Conformational Analysis of the Erythromycin Analogues Azithromycin and Clarithromycin in Aqueous Solution and bound to Bacterial Ribosomes

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The conformations of erythromycin A 9-ketone, azithromycin and clarithromycin free in aqueous solution and weakly bound to bacterial ribosomes are determined using ROESY and transferred NOESY ^{1}H NMR experiments.

Azithromycin **1** and clarithromycin **2** are semi-synthetic derivatives of erythromycin A **3**. Unlike erythromycin, both drugs exist as single isomers in aqueous solution.[1,2] They also have greater acid stability[1,3] and favourable pharmacokinetic properties.[3,4]

Erythromycin and its derivatives exert their antibacterial action by binding to the bacterial ribosome, specifically to the 50S subunit.[5] There appear to be two stages to this binding: a weak interaction which can be detected by NMR spectroscopy,[6–8] and a stronger interaction, detected by equilibrium dialysis and related methods.[9–11] The weak binding of erythromycin A to ribosomes has been characterised extensively by line broadening and transferred NOESY (TRNOESY) experiments,[6–8] the interaction being a fast exchange process on the NMR timescale. More recently we have shown that over a range of 10 erythromycin analogues, all the compounds with significant antibacterial activity and none of the inactive compounds exhibit this weak binding interaction.[12]

We have now analysed the conformations of azithromycin and clarithromycin in the weakly bound states and compared them with erythromycin A 9-ketone **3**. The corresponding analysis for the aqueous solution conformations has also been carried out.

Fully deuteriated ribosomes were used in NMR binding experiments to minimise the effects of spin diffusion. These were prepared as described previously.[8] Ribosomal core particles were prepared as described,[8] by treating deuteriated ribosomes with 2 mol dm^{-3} lithium chloride solution to remove the outer proteins, including L15 and L16 which are required for erythromycin binding.[13]

NMR spectroscopy was carried out at 30 °C on a Varian VXR600 instrument operating at 600 MHz. Solutions of **1**, **2** and **3** [each 4 mmol dm^{-3}, apparent pH 7.5 (±0.1), in 50 mmol dm^{-3} phosphate buffer in D_2O] were used in all experiments. ROESY spectra of the free drugs were acquired using data matrices of 2048 × 256 points with zero filling in F_1. The spectra were accumulated with mixing times of 130, 130 and 150 ms for erythromycin, azithromycin and clarithromycin, respectively. Another ROESY spectrum of clarithromycin was obtained with a mixing time of 250 ms and was qualitatively identical to the spectrum obtained using the shorter mixing time.

Samples for TRNOESY experiments were obtained by titrating ribosomes into the drug solutions until a doubling in linewidth was observed for the $H_3$15 triplet at δ 0.84. Typically, a final concentration of 1 μmol dm^{-3} ribosome was required, ribosomal activity varying slightly between batches. The control experiments were carried out using ribosomal cores at double the corresponding concentration of ribosomes so that the effect of non-specific binding could be monitored. TRNOESY spectra were accumulated using the same data matrix as the ROESY spectra with mixing times of 75–150 ms, optimised for the individual sample so as to minimise spin diffusion and maximise the signal-to-noise ratio. All 2D spectra were processed using a Gaussian window function and negative line broadening.

Molecular modelling was carried out on a Silicon Graphics Iris 4D workstation using Macromodel version 4.0 software.[14] ROESY and TRNOESY data were applied to azithromycin (derived from the crystal structure of 4″-deoxo-4″-α-amino azithromycin[15]), clarithromycin (derived from the crystal structure of (14*R*)-14-hydroxy-6-*O*-methyl erythromycin A[16]) and erythromycin A 9-ketone {derived from 9-[*O*-(2,5-dioxahexyl)oxime] erythromycin A hydrate[17]}.

The following constraints were used: large crosspeak = 2 ± 0.4 Å; medium crosspeak = 2.5 ± 0.5 Å; small crosspeak = 3.5 ± 1.5 Å, and suitable modifications were applied for constraints involving methyl and *N*-methyl groups.

A Monte Carlo search was set up with 20 000 steps for each structure. The structure of azithromycin in aqueous solution (derived from the ROESY data) is shown in Fig. 1. The antibiotic adopts the 'folded out'[18] conformation in which an NOE between H(11) and H(4) [as opposed to H(11) and H(3) in the 'folded-in' structure] is observed and the C(3)–C(5) fragment is 'folded out'. The two sugar rings are in the 'up–up' orientation, as seen in the crystal structure. The flexible left hand side of the macrolide ring [C(9)–C(15)] exhibits some deviation from the crystal structure: for example the methyl carbon C(20) is displaced by *ca.* 3 Å so that it points towards the left hand side of the molecule rather than towards C(6)OH, and C(15) has moved by *ca.* 1.5 Å towards C(13) and away from C(16).

Azithromycin adopts a 'folded in' conformation in $CDCl_3$;[19] the change to the 'folded out' conformation in polar solvents was also predicted.[19] The conformation of clarithromycin in

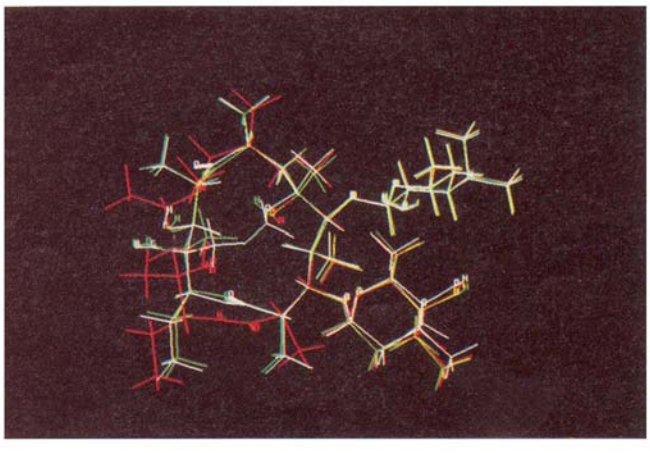
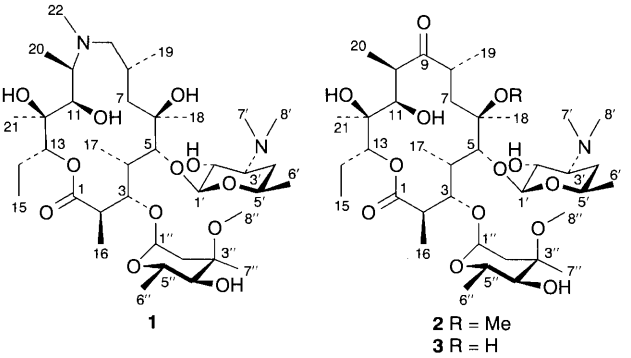


Fig. 1 ROESY derived structures of clarithromycin (white), azithromycin (red) and erythromycin (green) free in aqueous solution showing conformational homology in the C(3)–C(8) region



1

2 R = Me
3 R = H

aqueous solution has been previously reported[20] and our findings are in agreement. Clarithromycin also adopts the 'folded out' confirmation, as does erythromycin A ketone. Fig. 1 shows the three-dimensional structures of all three drugs. The regions C(3)–C(8) including the sugars are almost completely superimposable with variations in the 'left hand' side of the molecule. Active erythromycin analogues with quite substantial changes to this part of the molecule have been made.[21]

The TRNOESY spectra of erythromycin A 9-ketone, clarithromycin and azithromycin contained 95, 112 and 113 crosspeaks of which 82, 99 and 82, respectively, were due to hydrogens spatially proximate in the free drugs. When these data were applied to the structures of the drug and minimised using Macromodel, clarithromycin and azithromycin gave rise to the low energy structures shown in Fig. 2. The structure given for erythromycin represents one of two minima. The other structure shows a kinetically improbable distortion from the ROESY structure and fits the NOE data much less well than the structure shown.

The three structures representing the bound state of the drugs show greater similarity than the free drugs in the macrolide ring. The C(2)–C(6) portion superimposes well, the 'left hand side' C(9)–C(15) shows some variation but superimposes better than in the free state. The position of the desosamine ring is similar in all the drugs. The most striking findings are the variability in conformation of the cladinose ring and the differences in position of the C(16) methyl group. The cladinose ring adopts quite different orientations in the three drugs, although in each case the two sugars are overall closer together than in the free state. This is especially interesting in view of the wide variety of sugar moieties found among the macrolide antibiotics.

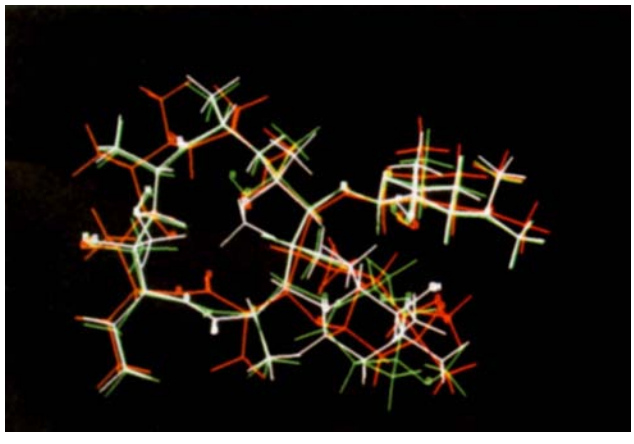


Fig. 2 TRNOESY derived structures of clarithromycin (white), azithromycin (red) and erythromycin (green) weakly bound to bacterial ribosomes showing conformationl homology in the C(2)–C(6) region, and profound differences in the cladinose residues

The weakly bound state of the macrolides observed here probably represents a first stage in an allosteric two stage binding process. We postulate that the weak binding of one molecule of macrolide facilitates the inhibitory binding of a second drug molecule. These data suggest that while active analogues of erythromycin A probably require desosamine or a similar sugar at C(5), the C(3) sugar may be varied.

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