9-epoxy tautomer is promoted in CD_3OD and [$^2H_7$]DMF but the major species (≥ ~75%) remains the C-9 ketone compound, as shown by ^{13}C n.m.r. spectroscopy. The ^3J values were measured on the C-9 ketone compound. Furthermore, in [$^2H_6$]DMSO especially, the low value of $^3J_{2,3}$ was indicative of some degree of fast conformational averaging around the C-2–C-3 bond. It was thus concluded that the unexpected n.O.e.s (n.O.e.[3]11 and n.O.e.[11]3) in $CDCl_3$ solution were due to a minor conformer of (**1**) in fast equilibrium with the major conformer (third explanation). The major conformer was very similar to the crystalline-state conformation (**A**) (with the exceptions detailed above) but the minor conformation has the macrolide ring in a conformation with $r_{3,11}$ < 3 Å.

Thus, erythromycin A in solution exists as a mixture of two conformers in fast equilibrium. The major conformer (probably >90%) is very similar to the crystalline-state conformation (**A**) except that the orientation of the two sugar groups is slightly different, with $r_{5',5''}$, $r_{8'',3'}$ and (probably) $r_{6'',5}$ longer in solution than in the crystal structure. The C-6 to C-9 region of (**1**) is conformationally unstable. Whilst this was suggested in previous studies, no conclusive evidence was shown.[7] The existence of a minor conformation of (**1**) in fast equilibrium with the major solution-state conformation is interesting and contradicts the often repeated dogma that all erythromycin molecules exist in a single stable conformation based upon an alternate diamond-lattice. Studies on several derivatives of (**1**) (to be reported in full shortly) have conclusively shown that a conformational equilibrium does indeed exist.[16]

Molecular Flexibility of (**1**).—(i) *Crystal structure predictions.*

* Thought to be the 6,9-hemiacetal tautomer of (**1**) (6-deoxy-9-deoxo-9-hydroxy-6,9-epoxyerythromycin A).[20]

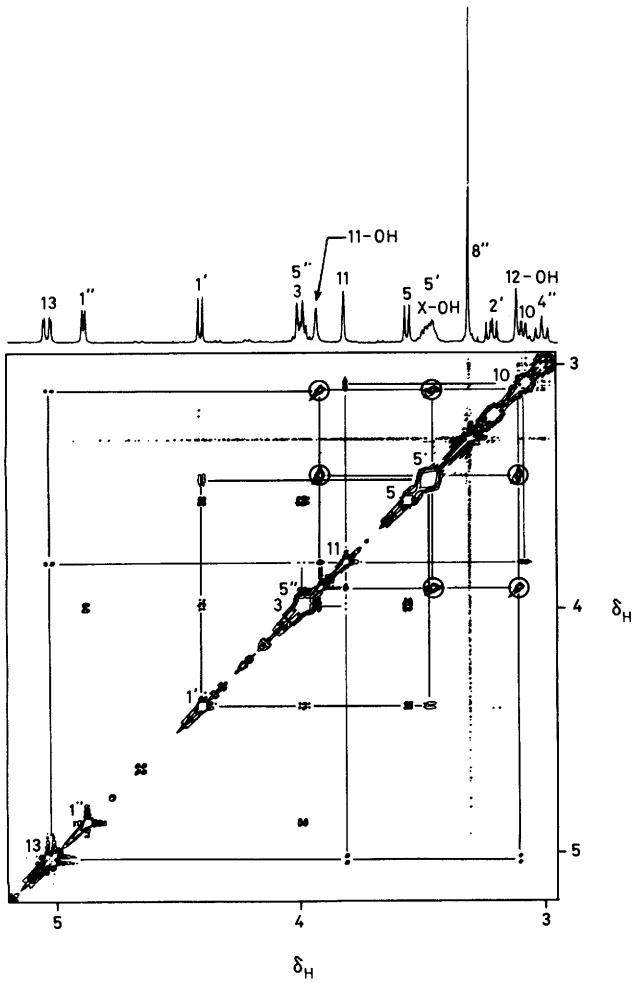


Figure 4. Contour plot of the low-field portion of the 400 MHz phase-sensitive 2D ^{1}H NOESY spectrum of (**1**) in $CDCl_3$–TMS underneath the corresponding 1D ^{1}H n.m.r. spectrum. Cross-peaks enclosed in circles (negative phase) are due to chemical exchange connectivity, the other cross peaks (positive phase) are due to (positive) n.O.e. connectivity. Some of the n.O.e. and chemical exchange connectivities are traced out with bold horizontal and vertical lines

The aims of this work were to use simple [19] energy calculations to:

(a) determine the flexibility of the sugar rings about the two glycosidic bonds joining each sugar to the lactone ring and

(b) to determine the rotational freedom of each methyl group on the basis of the crystal structure of the hydroiodide dihydrate of (**1**).

The sugar rotation calculations were performed with a version of the crystal structure incorporating partial atomic charges calculated using CNDO methods.[21] The methyl group rotations were performed without charges. Ramachandran calculations of total energy as a function of rotation (10° intervals) about the C-3–O-3, O-3–C-1″ and C-5–O-5, O-5–C-1′ bonds for cladinose and desosamine respectively gave the following results. Up to 1, 5, 10, and 20 kcal mol^{-1} above the mininum energy level, the approximate ratios of available conformational space for desosamine: cladinose were 3:1, 6:2, 7:5, and 11:10 respectively. Thus, on the basis of conformation (**A**), the desosamine sugar would be predicted to have more conformational freedom than the cladinose sugar but the difference becomes smaller as more energy becomes available. However, in absolute terms the motions of both sugars are very

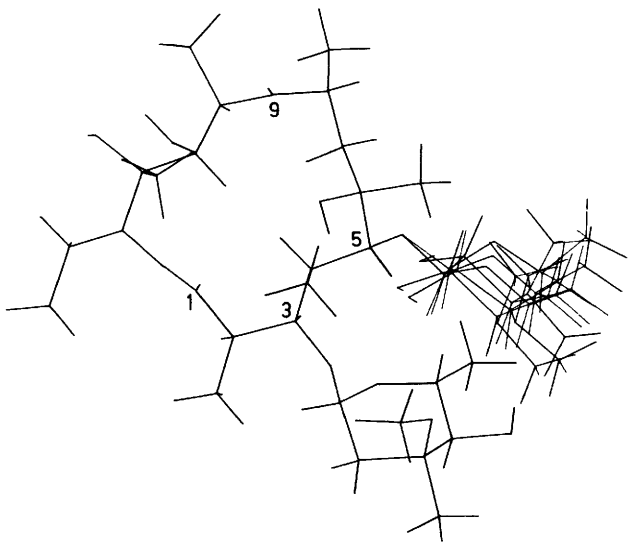


Figure 5. Stick representation of (**A**), showing the conformational space calculated to be available to the desosamine sugar ring by rotation about the C-5–O-5 and O-5–C-1′ bonds, given up to 10 kcal mol^{-1} energy above the minimum

restricted. The desosamine C-5–O-5 and O-5–C-1′ bonds were calculated to librate over only ~10 and ~30° respectively given up to 10 kcal mol^{-1} above the minimum energy level. Figure 5 shows in stick representation this desosamine libration for up to 10 kcal mol^{-1}. The minimum-energy conformations found by rotation over both pairs of glycosidic bonds were identical to the corresponding crystal structure conformations.

Calculations of the energy barriers to methyl group rotation gave some surprising results. One methyl group (Me-16) was found to have a very high energy barrier to rotation (>20 kcal mol^{-1}) due to an extremely close approach of its protons to 1″-H ($r_{16,1''\text{min}}$ ~ 1.3 Å). Other methyl groups were shown to possess almost free rotation. Figure 6 shows a plot of the total energy E *vs.* torsion angle φ for the rotation of three methyl groups Me-16, Me-17, and Me-8″; representative of very hindered, intermediate, and almost free rotation respectively. The calculated energy barrier to rotation in the crystal structure, and the ^{1}H and ^{13}C n.m.r. relaxation times for each methyl group in (**1**) are given in Table 5.

(ii) *Solution-state experiments.* The aim of this work was to obtain experimental measurements of:

(a) the flexibility of the sugar moeities and

(b) the hindrance to rotation of the methyl groups of (**1**) in $CDCl_3$ solution and to compare these results with the theoretical predictions based on the crystal structure.

Carbon-13 spin-lattice relaxation times (T_1) were used to probe the motions of the protonated carbons of (**1**). In the limit of fast, isotropic motion and full dipolar relaxation the ^{13}C NT_1 values (N = no. of hydrogen atoms on carbon) of methine and methylene carbon atoms are directly proportional to the reorientation rate of the respective C–H vector and are hence excellent probes of molecular motion.[22] The ^{13}C NT_1 values of the lactone ring carbons of (**1**) were all within 10% of their averaged value and indicated that the motion of (**1**) was approximately but not exactly isotropic. This minor deviation does not affect the following qualitative arguments. The averaged ^{13}C NT_1 results showed that the desosamine sugar (0.46 ± 0.02 s) had more motional freedom than the cladinose sugar (0.41 ± 0.05 s) with respect to the lactone ring (^{13}C $NT_{1\,\text{av.}}$ 0.38 ± 0.03 s) in good agreement with both the

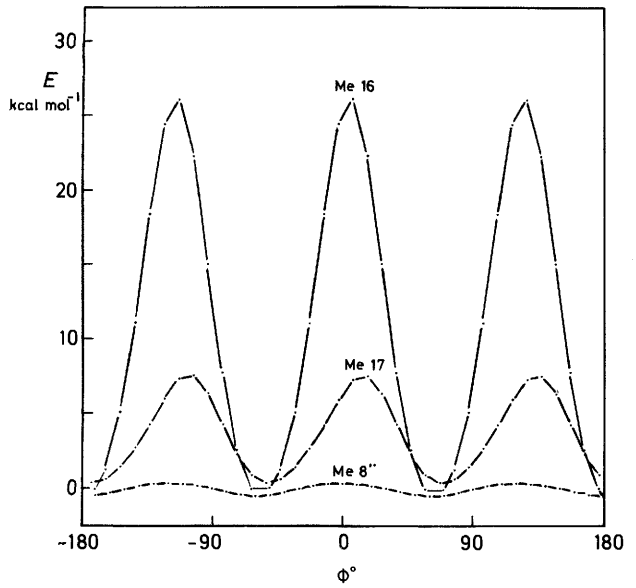


Figure 6. Total calculated energy E (kcal mol^{-1}) *versus* torsion angle φ (°) for rotation about the Me-16, Me-17, and Me-8″ groups in the crystalline-state conformation (**A**)

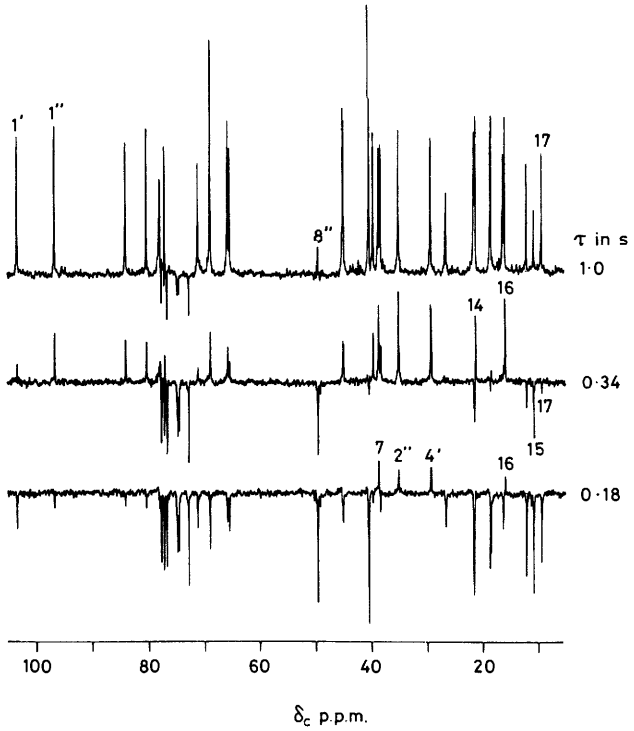


Figure 7. Partially relaxed 63 MHz ^{13}C n.m.r. spectra of (**1**) in $CDCl_3$ taken from an inversion-recovery T_1 experiment

Table 5. Calculated rotational energy barriers (E in kcal mol^{-1}, crystal structure) and experimental ^{13}C and ^{1}H n.m.r. relaxation times (T_1 in s, $CDCl_3$ solution) for the methyl groups in (**1**)

Methyl	E (kcal mol^{-1})[a]	^{13}C NT_1 (s)[b]	^{1}H T_1 (s)[c]	Class
16	F	0.78 ± 0.04	0.20	'Hindered'
21	C	1.48 ± 0.06	0.28	
19	B	1.56 ± 0.06	0.29	
17	C	1.80 ± 0.00	0.33	
7′, 8′	B	1.8 ± 0.1	0.35	
18	C	1.9 ± 0.2	0.30	'Intermediate'
7″	B	1.99 ± 0.05	0.38	
6″	B	2.0 ± 0.2	0.38	
20	B	2.1 ± 0.2	0.38	
6′	B	2.19 ± 0.02	0.42	
15	A	3.03 ± 0.02	0.52	'Free'
8″	A	3.4 ± 0.3	0.59	

[a] Theoretical calculations based on conformation (**A**). The results are given in terms of energy ranges: A (0—2), B(2—5), C(5—10), D(10—15), E(15—20), and F(20—30 kcal mol^{-1}). These ranges must be regarded as very approximate, since the hard-sphere calculations employed did not allow the molecule to relax as the methyl group was rotated hence the calculated barriers will be overestimated. [b] Experimental results (average ± half range, two determinations) on (**1**) in $CDCl_3$ solution; T_1 = spin-lattice relaxation time; N = 3, the number of protons on each carbon. The average calculated (DISNMRP) standard deviation of the NT_1 values was 0.09 ± 0.03 s (σ) and 0.04 ± 0.02 s (σ) in the two experiments. [c] Experimental results at 400 MHz for (**1**) in $CDCl_3$; single determination; probable error ±0.03.

theoretical calculations based upon the crystal structure and previous results.[11]

The ^{13}C NT_1 values of the methyl groups of (**1**) displayed a four-fold variation (Table 5). In exact agreement with the crystal structure calculations, Me-16 had a remarkably short NT_1 value indicative[23] of great steric hindrance to rotation (Figure 7). However, Me-15 and Me-8″ had very long NT_1 values indicative[23] of almost-free rotation and also in exact agreement with the crystal structure calculations. The remaining methyl groups had intermediate ^{13}C NT_1 values of 1.5—2.2 s in agreement with the intermediate energy barriers to methyl group rotation calculated for these methyl groups in the crystal structure. The ^{1}H T_1 values (Table 5) showed a three-fold variation between Me-16 (0.20 s, very hindered[23]) and Me-8″ (0.59 s, 'free' rotation[23]) and exhibited a trend which paralleled that of the ^{13}C NT_1 values.

In summary, there was a remarkable agreement between the theoretical predictions of sugar and methyl group motions in (**1**) [based upon conformation (**A**)] and the actual solution-state motions of (**1**) experimentally determined by the n.m.r. relaxation data. This agreement provided further evidence that the major solution-state conformation of (**1**) was very similar to (**A**).

Conclusions

A combination of n.m.r. spectroscopy techniques showed that the major conformation of (**1**) in $CDCl_3$ solution is similar but not identical to the crystalline-state conformation (**A**) of erythromycin A hydroiodide dihydrate. It was also shown that the major conformation is in fast exchange with a minor conformation possessing a lactone ring reorganised in the C-2 to C-9 region such that 3-H is closer to 11-H. However, the population of this minor conformation was low and it was therefore difficult to characterise with certainty. A good agreement was also found between the theoretical predictions of sugar and methyl groups motions in (**1**) and the motions determined in $CDCl_3$ solution by n.m.r. relaxation experiments. This agreement provided further evidence of the similarity between the major solution-state conformer of (**1**) and the crystalline-state conformation (**A**). It was concluded that n.m.r. spectroscopy is a powerful method for the determination of the solution conformations of macrolide antibiotics. Furthermore molecular modelling techniques were very useful for comparing these solution-state conformations with crystalline-state

models. Further work on the derivatives of (**1**) will be reported shortly.

Experimental

The erythromycin A base was obtained from K and K — Greef Chemicals Ltd., and was recrystallised from $CHCl_3$–hexane before use. ^1H N.m.r. experiments were conducted on either Bruker WM250 or AM400 n.m.r. spectrometers using 5 mm probes and a sample concentration of *ca.* 10 mg/0.5 ml. The samples were at ambient temperature (~21 °C) unless otherwise noted and were not degassed. Typically, ^1H n.m.r. spectra at 400 MHz were acquired over 2 717.39 Hz using 16 384 data points. The ^1H n.O.e. difference spectra were acquired automatically using a modification of the method of Hall and Saunders[17] and separate storage of the F.I.D. (free induction decay) corresponding to each irradiation. Typically, 8—10 irradiations would be performed in one experiment with automatic cycling through the frequency list, using four dummy scans and 16 scans at each frequency. The pulse sequence utilised a pre-irradiation delay (3—4 s) followed by a sub-saturating irradiation period (3—4 s) and then data acquisition with the decoupler gated off. Each F.I.D. was multiplied by an exponential function corresponding to a line-broadening of 2 Hz prior to Fourier transformation. Difference spectra were obtained by the subtraction of the control (off-resonance irradiation) from every other spectrum. The total number of scans acquired for each spectrum was typically 600—1 400.

The ^1H T_1 experiment was conducted using a standard inversion-recovery pulse sequence (D1–180°–τ–90°–F.I.D.) with the relaxation delay D1 = 3.0 s and averaging 32 scans into a 16 K data block (acquisition time 2.556 s). The experiment was repeated for 20 values of the variable delay τ, ranging from 1.0 ms to 2.0 s. Values of T_1 were determined by the null-point method.[23]

The 2D phase-sensitive ^1H NOESY experiment was performed using the time-proportional-phase-increment method.[18] F.I.D.s were acquired (64 scans, four dummy scans) over 2 000 Hz into a 2 K data block for 512 incremented values of the evolution time, t_1. The raw data were zero-filled to a 2 K × 2 K matrix and processed with a 1 Hz line-broadening function in both dimensions. The mixing time of 0.45 s was subject to a random variation of 5% and the relaxation delay D1 was 2.5 s.

All ^{13}C n.m.r. experiments were conducted on a Bruker WM 250 n.m.r. spectrometer using a 50 mg/0.5 ml sample which was not degassed. The sample was evaporated from CD_3OD prior to dissolution in $CDCl_3$–TMS. The ^{13}C T_1 experiment used an inversion-recovery pulse sequence, with a relaxation delay D1 = 3.5 s and averaging 2 000 scans into a 16 K data block (acquisition time 1.016 s). The experiment was repeated for 15 values of τ ranging from 1 ms—2.0 s. The whole experiment was repeated under slightly different conditions (D1 = 2.5 s, 14 τ values from 1 ms—1.0 s, 900 scans) and the T_1 values were averaged. The T_1 values were calculated by fitting the experimental signal intensity data to the equation:

$$I = I_0 - A \cdot e^{-\tau/T_1}$$

where I = signal intensity for delay τ
I_0 = normalised signal intensity with complete relaxation
A = the ^{13}C 180° flip angle in units of π/2

The fitting was done using the Bruker DISNMRP program. The $^{13}C\{^1H\}$ n.O.e. values for all the protonated carbons were measured by the acquisition of decoupled with n.O.e. and decoupled without n.O.e. ^{13}C n.m.r. spectra, using D1 = 3.5 s. For protonated carbons the average n.O.e. was 1.7 ± 0.3 (σ) indicating that the relaxation of these carbons was dominated by dipolar ^{13}C—^1H relaxation.

The crystal structural co-ordinates of erythromycin A hydroiodide dihydrate were supplied by Dr. H. H. Mills.[24] Partial atomic charges were calculated using a CNDO program.[21] All the interatomic distance and energy calculations on the crystal structure were performed using the MODEL program.[19]

Acknowledgements

Our thanks are due to Dr. E. Hunt for the sample of (**1**), to Drs. E. Hunt and P. Finn for helpful advice and to Miss A. Bentley for careful typing.

References

1 D. R. Harris, S. G. McGeachin, and H. H. Mills, *Tetrahedron Lett.*, 1965, 679.
2 T. J. Perun and R. S. Egan, *Tetrahedron Lett.*, 1969, 387.
3 L. A. Mitscher, B. J. Slater, T. J. Perun, P. H. Jones, and J. R. Martin, *Tetrahedron Lett.*, 1969, 4505.
4 T. J. Perun, R. S. Egan, P. H. Jones, J. R. Martin, L. A. Mitscher, and B. J. Slater, *Antimicrob. Agents Chemother.*, 1969, 116.
5 R. S. Egan, T. J. Perun, J. R. Martin, and L. A. Mitscher, *Tetrahedron*, 1973, **29**, 2525.
6 R. S. Egan, T. J. Perun, J. R. Martin, and L. A. Mitscher, *J. Am. Chem. Soc.*, 1975, **97**, 4578.
7 R. S. Egan, Ph.D. Thesis, University of Illinois at the Medical Center, 1971.
8 J. G. Nourse and J. D. Roberts, *J. Am. Chem. Soc.*, 1975, **97**, 4584.
9 S. Ōmura, A. Neszmélyi, M. Sangaré, and G. Lukacs, *Tetrahedron Lett.*, 1975, 2939.
10 A. Neszmélyi and H. Bojarski-Dahlig, *J. Antibiotics*, 1978, **31**, 487.
11 M. S. Puar and R. Brambilla, *J. Chem. Soc., Perkin Trans. 2*, 1980, 1487.
12 S. Ōmura, 'Macrolide Antibiotics, Chemistry, Biology and Practice,' Academic Press, 1984.
13 J. R. Everett and J. W. Tyler, *J. Chem. Soc., Perkin Trans 1*, 1985, 2599.
14 J. H. Noggle and R. E. Schirmer, 'The Nuclear Overhauser Effect Chemical Applications,' Academic Press, New York, 1971.
15 T. J. Perun, 'Drug Action and Drug Resistance in Bacteria 1. Macrolide Antibiotics and Lincomycin,' ed. S. Mitsuhashi, University Park Press, Baltimore, 1971, p. 123.
16 J. R. Everett and J. W. Tyler, *J. Chem. Soc., Chem. Commun.*, 1987, 815.
17 J. K. M. Saunders and J. D. Mersh, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1982, **15**, 353.
18 G. Bodenhausen, H. Kogler, and R. R. Ernst, *J. Magn. Reson.*, 1984, **58**, 370.
19 E. K. Davies, CHEMGRAF, Program for Molecular Modelling, Chemical Crystallography Laboratory, Oxford University, developed and distributed by Chemical Design Ltd., Oxford, 1985.
20 R. C. Pandey and K. L. Rinehart, Jr., *J. Antibiotics*, 1976, **29**, 1035.
21 P. A. Dobosh, QCPE No. 141, 1968.
22 G. C. Levy, *Acc. Chem. Res.*, 1973, **6**, 161.
23 W. Chazin and L. D. Colebrook, *Magn. Reson. Chem.*, 1985, **23**, 597.
24 H. H. Mills, personal communication.

Received 9th February 1987; *Paper* 7/231