The conformations of the macrolide antibiotics erythromycin A, azithromycin and clarithromycin in aqueous solution: a ^{1}H NMR study †

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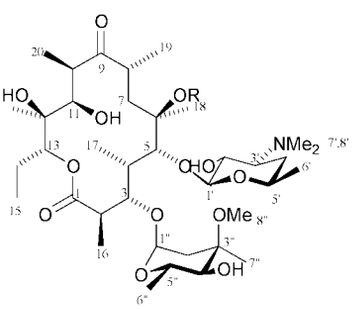
The conformations of the macrolide antibiotics erythromycin A, clarithromycin and azithromycin in aqueous solution have been determined. All three drugs were found to exist predominantly in a "folded-out" conformation with the sugars in the chair–chair up–up form. In the case of clarithromycin this result is in agreement with the published literature; previous reports suggest that the erythromycin A sugars adopt boat conformations and that azithromycin has a significant folded-in contribution. The discrepancies are discussed in full. Erythromycin A 9-ketone was found to be flexible in the C6–C12 region. We present evidence for a new minor conformation (the 8-*endo* folded-out conformation) intermediate between the folded-out and folded-in conformations. Evidence for traces of other minor conformations is presented for erythromycin and azithromycin, whereas clarithromycin is conformationally very stable.

Erythromycin A (**1**) is the best studied of the macrolide group of antibacterial agents. It is of very considerable clinical importance against a wide range of bacteria, and it is particularly implicated in the treatment of deep-seated Gram positive infections.

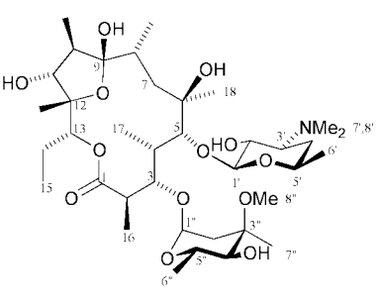
The semisynthetic analogues of erythromycin, clarithromycin (**2**) and azithromycin (**3**) have similar clinical indications to the parent compound, but advantages in use. In particular, they are much less sensitive to acid than erythromycin so fewer, more widely spaced oral doses are used.[1,2]

Erythromycin and its analogues inhibit bacterial protein synthesis at the translocation stage.[3] Erythromycin binds tightly to bacterial ribosomes, the dissociation constant being in the region 10^{-7}–10^{-9} M.[4–6] The binding site is located on the 50S subunit, and proteins L15 and L16 are required for binding.[7] Erythromycin protects specific regions of 23S RNA in footprinting studies,[8] but protein L22 is labelled by photoactive analogues of erythromycin.[9] A recent study of the crystal structure of protein L22[10] has resulted in a proposal that erythromycin acts by blocking the tunnel on the ribosome through which the polypeptide chain leaves. This proposal reconciles many of the previous results on the mode of action of erythromycin, but not all of the accumulated data fit comfortably within this model; for example, it has been reported that isolated L15 is able to bind erythromycin A, albeit weakly (K_d = 2×10^{-5} M).[7] Recently, however, a second mode of action of erythromycin A has been identified. The drug has been shown to inhibit the assembly of 50S ribosomal subunits.[11,12] This is especially interesting in view of the report that proteins L15 and L16 function as assembly proteins,[13] and an understanding of the mode of action of this important drug now seems tantalisingly close.

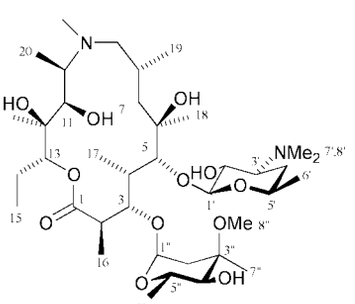
Erythromycin A exists in aqueous solution as a mixture of the 9-ketone (**1a**) and the 9,12-hemiacetal (**1b**).[14] The 9-ketone



1a: R=H
2: R=Me



1b



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† ROESY connectivities for clarithromycin, erythromycin A 9-ketone, erythromycin A 9,12-hemiacetal and azithromycin are available as supplementary data. For direct electronic access see http://www.rsc.org/suppdata/p2/b0/b003000g/

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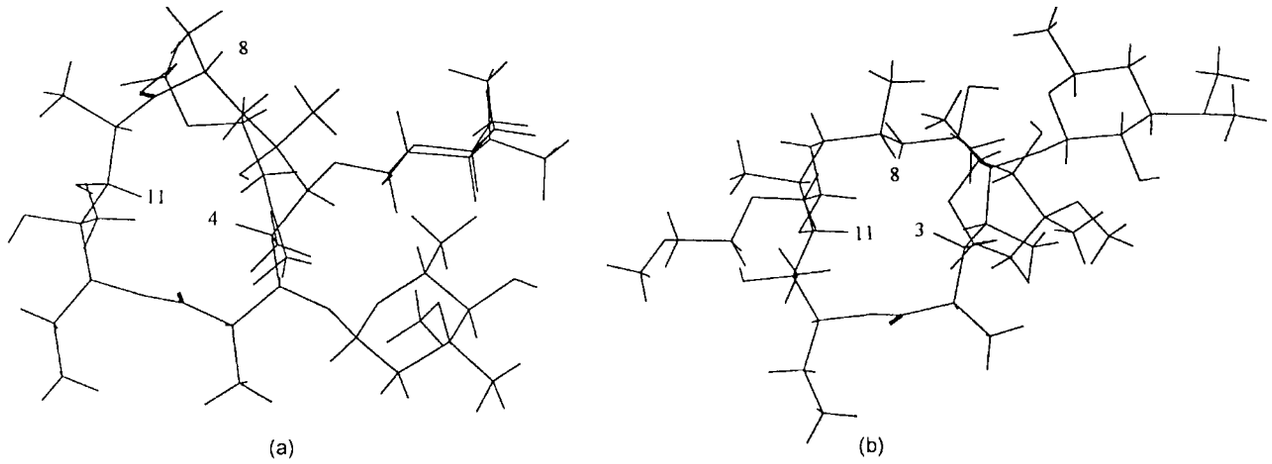

Fig. 1 Folded-out and folded-in conformations of macrolide antibiotics. (a) The crystal structure of erythromycin A [9-(*O*-2,5-dioxahexyl)oxime] hydrate, a folded-out macrolide, showing close approach of H4 and H11. (b) The crystal structure of dirithromycin, a folded-in macrolide, showing close approach of H3, H8 and H11.

(only) is able to take part in a second (weak) mode of binding to bacterial ribosomes.[15,16] This interaction is detectable by line broadening or transferred NOESY NMR spectroscopy.[17] The interaction is specific, and is associated with the 50S subunit of the bacterial ribosome.[15]

In an attempt to understand the common features that make these three drugs active, we have carried out a detailed NMR study of erythromycin A, clarithromycin and azithromycin free in aqueous solution and weakly bound to bacterial ribosomes. In this paper we describe the first part of this study.

In 1987, Everett and Tyler reported an analysis of the conformation of erythromycin A 9-ketone in $CDCl_3$ solution, using NOE difference and other NMR methods.[18] They demonstrated that the drug exists predominantly (over 90%) in the "folded-out" conformation (Fig. 1a). A small NOE between H3 and H11 alerted these authors to the presence of a second conformer in equilibrium, and they postulated that this is the folded-in conformer (Fig. 1b), which resembles the crystal structure of dirithromycin.[19] As can be seen in Fig. 1, the folded-out conformer is characterised by a close approach of H11 and H4 and a large dihedral angle between H2 and H3. Conversely, the folded-in conformation shows a close approach of H3 and H11 and a dihedral angle between H2 and H3 of close to 90°.

In their analysis of the conformation of azithromycin in $CDCl_3$, Lazarevski and co-workers demonstrated a $J_{H2,H3}$ of 3.6 Hz at 20 °C, rising to 4.5 Hz at 45 °C, and suggested that azithromycin exists predominantly in the folded-in conformation, the equilibrium shifting slightly towards folded-out on heating.[20] Steinmetz and co-workers have already carried out a fairly detailed analysis of the conformation of clarithromycin in aqueous solution, using a NOESY spectrum run at 300 MHz to obtain estimates of internuclear distances.[21] They concluded that clarithromycin exists as a single folded-out conformer in aqueous solution.

Gharbi-Benarous and co-workers have adopted a slightly different approach in their analysis of the conformations of roxithromycin and its metabolites.[22–24] Molecular dynamics have been used to identify likely conformations of macrolides in solution, and NMR spectroscopy to select from among these conformations. Again, variants on the folded-out and folded-in conformations were identified.

Everett and Tyler[18] presented detailed analysis of the conformations of the sugars in erythromycin A and concluded that they both adopted predominantly chair conformations, oriented "up–up", that is with H1′ of desosamine approaching H5″ of cladinose. This work assumed almost biblical authority over the following decade as researchers around the globe found that the chair–chair up–up conformation was independent of solvent and of modifications to the macrolide ring. In 1998, Commodari *et al.*,[25] however, published an analysis of the solution structures of the ketone and 9,12-hemiacetal forms of erythromycin A in 90% water, concluding that the sugars adopt the boat conformation. So far, there has been no independent support for this suggestion.

Studies of macrolide conformations by NMR methods are subject to a number of difficulties, especially in aqueous solution. Firstly there is considerable signal overlap in the important regions of the spectrum. The presence of an 11–3 crosspeak is considered diagnostic of a folded-in conformation, for example, but in both clarithromycin and erythromycin 9-ketone, H3 and H11 resonate within 0.02 ppm. A second problem is that, at 500–600 MHz, the NOEs for these drugs are close to zero. Even semi-quantitative analysis of NOESY spectra becomes dangerous. We have therefore used the rotating frame ROESY experiment[26] in which all the Overhauser signals are positive. This experiment requires careful adjustment of the spin-lock field. If this is too small the signal is strongly offset-dependent, if it is too great through-bond TOCSY crosspeaks (of opposite sign to the ROESY signals) are strongly encouraged, vitiating any quantitative analysis. For example, in azithromycin, H5 overlaps with H11 and a strong H5–H4 TOCSY signal can prevent the observation of the H11–H4 Overhauser crosspeak.

We now report a comparison of the conformations of erythromycin A and the analogues clarithromycin (**2**) and azithromycin (**3**) free in aqueous solution.

Results

Variable temperature measurements

The assignments of the ^1H spectra of clarithromycin, erythromycin A and azithromycin in aqueous solution have been published.[14,27,28] The large axial coupling constants in the sugar rings of the three drugs provided strong evidence that the sugars remain in the chair conformations seen in the crystal structures.[29–33]

Conformational analyses of macrolide antibiotics in solution have generally indicated "folded-out" or "folded-in" conformations of the macrolide ring or mixtures of these (Fig. 1). These conformations differ in the torsion angle H2H3 and hence in the corresponding scalar coupling constant. Folded-out conformers give $J_{H2,H3}$ values of nearly 10 Hz, folded-in only 2–3 Hz. Mixtures of conformers show intermediate values. Where mixtures of conformers exist, it is reasonable to expect

that the relative populations will be temperature-dependent. It has already been shown that $J_{H2,H3}$ for clarithromycin in aqueous solution is large and unchanged on heating, indicating close to 100% folded-out conformation.

We measured $J_{H2,H3}$ for azithromycin and erythromycin A 9-ketone over the range 20–80 °C. The results are shown in Table 1. Over this temperature range $J_{H2,H3}$ for both drugs are intermediate between the values expected for the folded-out and folded-in conformations, but change by less than 0.5 Hz with temperature. This experiment therefore provides no evidence for or against an equilibrium between two conformers; if such an equilibrium exists, the populations of the two conformations are almost temperature-independent in aqueous solution.

3-Dimensional structures of clarithromycin, erythromycin A and azithromycin

Conformations of the macrocyclic rings. *Clarithromycin.* Steinmetz *et al.*[21] used a NOESY spectrum run at 300 MHz to determine the conformation of clarithromycin in D_2O solution. Using the ROESY technique we obtained very similar results but with some additional crosspeaks (22–6″, 10–19 and 17–8″) presumably because of improved sensitivity of the 500 MHz ROESY spectrum. (Raw data are available in the supplementary material.)

The strategy used to derive structures from the NMR data was, as far as possible, to rely on the experimental NMR data, rather than on the results of modelling calculations. Given the wealth of NMR signals (67 pairs of peaks) available in the ROESY spectra, it was possible to carry out calculations in which the NMR data, rather than the modelling parameters, defined the final structures. In each case the final structures were examined for inconsistencies with the NMR data, in particular for the absence of signals that should be present.

In order to convert the ROESY data to a structure, we constructed a "folded-out" clarithromycin from the crystal structure of (14*R*)-14-hydroxy-6-*O*-methylerythromycin A[32] and a "folded-in" clarithromycin using the crystal structure of dirithromycin.[19] Both structures were subject to unconstrained local minimisation using the molecular modelling program Macromodel.[34] The energies of the water-solvated structures were computed as −173.5 kJ mol^{-1} (folded-out) and −173.4 kJ mol^{-1} (folded-in) and no prediction of the solution structure could be made from this modelling alone.

Table 1 Temperature dependence of $J_{H2,H3}$ of erythromycin A 9-ketone and azithromycin in aqueous solution

Temperature/°C	Erythromycin $J_{H2,H3}$/Hz	Azithromycin $J_{H2,H3}$/Hz
20	8.70	5.49
40	8.70	5.55
60	8.69	5.63
80	Not measured	5.80

Distance constraints for the folded-out and folded-in conformers were now predicted (see Table 2). Everett and Tyler[18] used the 3–11 and 4–11 correlations in this context, but because of the overlap of H11 and H3, the presence or absence of a 3–11 crosspeak could not be determined in this case. All the crosspeaks diagnostic of a folded-out conformation (4–11, 5–18, 8–18 and 15–16) are present in the ROESY spectrum of clarithromycin. Of the signals (other than 3–11) diagnostic of a folded-in conformation (3–8, 4–18, 8–11 and 16–17), none is present. This provides very clear support for the conclusion of Steinmetz *et al.*,[21] that clarithromycin exists overwhelmingly in the folded-out conformation in aqueous solution.

Finally distance constraints derived from the ROESY data were applied to both folded-out and folded-in clarithromycin, and Monte Carlo conformational searches with water solvation were carried out. The constraints applied (see Experimental) were based on known distances within the molecule. After 1000 steps the two calculations had converged to very similar minima, which closely resembled the starting folded-out conformation (see Fig. 2). The H2H3 torsion angle of 169° in the structure of minimum energy is consistent with the observed $J_{H2,H3}$ of 9.7 Hz. The only ROESY crosspeaks expected for this structure which did not appear were 4–7s, 19–21 and 5′–6″. The first two of these are far too close to the diagonal to be observable; the third overlaps with 5′–6′. Table 3 gives details of this structure.

Erythromycin A 9-ketone. Erythromycin A was analysed similarly. This drug exists in aqueous solution as a mixture of two isomers (**1a** and **1b**). The less abundant isomer is conformationally quite different from clarithromycin and azithromycin, and probably lacks antibacterial activity.

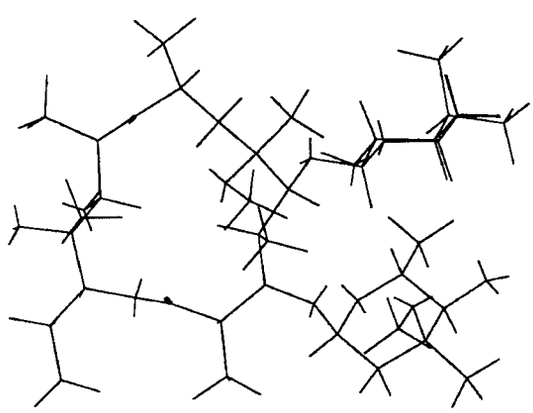

Fig. 2 The structure of clarithromycin in aqueous solution derived from ROESY NMR spectroscopy. A single folded-out structure is found.

Table 2 Predicted distances in folded-out and folded-in conformers of clarithromycin, erythromycin A 9-ketone and azithromycin

	Internuclear distance/Å					
	Clarithromycin		Erythromycin		Azithromycin	
Contact (HH)	Folded-out	Folded-in	Folded-out	Folded-in	Folded-out	Folded-in
3–8	5.8	2.1	5.9	2.1	5.6	2.2
3–11	3.8	2.5	4.0	2.5	3.4	2.8
4–11	2.4	3.3	2.7	3.3	3.2	3.9
4–18	4.6	2.1	4.3	2.2	4.4	2.2
5–18	2.4	3.8	2.4	3.8	2.7	3.8
8–11	4.2	2.3	4.2	2.4	4.7	2.2
8–18	2.2	4.5	2.3	4.6	1.9	4.5
15–16	2.6	3.3	2.7	3.3	3.3	3.1
16–17	4.0	2.3	4.2	2.4	2.7	2.3

Table 3 Structural details of the conformers of clarithromycin, erythromycin 9-ketone and erythromycin in aqueous solution

Compound	Energy/ kJ mol^{-1}	Approx. % Abundance	Torsion angle/°				Internuclear distance/Å									
			H2,H3	H3,H4	H4,H5	H7s,H8	H3–H8	H3–H11	H4–H11	H4–H18	H5–H18	H8–H11	H8–H18	H15–H16	H16–H17	H9r–H11	H11–H22
Clarithromycin folded-out	−184.6	100	170	−73	137	167	5.8	3.8	2.3	4.6	2.4	4.1	2.2	2.6	4.1	—	—
Erythromycin A 9-ketone																	
Folded-out	−218.9	>90	164	−81	146	175	5.7	3.7	2.6	4.5	2.3	4.3	2.3	2.6	3.9	—	—
Folded-in	−220.0	0	103	−66	119	53	2.2	2.4	3.3	2.2	3.8	2.3	4.6	3.4	2.3	—	—
8-*endo*-folded-out	−201.0	<10	132.5	−83	150	95	4.8	3.9	2.6	4.5	2.3	2.6	2.7	3.0	2.8	—	—
Cyclisable **A**	−210.2	Trace	111	−80.5	146	175	4.3	6.3	5.8	4.6	2.4	5.1	2.6	3.4	2.4	—	—
Cyclisable **B**	−208.6		118	−76	145	−176	5.7	6.0	5.1	4.6	2.4	5.5	2.3	3.1	2.5	—	—
Erythromycin A 9,12-hemiacetal	−272.0	100	96	−70.5	127	80	2.6	5.5	6.1	2.1	3.8	5.4	4.7	3.9	2.2	—	—
Azithromycin																	
Folded-in	−202.80	Trace?	99	−64	121	52	2.3	2.6	3.7	2.1	3.8	2.2	2.9	3.4	2.3	2.6	2.9
(11–4) Folded-out **A**	−220.07	>90	109	−71	134.5	151	5.6	3.2	2.7	4.5	2.4	4.9	2.2	3.4	2.4	3.7	3.6
(11–3) Folded-out **B**	−217.8		105	−68	140	149	5.4	2.9	2.9	4.6	2.3	4.9	2.1	3.4	2.4	3.8	3.4

Fig. 3 ROESY NMR spectrum of erythromycin A (8 mM) in D_2O buffered with 50 mM sodium phosphate, apparent pH 7.4, at 45 °C.

Everett and Tyler[18] analysed the conformation of erythromycin A 9-ketone in $CDCl_3$ solution and provided evidence for the drug existing in a mixture of folded-out (over 90%) and folded-in conformations. We now used the ROESY technique to obtain conformational information about the drug in aqueous solution. These data are shown in the Supplementary Material. The 2-dimensional NMR technique allowed somewhat better resolution in the crowded methyl region, but otherwise our data are similar to those of Everett and Tyler, and similar to our data for clarithromycin. Of the 116 crosspeaks in the spectrum only 16–17, 8–11 and their partners would not be expected from the crystal structure.

The simplest conclusion would be that erythromycin A 9-ketone adopts predominantly a folded-out conformation in aqueous solution with some folded-in structure responsible for the 16–17 and 8–11 crosspeaks. To test this hypothesis, a "folded-in" erythromycin A was constructed using Macromodel from the dirithromycin crystal structure[19] in the same way as for clarithromycin. This structure, like that of "folded-in" clarithromycin, showed close contacts (2–2.5 Å) for the H3–H8, H3–H11, H4–H18, H8–H11 and H16–H17 pairs (Table 2, Fig. 1b). Thus, of the five expected signals for folded-in erythromycin 9-ketone, only two were observed in the ROESY spectrum.

The ROESY spectrum was repeated at 45 and 60 °C. It was reasoned that at higher temperature H3 and H11 might separate sufficiently for a crosspeak to be observable. Further, any broad unobservable 3–8 or 4–18 crosspeak might sharpen and become visible. The spectrum acquired at 60 °C was of poor quality with broad signals. By contrast, the spectrum acquired at 45 °C was very clean with sharp crosspeaks (Fig. 3). Chemical exchange signals (of opposite sign to the ROESY crosspeaks) could be seen, indicating the onset of intermediate exchange between the ketone and hemiacetal, and explaining the line-broadening in the 60 °C spectrum. However, at 45 °C the signals due to H3 and H11 did not separate significantly, and no 3–8 or 4–18 crosspeak was seen. Several additional signals did appear, relative to the 30 °C spectrum. These corresponded to 3–4, 4–5, 5–17, 5–6″, 5–1′, 8–7r, 16–15, 17–8″, 18–6″, 1′–8″, 3′–8″, 11–20 and 13–21 and their partners. All but the last two of these are expected as small peaks from the crystal structure of erythromycin A.

The evidence for a folded-in conformation of erythromycin A in equilibrium with a folded-out remained sparse. Several modelling calculations were therefore carried out according to

Table 4. The small 11–20 and 13–21 crosspeaks could not be satisfied by any known conformation of erythromycin and these were not considered in calculations 1–4. Calculations 1 and 2 were designed to estimate the structures and relative energies of the proposed "folded-out" and "folded-in" conformers, so the signals characteristic of the folded-out conformation were omitted from calculation 2 and those characteristic of the folded-in conformation from calculation 1. It was possible, however, that the drug was present as a single conformer, at least at 30 °C. In calculation 3, therefore, all the constraints were applied. Calculation 4 was used to determine whether a modified folded-in structure, which did not give rise to a 4–18 crosspeak, was possible. Calculation 4 was unsuccessful; the folded-in structure requires a close approach of H4 and $H_3$18. Calculations 1 and 2 gave structures of very similar energies, suggesting that a folded-out–folded-in equilibrium would be possible, were it not for the lack of the 3–8 and 4–18 crosspeaks in the ROESY spectrum. Calculation 3 gave rise to a structure of slightly higher energy than the folded-out and folded-in structures which satisfied all the constraints. This structure is essentially a folded-out structure but is twisted at C8–C9 relative to that structure so that H8 is *endo* rather than *exo* to the macrolide ring (see Fig. 4). Folded-in macrolide structures are also 8-*endo* so the 8-*endo*-folded-out structure can be regarded as an intermediate between folded-in and 8-*exo*-folded-out.

The 8-*endo*-folded-out structure cannot in fact be the sole or major conformer of erythromycin A in aqueous solution. The H2H3 torsion angle of 132.5° is not consistent with the observed $J_{H2,H3}$ of 8.7 Hz, and the H7sH8 torsion angle of 95.2° is not consistent with the corresponding scalar coupling constant of 9.2 Hz. This structure is, however, a very good candidate for the minor conformer, with the normal 8-*exo*-folded-out structure as the major conformer. The 8-*endo*-folded-out structure is closely similar to the folded-out structure, so a very low energy barrier between the two is anticipated and the temperature independence of scalar couplings (and of the relative populations of conformers) is easily reconciled. Further, the 8-*endo*-folded-out conformation fits the ROESY data better than the folded-in conformation. It is likely therefore that in aqueous solution erythromycin A 9-ketone consists predominantly of the folded-out conformer with perhaps 10% 8-*endo*-folded-out conformer (Fig. 4).

The appearance of 11–20 and 13–21 crosspeaks at 45 °C was interesting. None of the normal (folded-out, folded-in or 8-*endo*-folded-out) conformers of erythromycin A 9-ketone is set up to cyclise in a 12–9 direction to give the 9,12-hemiacetal. In

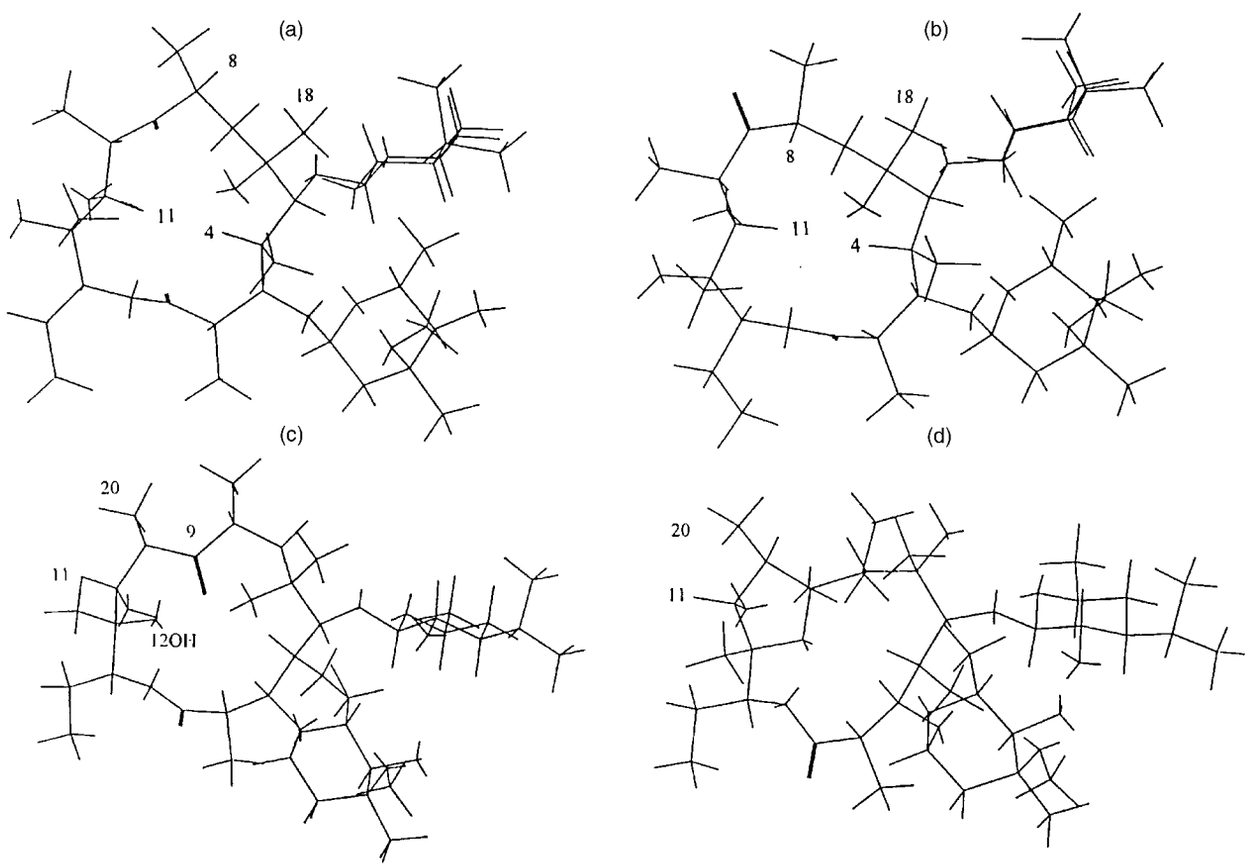

Fig. 4 The structure of erythromycin A in aqueous solution derived from ROESY NMR spectroscopy. (a) Folded-out erythromycin A 9-ketone (approx. 90% of total 9-ketone). H8 is *exo* to the macrolide ring. (b) 8-*endo*-folded-out erythromycin A 9-ketone (approx. 10% of total 9-ketone). (c) Cyclisable erythromycin A 9-ketone, present in trace amounts. The lower energy of two families of cyclisable conformers identified by modelling is shown. (d) Erythromycin A 9,12-hemiacetal (represents about 25% total erythromycin A at 30 °C).

Table 4 Monte Carlo searches on erythromycin A 9-ketone

Calculation	Starting "crystal" conformation	Distance constraints applied	Torsion constraints applied	Result
1	Folded-out and folded-in	All except 3–11, 16–17, 11–20, 13–21	H2H3 140–180°	Folded-out
2	Folded-in	All except 4–11, 5–18, 8–18, 15–16, 11–20, 13–21	None	Folded-in
3	Folded-out	All except 11–20, 13–21	None	8-*endo*-folded-out
4	Folded-in	As calculation 2 but H4–H18 > 2.8 Å	None	No minimum achieved
5	Folded-out	All including 11–20, 13–21, but no other constraints to H11; O12–C9 also included	None	Cyclisable

Table 5 Monte Carlo searches on azithromycin

Calculation	Starting conformation	Distance constraints applied	Result
1	Folded-out	All except 3–11, 4–18, 8–11, 9r–11, 11–22, 16–17	Folded-out, 11 approaches 4
2	Folded-out	All except 2–11, 4–11, 4–18, 8–11, 9r–11, 11–22, 15–16	Folded-out, 11 approaches 3

all these structures the oxygen on C12 is more than 4 Å from C9, whereas the oxygen on C6 is much closer. A conformer in which H11 approaches $H_3$20 and H13 approaches $H_3$21 will have the C12 oxygen pointing into the macrolide ring and will therefore be cyclisable in a 12–9 direction. Such a conformer is therefore expected in aqueous solutions of erythromycin A, but need not necessarily be present in detectable amounts.

The presence of 11–20 and 13–21 crosspeaks could not immediately be taken as evidence of a cyclisable conformer. Erythromycin A 9,12-hemiacetal was expected to give rise to these crosspeaks and chemical exchange occurred in the ROESY spectrum at 45 °C. While the chemical exchange signals which could be securely assigned did not have an Overhauser component (they were all of the form H2k–H2h, where k = ketone, h = hemiacetal) this could not be precluded. The ROESY spectrum was therefore repeated at three different mixing times. The 11k–20k and 13k–21k crosspeaks persisted at mixing times of 250, 150 and 100 ms, suggesting that they were ROESY, rather than chemical exchange crosspeaks, and indicating the presence of a cyclisable conformer in detectable amounts.

Calculation 5 of Table 4 was set up to determine the structure of the cyclisable conformer. The calculation gave rise to two families of low energy conformers (Fig. 4, Table 3). We conclude that one or both of these conformers is present in trace amounts in aqueous solutions of erythromycin A.

The modelling calculations clearly indicate that the cyclisable and 8-*endo*-folded-out conformations are discreet, low energy structures. (The folded-out and cyclisable conformers are also seen when erythromycin A 9-ketone is subject to unconstrained conformational search.) Of course, the NMR data alone could in principle allow for a whole range of transient intermediate conformers, but there is no direct evidence for these.

The evidence presented here suggests therefore that erythromycin A 9-ketone is present in aqueous solution in the conformational mixture folded-out > 8-*endo*-folded-out > cyclisable.

Erythromycin A 9,12-hemiacetal. In aqueous solution, erythromycin 9,12-hemiacetal represents less than 30% erythromycin A at room temperature, falling to about 10% at 50 °C. The ROESY signals for this isomer were poorly resolved, in many cases overlapping with the 9-ketone signals at both 30 and 45 °C. The few crosspeaks which could be distinguished are presented in the Supplementary Material.

Erythromycin A 9,12-hemiacetal was modelled from the 9-ketone and the constraints derived from the ROESY data applied. One family of conformers dominated the calculation and the lowest energy of this family is shown in Fig. 4. Details of the structure are shown in Table 3.

It should be noted that the data used to define this structure were sparse, and the result should be regarded as approximate.

Azithromycin. Azithromycin has a much smaller value for $J_{H2,H3}$ than either erythromycin A 9-ketone or clarithromycin, which might suggest the presence of a substantial amount of the folded-in conformer. The crystal structure recorded by Djokic *et al*,[31] however, is a folded-out structure with an H2H3 torsion angle of only 108°, showing that folded-out azithromycin enjoys some flexibility in the right-hand side of the macrolide ring.

A model of folded-in azithromycin was constructed in the same way as folded-in clarithromycin and the folded-out structure was modelled from the crystal structure in the usual way. The key parameters for these predicted structures are shown in Table 2.

The ROESY spectrum of azithromycin was acquired at 30 °C. Signal overlap for this drug is worse than with erythromycin, with particular problems arising from the overlap of the signals due to H5, H10 and H11. The experiment was therefore repeated at 45 °C. At this temperature, separation of H10 from H11 and H5 was achieved, allowing the clarification of some assignments. Otherwise, there were very few differences in the crosspeaks seen in the two spectra, and a summary is shown in the connectivity table in the Supplementary Material. The presence of both 3–11 and 4–11 crosspeaks would support the notion that azithromycin exists as a mixture of folded-out and folded-in conformers, were it not for the absence or near absence of all the other crosspeaks (3–8, 4–18, 8–18 and 16–17) required for a folded-in conformation.

Modelling was carried out using the azithromycin[31] crystal structure as a starting point. Initially all the constraints derived from the ROESY data were added to the structure. The low energy minimum, however, was some 50 kJ mol^{-1} higher than the starting point, suggesting that multiple conformations were present, although these might be very similar. Separate calculations were therefore set up, as shown in Table 5.

Calculations 1 and 2 accommodated all but a few small crosspeaks, most of which did not appear in the spectrum run at 45 °C. The principal difference between these two calculations is that the H4–H11 constraint is included in one, the H3–H11 constraint in the other. Both calculations converged well to give the very similar folded-out structures shown in Fig. 5a–c. The relative sizes of the 3–11 and 4–11 crosspeaks are hard to judge because of overlap with 4–5 and, especially, 3–5. The data indicate, however, that these two conformations make up over 95% of the total azithromycin in aqueous solution, and that the conformation in which H11 approaches H4 [(11–4)folded-out] is more abundant than the conformation in which H11 approaches H3 [(11–3)folded-out]. See Table 3 for structural details.

The only signals of significant size not to be accommodated by these two structures are 9r–11 and 11–22. In the structure given by calculation 1, these distances are both a little outside 3.5 Å. H11 is a tall signal with low multiplicity and is perhaps capable of long-range ROE-couplings. Alternatively, these constraints are easily accommodated by reversible epimerisation at N9 to give the *S* stereochemistry.

The 8–11 and 4–18 crosspeaks were very small and were seen only at 30 °C. These could only be accommodated by a folded-in structure, but the expected 3–8 crosspeak was not seen. It is possible that trace amounts of folded-in azithromycin are present, but this has by no means been conclusively demonstrated.

Finally, the H2H3 torsion angle was varied on the (11–4)folded-out structure. The angle could be increased up to 160° with no significant increase in energy. We conclude that azithromycin is somewhat flexible in the C2–C4 region. In aqueous solution azithromycin exists overwhelmingly in a folded-out conformation. The structure is somewhat flexible allowing extremes in which H11 approaches either H3 or H4. The structures are shown in Fig. 5.

Conformations of the desosamine and cladinose sugars. The ROESY spectra for clarithromycin, erythromycin A 9-ketone

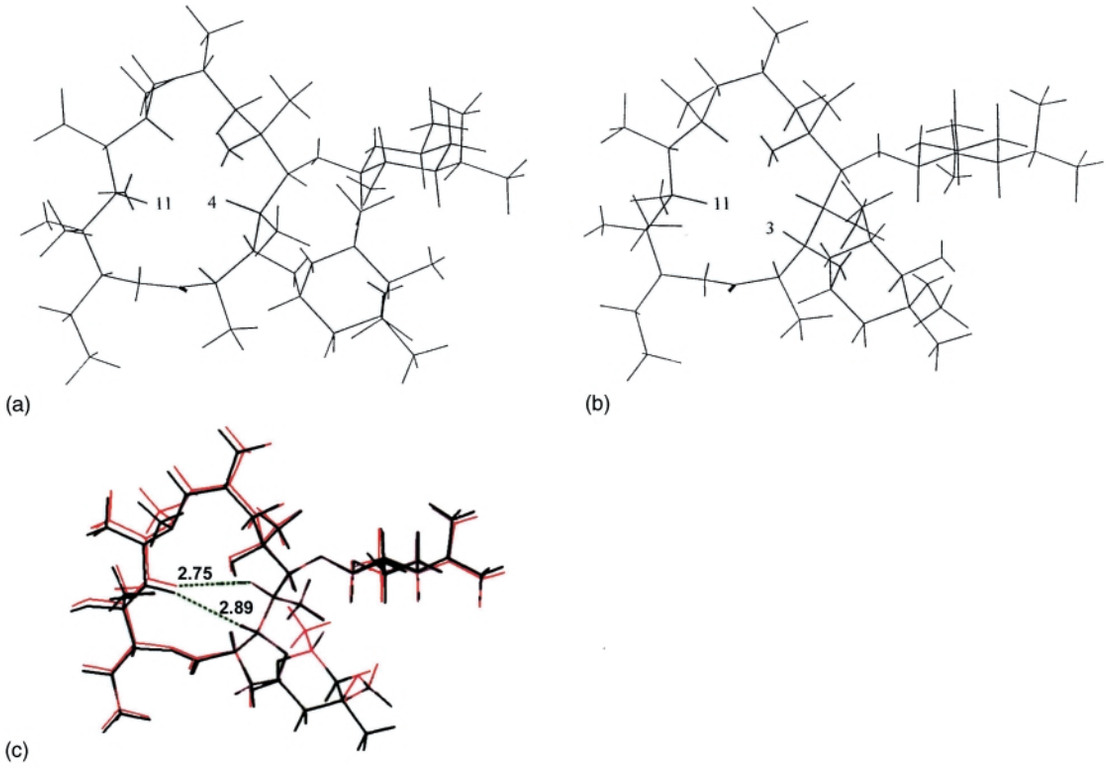

Fig. 5 The structure of azithromycin in aqueous solution derived from ROESY NMR spectroscopy. (a) (11–4)Folded-out azithromycin. (b) (11–3)Folded-out azithromycin. (c) Superimposition of (11–4)folded-out azithromycin (red) with (11–3)folded-out azithromycin (black). Folded-out structures represent at least 90% of total azithromycin under these conditions.

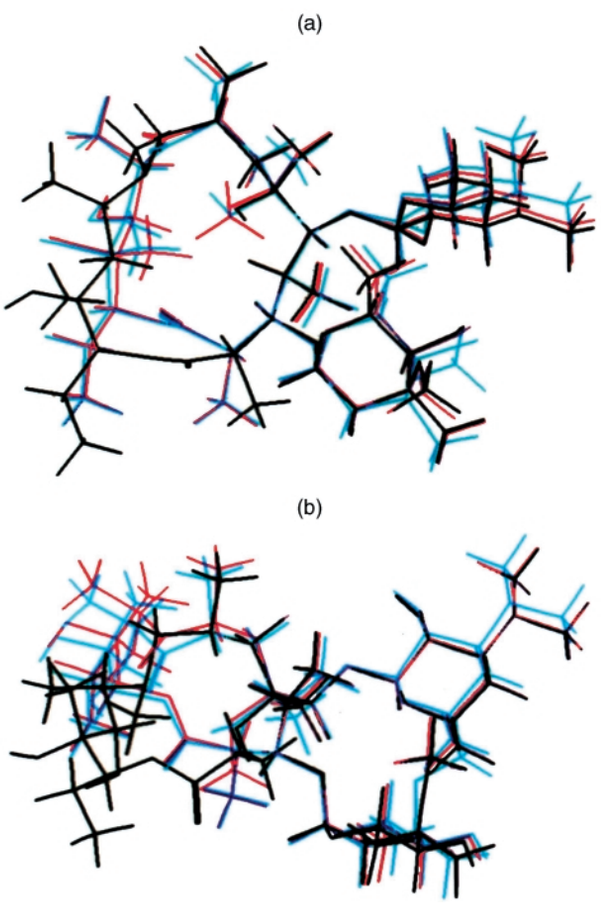

Fig. 6 Superimposition of folded-out erythromycin A 9-ketone (blue), clarithromycin (red) and (11–4)folded-out azithromycin (black) in two different orientations, showing conformational homology in the C3–C7 region.

and azithromycin show all the expected crosspeaks for sugars in the chair–chair, up–up conformation. These include 2′–4′*proR*, 1′–3′, 1′–5′ and 3′–5′ which indicate a chair conformation for the desosamine sugar and 2″*proR*–4″, 2″*proS*–7″, 4″–7″ and 4″–6″ indicating a chair conformation for the cladinose sugar. The large diaxial coupling constants found in the original assignments also suggest that chair conformers predominate.[14,27,28] Commodari *et al.*[25] suggested that their use of 90% water rather than D_2O may account for the boat conformations they observe. However, our ROESY spectrum for erythromycin A (Fig. 3) is visually very similar to theirs. We note, however, the close proximity of signals due to H4 and H4′*proS* and the relatively low resolution of the ROESY spectra reported. H2H4′ and H3H4′ crosspeaks are crucial to their analysis; we do not observe these signals but do see H2H4 and H3H4 crosspeaks, which Commodari *et al.*[25] do not. Further, the signals marked C2″e–C4′a and C3–C10 on their spectrum, we assign to H4′–H4′ and H11–H10 respectively.

Comparison of the conformation of clarithromycin, erythromycin A 9-ketone and azithromycin. As discussed above, the three drugs all exist predominantly in the folded-out conformation in aqueous solution, and the dominant conformation closely resembles the crystal structure. In Fig. 6, the three structures, using (11–4)folded-out azithromycin, are superimposed. It can be seen that the C3–C7 portions of the three drugs are superimposable, whereas the "left-hand" side of the azithromycin molecule is oriented quite differently from that of the other two drugs. In the extreme, C15 of azithromycin is 3 Å from C15 of erythromycin A 9-ketone when the sugars are superimposed.

Conclusions

This study has shown that clarithromycin, azithromycin and erythromycin A 9-ketone all exist overwhelmingly in the

folded-out conformation in aqueous solution. This is irrespective of the H2H3 torsion angle and we believe that caution should be exercised in using this parameter as an indicator of folded-out : folded-in ratios. The sugar rings are found to be in the Everett–Tyler chair–chair up–up conformations.

For erythromycin A and azithromycin there is evidence of some conformational flexibility. In azithromycin, the left-hand portion of the macrolide ring is sufficiently mobile to allow close approach of H11 to both H3 and H4 without the necessity for a major conformational change.

For erythromycin A, there is good evidence that a modified folded-out conformation in which H8 approaches H11 (the "8-*endo*-folded-out" conformation) exists in equilibrium with the normal folded-out conformation. In addition, trace amounts of a conformation in which H11 approaches $H_3$21 may be present. In this conformation, cyclisation to the 9,12-hemiacetal will be favourable. These minor conformations are not, we believe, exhibited in macrolide crystal structures currently available.

Finally, the superimposition of the dominant conformers of the three drugs suggests a key role for the sugar residues in binding to ribosomes. Although the left-hand portion of azithromycin appears in two dimensions to resemble erythromycin A 9-ketone and clarithromycin the three-dimensional structures resemble one another closely only on the right-hand side of the molecule, including the two sugar residues.

Experimental

^{1}H NMR spectra were measured on either a Varian VXR 600S operating at 600 MHz or a Varian Unity 500 operating at 500 MHz. ROESY spectra were obtained using 4, 8 or 12 mM solutions of the drugs in buffered D_2O (50 mM sodium phosphate, apparent pH 7.5). ROESY spectra were acquired at 30 or 45 °C using data matrices of 2048 × 1024 complex points and mixing times of 100–250 ms. In each case the spectrum analysed initially was run at 30 °C with a mixing time of 150 ms. Variations are described in Results. Typically, spectra were run with a sweep width of 3.5 kHz (at 600 MHz) with a spin lock field of 2.2 kHz, optimised empirically. The spectra were processed using a Gaussian window function with zero-filling in F_1.

The conformational analysis was carried out using a Silicon Graphics Iris 4D Indigo work-station, using Macromodel version 4.0 or 4.5 software.[34] Crystal structures of erythromycin A [9-(*O*-2,5-dioxahexyl)oxime] hydrate,[29] 4″-deoxo-4″-α-amino-azithromycin[31] and (14*R*)-14-hydroxy-6-*O*-methylerythromycin A[32] were obtained from the Cambridge crystallographic databank and modified manually to give erythromycin A, azithromycin and clarithromycin respectively. Each modified structure was minimised using the TNCG (Truncated Newton Conjugate Gradient Minimisation) method to find a local minimum which was used as the starting point for conformational analysis using the ROESY spectra.

The crosspeaks from the ROESY spectra were designated small (2.5 ± 1 Å), medium (2.3 ± 0.5 Å) or large (2.0 ± 0.4 Å) by comparison with peaks due to pairs of hydrogens of fixed internuclear distance. Distance constraints from each ROESY spectrum were applied to the appropriate modified crystal structure and minimised using the TNCG method. Monte Carlo conformational searches were carried out using an MM2* force field and the water solvation option over 1000–4000 steps until the global minimum had been found 20 times and no further minima were discovered.

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