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Theoretical and experimental investigation on clarithromycin, erythromycin A and azithromycin and descladinosyl derivatives of clarithromycin and azithromycin with 3-*O* substitution as anti-bacterial agents†

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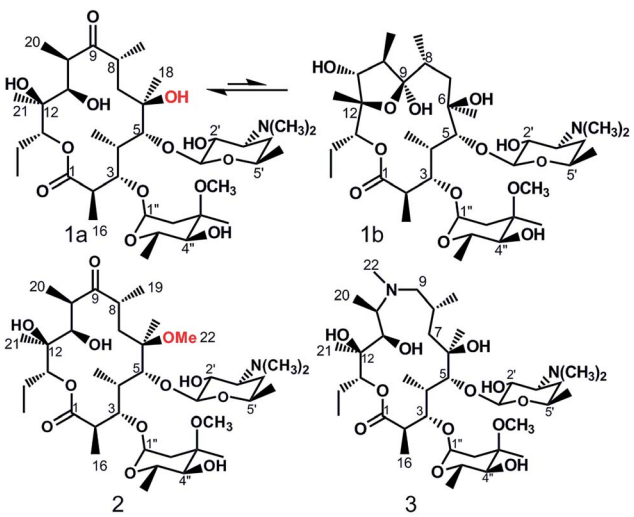
Erythromycin A, clarithromycin and azithromycin are the three most important macrolide antibiotics and are all very widely used in the clinic. They act on the bacterial ribosome inhibiting protein synthesis. We have performed both NMR (transferred NOESY) and modelling studies in order to determine the conformations of these antibiotics when they are bound to ribosomes. All three drugs exhibit the "folded-out" conformation in the bound state, but the dominant conformation of azithromycin is not completely superimposable on the clarithromycin and erythromycin A 9-ketone structures. Modelling suggests that clarithromycin (based on a 14-membered ring) and its 3-*O*-substituted descladinosyl derivatives are conformationally rigid molecules; these are compounds which generally exhibit more activity against Gram-positive bacteria. Azithromycin (based on a 15-membered ring) and its 3-*O*-descladinosyl derivatives are flexible *in silico* and show more activity against Gram-negative bacteria in culture.

Introduction

The first macrolide antibiotic, erythromycin A, was isolated in 1952 from a culture of *Saccharopolyspora erythraea*[1] and structurally described in 1957.[2,3] In aqueous solution, the 9-ketone (1a) equilibrates with the 12,9-hemiacetal (1b), which appears to have no antibacterial activity.[4,5] The second generation macrolides, clarithromycin and azithromycin are semi-synthetic derivatives of erythromycin A, and show increased acid stability and broader spectra of antibacterial activity. In general, clarithromycin is more active against Gram-positive bacteria but azithromycin has Gram-negative activity, which clarithromycin does not share.[6] In combination with existing antimalarial drugs (artesunate and quinine), azithromycin also shows mild antimalarial activity.[7]

The macrolide antibiotics inhibit bacterial protein synthesis by binding reversibly to the 50S subunit of the bacterial ribosome. They block the exit tunnel of the ribosome near the peptidyl transferase centre, and thus inhibit the elongation of the peptide chain.[8]

The three-dimensional structures of erythromycin A and its derivatives have been analyzed in three distinct situations: in the free drug (both in solution[9] and in the crystalline state[10]), in the bound state (by X-ray crystallography of drug-ribosome complexes),[11–14] and in an intermediate weakly-bound state, detected by NMR spectroscopy, of drug-ribosome mixtures.[15–17]



1a: Erythromycin A 9-ketone 1b: Erythromycin A 12,9-hemiacetal
2: Clarithromycin
3: Azithromycin

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Table 1 Key internuclear distances in crystal structures of folded-out (erythromycin A) and folded-in (dirithromycin) macrolides

	Internuclear distances (Å)	
Contact	Erythromycin A	Dirithromycin
H4–H11	2.51	3.33
H15–H16	3.28	3.81
H3–H8	5.34	2.30
H3–H11	3.48	2.46
H4–H18	4.02	2.71
H5–H18	2.66	3.33
H16–H17	3.84	2.51

Everett *et al.* (1990) described two conformational states available to the macrolide antibiotics: the folded-in conformation in which H3 approaches H11 and H4 approaches Me-18, and the folded-out conformation, in which H4 approaches H11 and H5 approaches Me-18 (Table 1).[18] Erythromycin 9-ketone, clarithromycin and azithromycin have all been shown to adopt folded-out conformations, both in the free state and when bound to ribosomes (see Fig. 1).

The conformations adopted by these drugs in the intermediate weakly bound state have been the subject of much investigation, but also some confusion. The transferred NOESY experiment is prone to artefacts (especially from spin diffusion). In addition, Everett *et al.*[18] showed that close approach of H11 to either H4 or H3 was diagnostic of a folded-out or folded-in conformation respectively for 14-membered macrolides, providing a convenient short-cut to the conformational analysis of 14-membered macrolides by NMR spectroscopy. However, this relation does not necessarily hold for 15-membered macrolides, which retain sufficient flexibility to be able to assume a folded-out conformation even when H3 approaches H11. Several other contacts, especially 4–18 and 5–18, are required to distinguish between folded-out and folded-in conformers.

Neither NMR nor X-ray crystallographic studies have the predictive potential of molecular modelling, and it would be useful to be able to investigate the conformation of a macrolide without having to synthesize it. An attempt to develop a systematic protocol for describing the conformations of macrolides was described in 2006, using oleandomycin and some of its derivatives as examples. Unconstrained molecular dynamics suggested that the drug forms a mixture of folded-out and folded-in conformations in both aqueous and chloroform solutions, and this conclusion was supported by 2D ROESY NMR spectroscopy.[15] Other molecular modelling approaches have generally depended upon NMR results and were not designed to be used independently.[16,17]

The main purpose of the work described here was to develop molecular modelling protocols for conformational characterization that would apply to 15-membered as well as 14-membered macrolides. We have also described systematically the intermediate weakly bound state for erythromycin, clarithromycin and azithromycin, and correlated these findings with *in vitro* anti-bacterial activity.

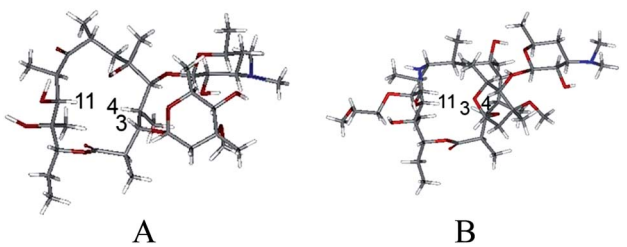


Fig. 1 Crystal structures of (A) erythromycin A[10] (folded-out conformation) and (B) dirithromycin[18] (folded-in conformation).

Results

The conformations of erythromycin A, clarithromycin and azithromycin bound weakly to bacterial ribosomes

Information about the conformation of a bound ligand in fast exchange with free ligand can be obtained using the transferred NOESY (TRNOESY) NMR experiment. This experiment relies on the fact that NOE build-up is fast in large complexes and slow (compared with the dissociation rate of the complex) in small ligands. NOE information is therefore transferred from bound to an excess of free drug, so that experiments show the chemical shifts of the free drug, but Overhauser effects characteristic of the conformation(s) of the bound drug. For macrolide antibiotics associated with ribosomes, ribosomal core particles replace whole ribosomes in control experiments (controlling for non-specific binding). Because of their intermediate size, 14- and 15-membered macrolides have solution NOEs close to zero (at 500–600 MHz and short mixing time).

Clarithromycin

The ^{1}H NMR spectrum of clarithromycin in aqueous solution has been described,[19] and in the presence of bacterial ribosomes, clarithromycin gives the TRNOESY spectrum shown as Fig. S1A in the ESI.† The spectrum contains 75 pairs of signals, consistent with a folded-out conformation of clarithromycin (Table S1 in the ESI†). Thirty-three of these signals are not seen in the ROESY spectrum of the same drug but are completely consistent with the folded-out structure. Only the weak long-range signal (7′8′–8″) gives additional information, constraining the relative positions of the two sugars. The crystal structure of clarithromycin is closely similar, although the H4–H11(3.04 Å) and the H7′H8′–H8″ (7.71 Å) distances obtained by molecular modelling (molecular mechanics) are rather long.

Erythromycin A

Fig. S1B in the ESI† shows the TRNOESY spectrum of erythromycin A. There are 83 pairs of signals belonging to erythromycin A 9-ketone (Table S1, ESI†). Up to four small signals (depending on processing) due to the 9,12-hemiacetal, and due to chemical exchange between the two isomers, are also seen in this spectrum, but it is clear that the 9-ketone binds to ribosomes and the 9,12-hemiacetal does not. Fig. 2 shows the global minimum

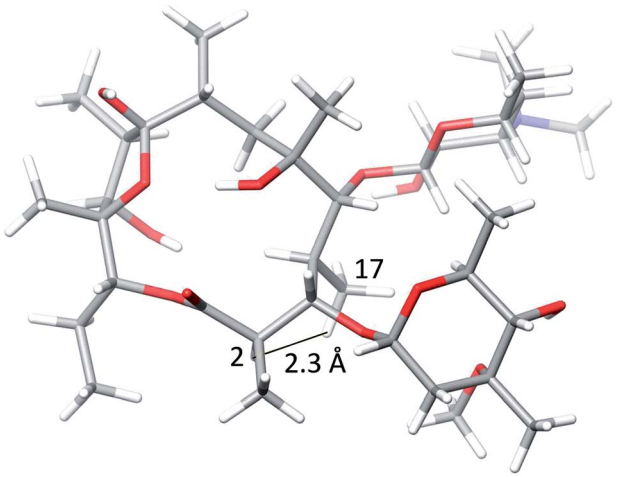


Fig. 2 Erythromycin A 12,9-hemiacetal: global minimum from unconstrained Monte Carlo search.

of an unconstrained Monte Carlo search on erythromycin A 9,12-hemiacetal, showing close approach of H2 to $H_3$17.

Most of the ketone signals are equivalent to those found in the clarithromycin TRNOESY spectrum suggesting that the dominant conformation of erythromycin is the same folded-out conformation as adopted by clarithromycin. The differences between the TRNOESY spectra of clarithromycin and erythromycin are concentrated in two areas of the molecule. Clarithromycin shows more intra-sugar cross-peaks for the desosamine sugar, but the extra peaks are all very small and consistent with spin diffusion. The erythromycin spectrum shows an H8–H11 cross-peak, and $H7_{proS}$–$H_3$18 and $H7_{proS}$–$H_3$19 connectivities are also present. This is consistent with erythromycin retaining some flexibility in the top portion of the molecule and thus the ability to form an 8-*endo* folded-out conformer, even when bound to ribosomes.

TRNOESY data were previously published by Bertho *et al.*[17] Differences between our results and theirs are indicated in Table 1 of the ESI.† The additional peaks that they report can be attributed to spin diffusion, because of the use of non-deuterated ribosomes and a relatively long mixing time.

Azithromycin

The TRNOESY spectrum of azithromycin in the presence of bacterial ribosomes (analysed with the aid of published assignments[20]) is presented in Fig. S1C of the ESI,† together with tabulated data (Table 2 of the ESI†). The vast majority of cross-peaks are identical with those seen for clarithromycin, suggesting that azithromycin, from a range of available conformations, adopts the clarithromycin-like folded-out conformation when bound to ribosomes.

Azithromycin enjoys some flexibility in the H2–H5 region so that H3 can approach H11 without significant effects on the rest of the molecule. The large H2–H4 cross-peak is a result of the same rotation. This signal is small in both the erythromycin and clarithromycin spectra. Superimposed folded-out structures of clarithromycin (global minimum from the unconstrained conformational search in Macromodel) and azithromycin (global minimum from the constrained search in Macromodel) are shown in Fig. S2 in the ESI.† Another small difference between the two drugs is that there is clear evidence of rotation about the C14–C15 bond of azithromycin, such that $H_3$15 can approach both $H_3$16 and $H_3$21. This can also be seen in the crystal structure of azithromycin bound to *Deinococcus radiodurans* ribosomes; in this structure two molecules of azithromycin bind, one with C15 approaching C21 and the other with C15 (more conventionally) approaching C16.

Comparison of the binding modes of clarithromycin, erythromycin and azithromycin

The dominant bound conformations of clarithromycin, erythromycin A and azithromycin are very similar. However, clarithromycin shows almost no flexibility in the bound state whereas erythromycin shows some limited flexibility and azithromycin rather more. For all three drugs, the methyl group $H_3$16 gives a broad ^{1}H NMR signal. When clarithromycin is

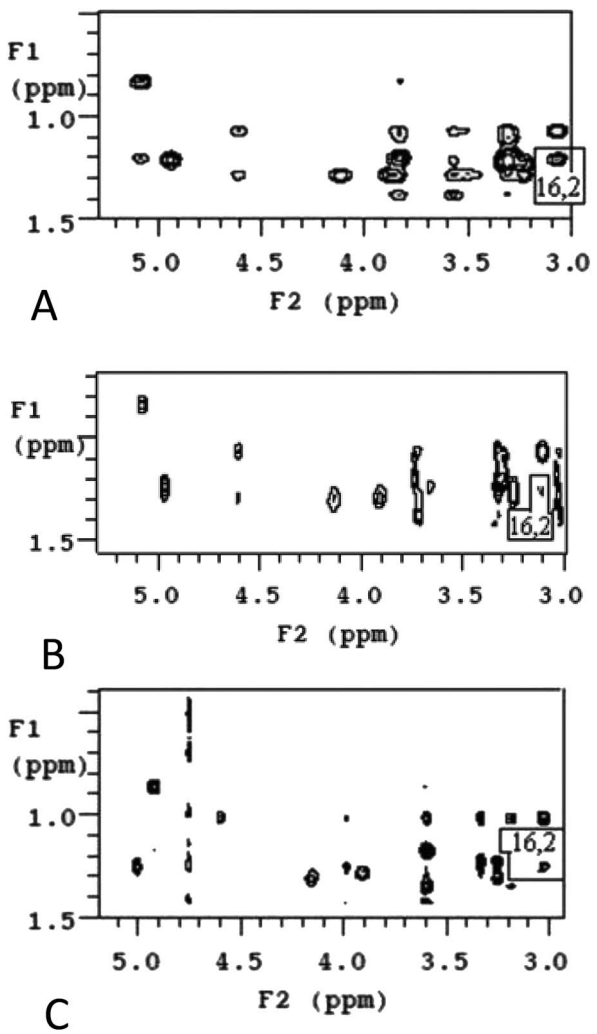


Fig. 3 Comparison of the TRNOESY NMR spectra of (A) erythromycin A, (B) clarithromycin and (C): azithromycin, showing the size of the 16,2 crosspeak.

bound to ribosomes, the TRNOESY H16–H2 cross-peak almost disappears, whereas in the TRNOESY spectra of the more flexible azithromycin and erythromycin A, this signal is clearly visible. Fig. 3 shows this region of the TRNOESY spectra of the three drugs.

Unconstrained molecular mechanics and molecular dynamics searches on clarithromycin, erythromycin A, azithromycin

We now compared results of unconstrained modelling using two conformational analysis approaches: a Monte Carlo Multiple Minima (MCMM) search and a molecular dynamics-based simulated annealing (MDSA) method. Unconstrained MCMM calculations were carried out with Macromodel version 8 using the AMBER* force field[21] and water as a solvent (GBSA solvent model).[22] Molecular dynamics calculations were performed in Sybyl 7.3 (Tripos, St. Louis) using simulated annealing with energy minimization using Tripos force field and water solvation model.[23] The starting structures for both modelling protocols were crystal structures of the macrolides alone (clarithromycin,[24] erythromycin A[10] and azithromycin[25]) available in the Cambridge Crystallographic Database.

Molecular mechanics calculations are generally preferred for small molecules: the exploration of conformational space is often comprehensive and very accurate predictions of lowest energy structures can be obtained. Molecular dynamics calculations are generally preferred for large molecules. The exhaustive exploration of conformational space is simply not practical for large, flexible molecules. Macrolide antibiotics do not fit comfortably into the description "small molecules" but neither are they macromolecules. For this reason we used both molecular dynamics and molecular mechanics approaches.

Clarithromycin

In the case of clarithromycin, molecular mechanics calculations appeared to be satisfactory and a calculation using the AMBER force field gave a folded-out global minimum which was found 19 times and is shown in Fig. 4A. All the NMR constraints are satisfied in this single conformer. The global minimum found by molecular dynamics (simulated annealing) experiments was almost identical with this structure, as shown in Fig. 4B. Key internuclear distances for these structures are shown in the ESI (Table S3†).

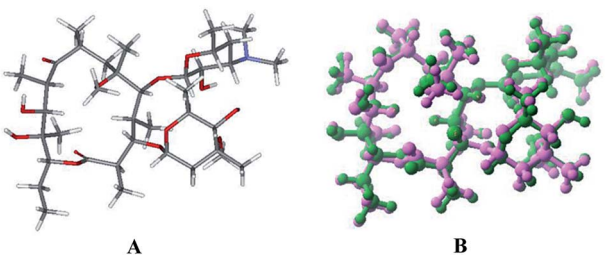


Fig. 4 (A) Global minimum of unconstrained Monte Carlo search on clarithromycin in water; (B) superposition of this global minimum (pink) and the lowest energy conformation (shown in green) obtained by simulated annealing. Both structures satisfy all the TRNOESY NMR constraints.

Erythromycin

Unconstrained molecular mechanics calculations suggest that the 6-*O*-Me group of clarithromycin has a profound effect on its conformational behaviour. Erythromycin A, which carries a hydroxyl group at C-6, shows four different conformers within 1 kcal mol^{-1} of the global minimum: Fig. 5 shows a folded-out conformer, an 8-*endo*-folded-out conformer (in which H8 approaches H11)[9] in two versions differing in their H4–H11 distances, and a 7-*endo*- folded-in conformer in which H11 approaches both H3 and H7 (but not H8). On the basis of the populations of structures within 1 kcal mol^{-1} of the global minimum, we would expect the folded-out conformer to predominate (about 75% of the minima found belong to this family). On the same basis, the 7-*endo*-folded-in conformer would be present at less than 5%.

Simulated annealing gave very similar results, with a folded-out structure again appearing at lowest energy and 8-*endo*-folded-out and 7-*endo*-folded-in structures appearing within 1 kcal mol^{-1}. The orientation of the cladinose sugar is slightly different in the global minimum obtained by simulated annealing from that obtained by molecular mechanics (see Table S4 in the ESI† for details), so that the superposition of the two sets of modelling results (Fig. 5E) is less good than for clarithromycin (Fig. 4B).

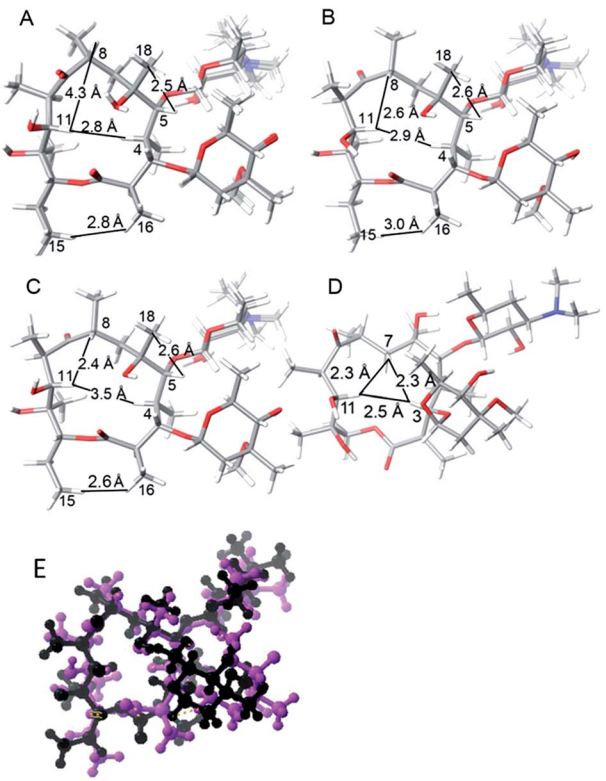


Fig. 5 Families of conformers of erythromycin A within 1 kcal mol^{-1} of the global minimum found by Monte Carlo searching: (A) folded-out; (B) and (C) variants of the 8-*endo*-folded-out conformation; (D) 7-*endo*-folded-in conformer. (E) Superposition of this global minimum (pink) and the lowest energy conformation (shown in black) obtained by simulated annealing.

The TRNOESY NMR spectrum for erythromycin A shows H2″s–H4″ and H8″–H2″r peaks which may be assumed to be due to spin diffusion. Otherwise, all the cross-peaks are satisfied by the 8-*endo*-folded-out conformer and, with the exception of H8–H11, by the global minimum folded-out conformer.

Azithromycin

An unconstrained molecular mechanics calculation gave rise to a global minimum structure (Fig. 6A) with an energy of 30.6 kcal mol^{-1}. This structure does not resemble any known conformation for macrolide antibiotics, neither does it satisfy the NMR constraints (see Table S5 in the ESI†). Interestingly, it closely resembles the 7-*endo*-folded-in erythromycin, which was found *in silico* (see Fig. 5). As in the conventional folded-in conformation, H11 approaches H3, but whereas a conventional folded-in conformation shows a close approach of H8 to H3, this conformation has H7 approaching H3 and H4.

There were no familiar conformers within 2.4 kcal mol^{-1} of the global minimum; the H3–H11 and H4–H11 distances were all too large. In order to achieve a folded-out structure that would satisfy the NMR data, it was necessary to constrain the Monte Carlo search, setting H3–H11 to 3.5 ± 0.3 Å and H4–H11 to 2.5 ± 0.3 Å. This yielded a conformer of energy 35.3 kcal mol^{-1} that satisfied all the NMR constraints (Fig. 6B).

Molecular dynamics calculations were superficially more immediately successful, and the global minimum in the unconstrained calculation was a folded-out structure (Fig. 6C) in which the NMR constraints were all satisfied, except that the inter-sugar distance H1′–H5″ of 2.77 Å is long-below 2.5 Å is expected. The structure, however, is profoundly different from the crystal structure; the orientation of the sugar rings is quite unexpected.

A more conventional folded-out structure with H1′–H5″ of 2.35 Å, was found at 3.8 kcal mol^{-1} above the global minimum (Fig. 6D).

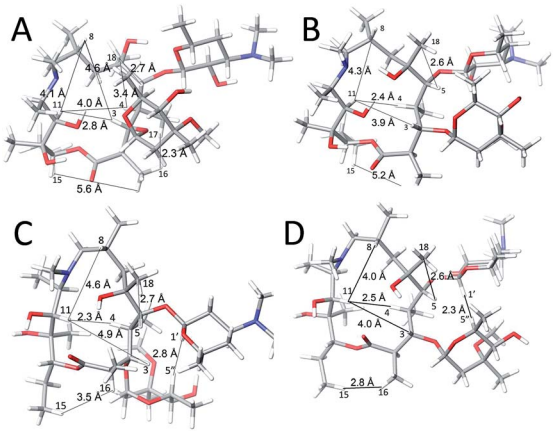


Fig. 6 Molecular modelling of azithromycin (A) Global minimum of unconstrained Monte Carlo search in water (B) global minimum of Monte Carlo search with H3–H11 and H4–H11 constrained. (C) Unconstrained molecular dynamics lowest energy structure. (D) Unconstrained molecular dynamics structure 3.8 kcal mol^{-1} above the lowest energy structure.

The conformational flexibility of azithromycin: ^{1}H NMR studies and molecular dynamics simulation

The failure of both molecular dynamics and molecular mechanics calculations to identify the global minimum of azithromycin found experimentally was disappointing. We considered the (highly remote) possibility that this relatively large molecule could adopt a metastable conformation in aqueous solution, above the global minimum. In order to explore this possibility, we heated an aqueous solution of azithromycin in an NMR tube over the temperature range 30 °C-90 °C and allowed the solution to cool, measuring the ^{1}H NMR spectrum at intervals. Fig. S3 of the ESI.† shows the (uninteresting) ^{1}H NMR spectra obtained in this experiment. There was no change in the spectra over the temperature range other than the expected slight temperature-dependent shifts. The final spectrum was identical with the starting spectrum.

The same experiment was carried out *in silico* (molecular dynamics calculations) starting from the folded-out crystal structure of azithromycin. It showed the same result as ^{1}H NMR experiment. In aqueous solution the conventional folded-out conformer of azithromycin is stable.

The properties of descladinosylclarithromycin and descladinosylazithromycin

As shown above, the second generation macrolides, although often assumed to be equivalent to one another, differ hugely in their conformational flexibility. The effect of the removal of the cladinose sugar was now explored. 5-Desosaminylerythronolide A (descladinosylerythromycin A) has little or no antibacterial activity,[26] and unconstrained molecular modelling shows a folded-out structure in which the desosamine sugar fails to superimpose upon that of erythromycin A (data not shown). Descladinosylclarithromycin (**4**) and descladinosylazithromycin (**5**) were constructed from the corresponding drugs[24,25] *in silico*, and were subjected to unconstrained Monte Carlo searches.

Fig. 7 shows how the global minimum structure of descladinosylclarithromycin is almost superimposable on that of clarithromycin. As expected, the more flexible azithromycin derivative did not superimpose on a folded-out macrolide.

The synthesis of compounds **4** and **5** by acid-catalysed degradation of the parent drugs is described in the ESI,† together with their ^{1}H and ^{13}C NMR data. Measurements of minimum inhibitory concentrations of **4** and **5** against an azithromycin-susceptible *Escherichia coli* K12 strain and against

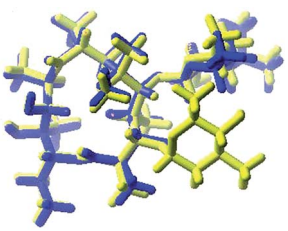
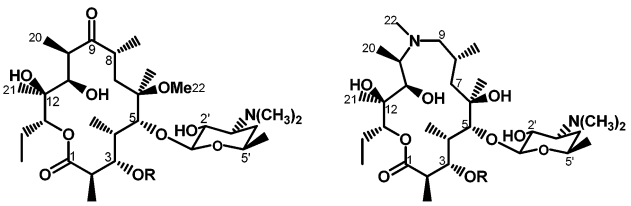
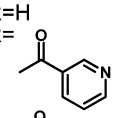


Fig. 7 Global minima of clarithromycin (yellow) and descladinosylclarithromycin (4, blue) in water, obtained from unconstrained molecular mechanics, superimposed.

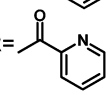
Table 2 Key internuclear distances in the global minima of compounds **6**, **9** and **12**. The global minima of **7** and **8** are essentially identical with **6** and those of **10** and **11** are essentially identical with **9**. (*S.a.* = *Staphylococcus aureus*, *H.i.* = *Haemophilus influenzae*)



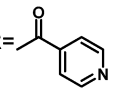
4: R=H
6: R=



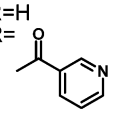
7: R=



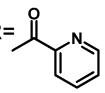
8: R=



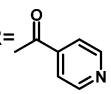
5: R=H
9: R=



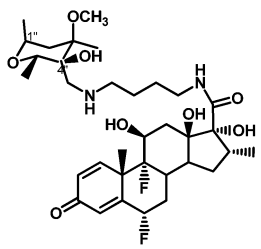
10: R=



11: R=



12: R=



	MIC (μg ml^{-1})		Key distances (Å) in the conformation representing the global minimum							
Cmpd	*S.a.*	*H.i.*	3–11	4–11	3–8	8–11	4–18	5–18	15–16	16–17
6	0.78	12.5	4.0	2.5	6.1	4.3	4.0	2.7	2.8	4.2
9	3.13	1.56	2.4	4.5	3.9	4.8	2.7	3.5	5.3	2.3
12	NA	NA	4.0	2.3	5.9	4.3	4.0	2.6	5.1	4.4

macrolide-susceptible *Bacillus cereus* and *Staphylococcus aureus* strains showed essentially no activity (see ESI Table S7†). This is the expected result for descladinoseazithromycin, but illustrates that, although descladinoseclarithromycin (**4**) adopts the same conformation as its antibacterial parent, this is insufficient for activity.

Unconstrained molecular mechanics on 3-*O*-substituted descladinosyl clarithromycin and 3-*O*-substituted descladinosyl azithromycin derivatives

Derivatives of **4** and **5**, substituted at the 3-oxygen have been reported and some have antibacterial activity.[27,28] Compounds **6–9**, together with the unreported **10–11** were now subjected to unconstrained Monte Carlo searches. The compounds, their activities and structural characteristics obtained using unconstrained molecular modelling are represented in Table 2. Compounds **6** (based on clarithromycin) and **9** (based on azithromycin) have significant antibacterial activity, but (as in the case of the parent drugs) the global minima found for these compounds are quite different from one another. Compound **6** is a proper folded-out structure. Derivatives of descladinosylazithromycin showed folded-out (active) conformers about 4.8 kcal mol^{-1} above the global minimum. Only when the azithromycin skeleton was substituted with a large steroid sidechain (compound **12**) did it give rise to a folded-out structure in the global minimum of a molecular mechanics calculation. The latter compound was, however, developed not as an antibacterial but as an anti-inflammatory agent!

Discussion

Molecular modelling calculations, both molecular mechanics and molecular dynamics are successful in finding global minima for the 14-membered macrolides. In the case of clarithromycin, the folded-out structure (Fig. 4) is the only structure to be found by modelling and the only structure indicated by NMR. The TRNOESY data for clarithromycin bound to bacterial ribosomes indicate that this drug has very limited conformational flexibility, hence the almost complete disappearance of the H2–H$_3$16 cross-peak. Erythromycin A is more flexible than

clarithromycin, and is able to adopt an 8-*endo*-folded-out as well as a conventional 8-*exo*-folded-out conformation. Molecular modelling predicts a minor 7-*endo*-folded-in conformer, but this has not been distinguished experimentally.

Azithromycin, the 15-membered macrolide, is, however, quite different. Molecular modelling indicates that a wide range of conformations is available to this drug. Although the preferred conformation (as indicated by NMR) is essentially the same folded-out structure as is seen for erythromycin A and clarithromycin, this drug is conformationally flexible, because of the increased size of the macrolide ring.

In three distinct situations (free solution,[9] weakly-bound to bacterial ribosomes in the present work, and tightly-bound to ribosomes detected by X-ray crystallography[14]) clarithromycin, erythromycin A 9-ketone and azithromycin adopt essentially the same conformation, as shown in Fig. 1. (The distorted conformation of erythromycin reported in early X-ray studies of drug-ribosome complexes[12] has been superseded[29]-it probably derived from the relatively low resolution of these studies and from their focus on the ribosome rather than the drug.). Molecular dynamics or molecular mechanics calculations can be used to predict whether a macrolide has this conformation available, but in the case of azithromycin, we have been unable to find a simple modelling protocol using commercially available software that successfully finds the lowest energy conformation seen experimentally. Choosing an appropriate solvent and solvent model is one of the key stages in Monte Carlo calculations. Implicit solvation represents a compromise solution in which explicitly represented water molecules are replaced by a continuum model that reflects the average behaviour of water molecules. Our choice of the GBSA solvent model (Generalized Born model augmented with the hydrophobic solvent accessible surface area (SA) term) and AMBER* force field gives good results for conformationally rigid molecules like clarithromycin, its 3-*O*-substituted descladinosyl derivatives and erythromycin A. However, approximations in the water model and force field have the potential to impair the prediction of biologically active conformations of conformationally flexible molecules like azithromycin and its 3-*O*-substituted descladinosyl derivatives. Where there is more flexibility, there are more opportunities for intra-solute interactions. These intrasolute interactions could be over emphasised using implicit solvent rather than explicit water molecules – the latter could more adequately screen and provide bridging hydrogen bond networks. Implicit solvent has shown inadequate shielding for salt bridges.[30,31]

At the present time, we can model clarithromycin derivatives and predict that only a molecule that adopts a folded-out conformation (Fig. 1) in the global minimum is likely to be active, whereas for azithromycin derivatives we are required to search for the active conformer among the range of structures indicated by modelling.

Macrolides are among the most efficacious and safe drugs in the clinic. A frustration is that they are all based on natural products containing many chiral centres. This means that systematic investigations of structure–activity relationships by directed modification of the skeleton are simply not feasible. It is conventional to think of the semi-synthetic antibiotics azithromycin and clarithromycin as broadly equivalent; indeed hospital antibiotic policies often permit one but not the other. Both are acid-stable, hydrophobic derivatives of erythromycin A, and both have excellent pharmacokinetic properties. However, it has long been known that these remarkable compounds have quite different spectra of activity. Azithromycin has the rare property of excellent anti-Gram-negative activity (and even anti-malarial activity), whereas clarithromycin generally has excellent activity against Gram positive organisms.

Molecular modelling, even with commercial packages, has advanced our understanding of the properties of these molecules. Hence we are able to postulate that the chemical basis of the clinical differences between these drugs may lie in their very different flexibilities. Azithromycin is conformationally labile, confounding the barriers that cells throw up to bar the entry of drugs. What clarithromycin lacks in subtlety it makes up for in focus: sensitive Gram positive infections are very sensitive indeed.

Experimental

Molecular modelling

Erythromycin A, clarithromycin and its 3-*O*-substituted descladinosyl derivatives, azithromycin and its 3-*O*-substituted descladinosyl derivatives were constructed using Macromodel 8 software[32] from the appropriate crystal structures. Structures were minimized using the Truncated Newton Conjugate Gradient (TNCG) method in order to obtain local minima. The Monte Carlo Multiple Minimum (MCMM) conformational search was used to find the global minima.[33] The GBSA model was used for water solvation. The search was set to 10 000 structures to be minimized and all structures within 6 kcal mol^{-1} energy range were stored.

Molecular dynamics calculations were performed using Sybyl 7.3.[34] Minimization was performed using the Tripos force field in water as a solvent (ε=80.4). The Gasteiger–Huckel charges were used throughout the calculation. Global minima of clarithromycin, erythromycin A and azithromycin were obtained from their crystal structures minimized by Tripos force field followed by simulated annealing [(clarithromycin; heated to 600 K for 1500 fs and then cooled to 0 K for 10 000 fs), (erythromycin A; heated to 1000 K for 750 fs and then cooled to 0 K for 10 000 fs) and (azithromycin; heated to 600 K for 2000 fs and then cooled to 0 K for 10 000 fs)]. The structures obtained were minimized again using the Tripos force field. The number of iterations for each minimization was 10 000 and the minimization simulation was terminated gradiently when the difference in energies between two conformations was no more than 0.005 kcal mol^{-1}.

The abundance of different conformers (in case of erythromycin A) was calculated according the simple formula:

$$\omega\%\ (\text{conformation type}) = \frac{N(\text{conformation type})}{N(\text{total})} \times 100,$$

where N (conformation type) is the number of structures of the same type (*e.g.* folded-out) within 1 kcal mol^{-1} of the global minimum and N (total) is the number of structures of all types within 1 kcal mol^{-1} of the global minimum.

NMR analysis

One and two dimensional NMR spectra were obtained by standard procedures, described in the ESI.†

Minimum inhibitory concentrations of clarithromycin, azithromycin and their derivatives against *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Serratia marcescens* and *Corynebacterium xerosis*

These were determined by standard methods[35] and are described in the ESI.†

Conclusions

Molecular dynamics and molecular mechanics calculations using commercially available software can be used to predict whether macrolide derivatives can adopt the active folded-out conformer required for ribosome binding. In addition, an indication of the likely flexibility of a given molecule can be obtained. For antibacterial 14-membered macrolides, this folded-out conformer is likely to be found at lowest energy in unconstrained searches. 15-membered macrolides adopt the same conformation in the active state, as shown by NMR and X-ray crystallographic measurements, but unconstrained molecular modelling cannot be relied on to find this conformer at lowest energy. Both NMR and modelling studies indicate that azithromycin is an intrinsically flexible molecule and can adopt numerous conformers. The flexibility correlates with the ability of the drug to target Gram negative and even malarial cells.

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