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Transferred nuclear Overhauser effect study of macrolide–ribosome interactions: correlation between antibiotic activities and bound conformations

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Abstract

The study of macrolide–ribosome interactions has been investigated using two-dimensional transferred nuclear Overhauser effect spectroscopy (TRNOESY). A new medically important macrolide antibiotic, roxithromycin, with the replacement of the 9-keto group in erythromycin by a 9-oxime chain, was studied in the complex state with the bacterial ribosome. Analysis of transferred nuclear Overhauser effect (TRNOE) experiment resulted in a set of constraints for all protons pairs. These constraints were used in structure determination procedures based on molecular modelling to obtain a bound structure compatible with the experimental NMR data. The results compared with the conformational analysis of the substrate in solution indicate that only one specific conformation is preferred in the bound state while in the free state the sugar ring moities were relatively disordered. The bioactive macrolide antibiotics studied roxithromycin and erythromycin which displayed a strong NMR response, are metabolized in RU39001 and erythralosamine respectively which do not retain antimicrobial activity. The inactive major metabolites were used to define if TRNOEs observation may be characteristic of a biological activity. These control experiments gave essentially blank TRNOESY spectra. This study shows that Mg^{2+} does not play a direct role for the low affinity binding site studied by TRNOE what is in agreement with an hypothesis of two distinct binding levels, with a low affinity binding level necessary for the tight binding one.

Keywords: NMR spectroscopy, transferred NOE, macrolides, antibiotics, ribosomes.

1. Introduction

Macrolide antibiotics can be classified structurally and functionally into two main groups represented by erythromycin A and spiramycin, respectively [1]. A new macrolide antibiotic, roxithromycin (**1**) is actually an important derivative of erythromycin in clinical macrolide therapy (Fig. 1) [2]. Despite many years of study by many investigators, many details about the macrolides mechanism of action remain incompletely determined or unknown [3,4]. Macrolide antibiotics like erythromycin A and roxithromycin inhibit protein biosynthesis in the elongation step by binding to 50S bacterial ribosome [5]. In addition to their inhibitory effect on translation, macrolides antibiotics like erythromycin can prevent the formation of the 50S ribosomal subunit in growing bacterial cells [6].

Roxithromycin (**1**) exerts its antibacterial effect through the disruption of protein synthesis inside the bacterial cell. This antibiotic interferes at the ribosome level where aminoacids carried by tRNA are incorporated into protein chains as specified by the bacteria's mRNA. Work on ribosomal preparation derived from *Escherichia coli* has demonstrated that roxithromycin binds specifically to the 50S ribosome component [7].

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The key reaction of the translation, peptide bond formation, is promoted by the catalytic centre of 50S (the peptidyl transferase centre), and the growing peptide chain attached at the donor P site undergoes peptide linkage with an aminoacyl-tRNA at the acceptor A site [5]. Direct visualization of A-, P-, and E-site tRNAs in *E. coli* ribosomes by 3-D cryoimaging have advanced our understanding of ribosomal structures and function [8]. The stage is set for detailed ligand binding experiments that explore the binding state of elongation factors and tRNA [9]. Ribosomal proteins L16, and especially L15 [10], as well as 23S-rRNA [4] play a key role in the binding of erythromycin A near the peptidyl transferase.

The inhibition stage in the macrolides mechanism of action, have mainly been attributed to a steric hindrance of the drug molecule bound to the polypeptide chain exit channel, once a certain number of peptide bonds have been formed [11,12]. The drug would block the exit channel, hindering the growing polypeptide [13]. The steric hindrance that the macrolide molecule imposes, can destabilize the peptidyl-tRNA during translocation from the A site to the P site, inducing the release of the tRNA molecule and thus inhibiting protein biosynthesis [14].

T. Tritton has been the pioneer in the use of proton nuclear magnetic resonance by differential broadening, to define the nature of the drug when it is bound to its ribosomal receptor in fast exchange [15].

Two different types of Macrolide-*E. coli* Ribosome specific interactions exist with the 50S ribosomal subunit: (1) strong interaction ($K_d = 10^{-7}-10^{-9}$ M) [16–18] classically measured by 'binding' test and (2) weak interactions ($K_d = 10^{-3}-10^{-5}$ M) characteristic of a biological activity which can be studied by transferred nuclear Overhauser effect measurements (TRNOE) [19–21]. It was postulated that the binding to the bacterial ribosome occurs in a two step process, the first step involving a low affinity preinhibition binding site and the second one being the strong interaction responsible for the protein biosynthesis inhibition [22].

If binding is sufficiently weak to allow exchange (fast on the time scale of spin-lattice relaxation rate), TRNOE NMR experiment [23–28] is well adapted to study the bound conformation in a ligand-receptor complex transferred to the free molecule via chemical exchange.

Taking advantage of the exchange between bound and free macrolide antibiotic, we have developed a study of macrolide-ribosome interactions of roxithromycin (**1**) and its major metabolite (**2**) (Fig. 1) to *E. coli* ribosomes, using two-dimensional transferred nuclear Overhauser effect spectroscopy (TRNOESY). The information present in TRNOE experiments focus on a plausible conformation on binding to the bacterial ribosome and essential for its antibacterial action.

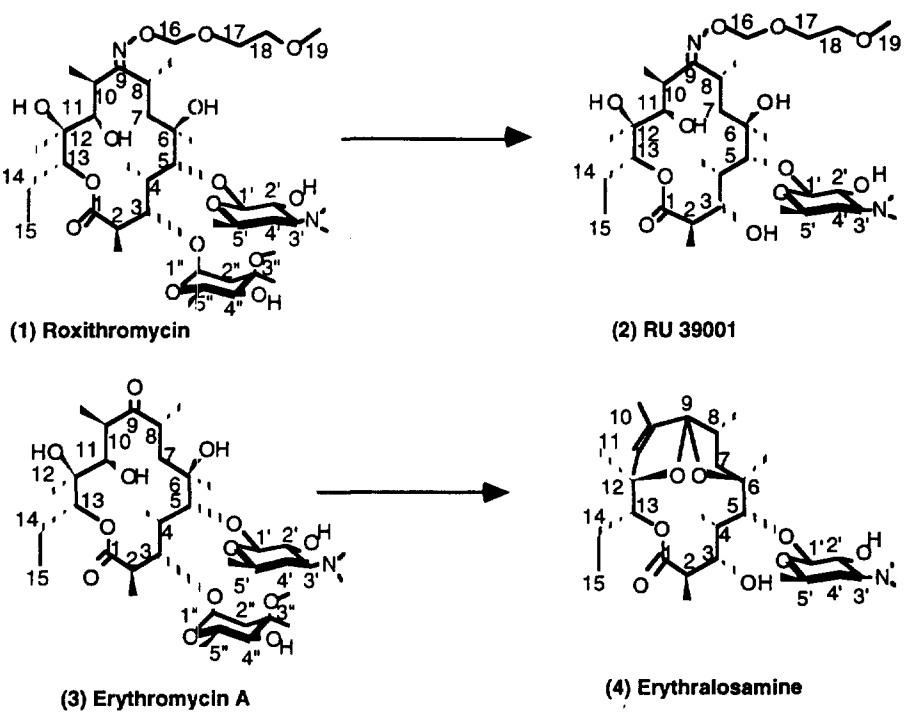


Fig. 1. Structures of macrolide antibiotics roxithromycin (**1**) and erythromycin (**3**) and their major metabolites RU39001 (**2**) and erythralosamine (**4**), respectively.

In view of the biological activity these results are compared to those obtained for erythromycin A (**3**) and one of its metabolite, erythralosamine (**4**).

2. Results

Because of their intermediate size, the 14-membered-ring macrolides, one of which is roxithromycin (**1**), display in solution typically NOEs which are close to zero (at 500 MHz with short mixing time). In TRNOESY spectrum (Fig. 2), cross peaks of the macrolide antibiotics in interaction with ribosome are strong and negative (characteristic of macromolecular correlation time domain). A TRNOE experiment with roxithromycin (**1**) is developed in presence of ribosomes (70S) and we observed a broadening of the signals. This experiment was used in order to develop the experimental parameters.

Physical parameters of crucial importance for the chemical exchange such as temperature, substrate-ligand ratio and *E. coli* ribosomal concentration have to be optimized in order to develop the TRNOESY experiment in the specific case of the macrolide-ribosome interaction.

1. Initially, to avoid ribosome denaturation at room temperature, the experiment was performed at 277 K. Lowering the temperature slows down considerably the chemical exchange between the free and the bound structure. In consequence, room temperature (293 K) seems better for fast exchange upon complex formation and leads to optimal conditions for TRNOESY experiments.
2. The line-broadening of roxithromycin (**1**) is proportional to the amount of ribosome. Low ribosomal concentration (0.8 μM) with a larger fraction of ligand (4 mM) causes a half line-broadening of roxithromycin (**1**) signals as TRNOE cross peaks well resolved. To the best of our knowledge, all the applications so far of transferred nuclear Overhauser effect spectroscopy involved ligand/receptor ratios less than 50. If we suppose that τ_c increases in proportion to the molecular mass of the complex, it would make the TRNOE observable even for a large excess of ligand (e.g. 1000:1 ratio or even more) [29]. Here, with a 5000 ligand/ribosome ratio associated with a large value of the k_{off}, transferred NOEs effects from macrolide-ribosome interactions can be observed only with these concentrations (0.8 μM ribosomes and 4 mM macrolide).
3. The tight interaction measured by the binding tests between macrolides and *E. coli* ribosomes is strongly Mg^{2+} dependent [30]. In the TRNOE NMR experiments, it was thus important to determine the effect of Mg^{2+} on the weak ribosomal binding site (the one which is observed by TRNOE NMR experiments). The experiments were performed with roxithromycin (**1**) in various phosphate buffers at apparent physiological pH 7.6: (i) potassium phosphate buffer 50 mM with KCl 200 mM and $MgCl_2$ 10 mM, (ii) potassium phosphate buffer 50 mM with KCl 200 mM, and (iii) TRIS buffer 10 mM with NH_4Cl 10 mM.

   The weak interaction between macrolide antibiotics and ribosomes observed by NMR was found to be Mg^{2+} independent, unlike the tight binding measured by equilibrium dialysis.
4. The spectrum of roxithromycin (**1**) with 50S ribosomal subunit is identical to that of macrolide in the presence of whole 70S ribosomes. This experiment displays selectively the structure in interaction with the weak binding site of 50S ribosomal subunit.

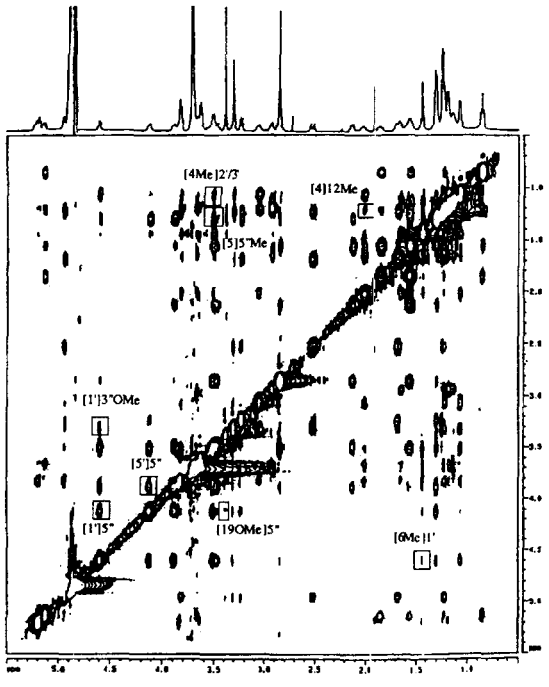


Fig. 2. Transferred NOESY spectrum of roxithromycin (**1**): mixing time 150 ms.

Roxithromycin (**1**) is metabolized [31] to RU 39001 (**2**) by an acidic cleavage of the bond between the macrolide itself and the cladinose sugar. In this case, no ketal formation is possible and the macrolide ring remains intact. This metabolite does not retain antimicrobial activity unlike its parent compound in vivo. Consequently, **2** is not able to take part in a binding interaction with bacterial ribosomes. The inactive RU 39001 (**2**) showed very little line broadening and at the same time gave essentially blank TRNOESY spectra.

We extended this study of *E. coli* ribosome–macrolide interaction to erythromycin A (**3**) and to one of its major metabolite, erythralosamine (**4**) (Fig. 1).

The NOESY spectrum of **3** shows an equilibrium at 293 K between two tautomer forms in aqueous solution [32], a major 9-ketone structure (65%) and a minor 9-12 hemiketal (35%). Decomposition of erythromycin under acidic conditions has been thought to proceed via the 6,9; 9,12-spiroketal [33]. Since the intramolecular cyclization of erythromycin destroys its antibiotic activity, the replacement of the keto group by an ether-oxime chain in order to reduce or completely block this degradative pathway, gave rise to roxithromycin. A previous TRNOE experiment with erythromycin A (**3**) displays selectively the ketone structure in interaction with the weak binding site of 50S ribosomal subunit [34]. This experiment was reproduced (Fig. 3) in order to test the experimental parameters and we observed a selective broadening of the ketone signal in presence of ribosomes (50S or 70S).

Erythromycin A (**3**) is extremely unstable towards acid and when administered orally undergoes dehydration in vivo leading to an inactive 6,9; 9,12-spiroketal metabolite (i.e. erythralosamine, **4**) (Fig. 1). When **3** was allowed to stand in aqueous acid solution, a cleavage of the cladinosyl moiety proceeded gradually with dehydration in the aglycone ring to yield **4** which was reported [35] as one of **3** metabolites. This metabolite **4** does not bind to bacterial ribosomes and do not exert antibiotic activity. The inactive compound **4** gave essentially blank TRNOESY spectra.

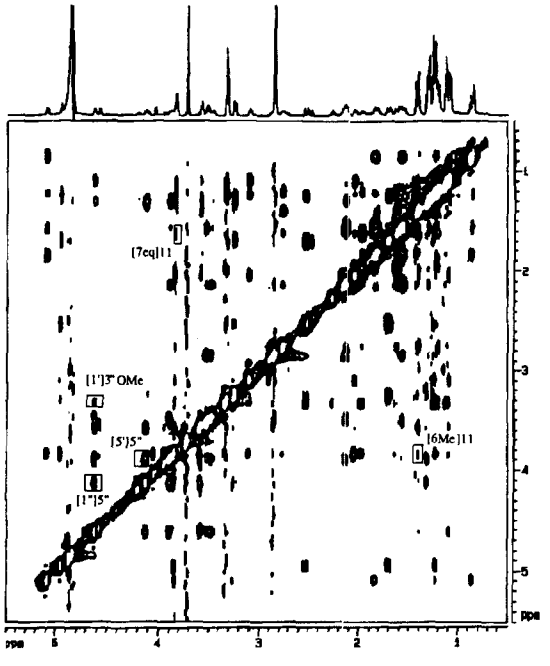


Fig. 3. Transferred NOESY spectrum of erythromycin (**3**): mixing time 150 ms.

3. Discussion

3.1 Biological mechanism

The weak interaction between macrolide antibiotics **1** (and **3**) and ribosomes observed by NMR was found to be Mg^{2+} independent but the tight interaction measured by the binding tests between **1** (or **3**) and *E. coli* ribosomes is strongly Mg^{2+} dependent [30]. It is known that cations are essential for macrolide-ribosome interaction [16,36]. [^{14}C]erythromycin binding is negligible in the absence of K^+ and is almost optimal at 100 mM. Neither Na^+ nor Li^+ can substitute for K^+. Although a minimal concentration of Mg^{2+} is required for optimal binding, at 0.1 mM Mg^{2+}, substantial [^{14}C]erythromycin binding to ribosomes is observed. Essentially, identical magnesium effects are obtained with 50S subunit. The binding is maximal from 1 to 10 mM Mg^{2+} and inhibitory at higher Mg^{2+} concentration. Similarly, the requirement of both Mg^{2+} and a monovalent cation (K^+or NH_4^+) have been reported for erythromycin binding to various bacterial species. Magnesium ions are also involved for ribosomal subunit association and its stabilization [37,38].

The weak interaction Mg^{2+} independent observed by NMR is in agreement with an hypothesis of two distinct binding levels, as was reported for ribosome-tetracycline interaction [39], with a low affinity binding level and the tight inhibition binding one. This weak binding observed by TRNOE experiments could be involved in the first step of recognition and selection of macrolide antibiotics by the ribosomal machinery. Mg^{2+} could have a crucial role for the second tight stage. The divalent ion acts probably into to strengthen macrolide binding and hence the inhibition of protein biosynthesis by macrolide antibiotics of the erythromycin A group. Thus, Mg^{2+} does not play a direct role for the low affinity binding site studied by TRNOE. It is possible that a single magnesium ion simultaneously binds a macrolide molecule and a negatively charged center on the ribosome. Mg^{2+} may form a connecting bridge between the drug and the ribosome. Another possibility is that Mg^{2+} could maintain a tertiary structure necessary for the second step of interaction.

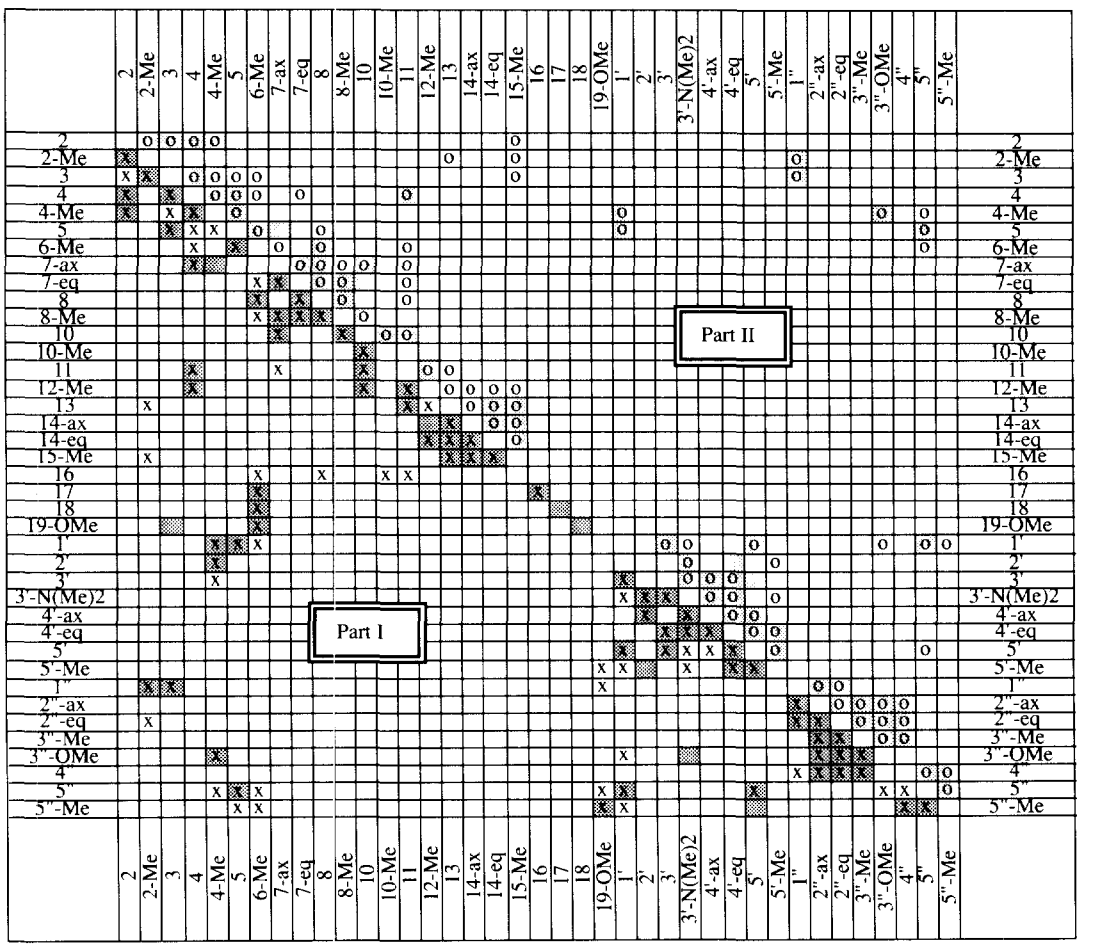
Compounds that are not able to take part in a weak binding interaction with bacterial ribosomes do not exert antibiotic activity, what was also observed by Barber et al. [19,22]. Thus, the weak binding site seems to be a necessary step for the strong interaction.

The present cell-free study applied to an interaction of (9S)-9-dihydroerythromycin A also gave rise to a blank TRNOESY spectrum in the presence of ribosomes, as it

was previously observed [20]. Unlike metabolites **2** and **4**, this compound is an antibiotic which inhibits cell-free protein synthesis and inhibits binding of erythromycin to ribosomes even if it is less active than erythromycin A. In fact, this compound is more active in a cell-free system probably due to its reduced permeability through the bacterial cell. This negative result also observed by Brennan et al [20] was relative to experimental conditions as TRNOEs could be observed for (9S)-9-dihydroerythromycin A when we lower the ligand:ribosome ratio (1000:1 ratio or even less) unlike metabolites **2** and **4**. The biological significance of what is being studied here by NMR is in no case the membranar permeability, but TRNOE NMR study detects interaction between macrolides antibiotic and the bacterial ribosome which is the target site of the macrolides antimicrobial action. As a result, this preliminary study of 14-membered-ring macrolide shall provide the basis for further TRNOE NMR studies of roxithromycin (**1**) and erythromycin (**3**) derivatives or other groups of macrolides antibiotics.

In conclusion, the bioactive macrolide antibiotics studied displayed a strong NMR response. For systems of interacting biomolecules like enzyme-substrate,

Table 1
NOEs (τ_m = 150 ms) for roxithromycin (**1**) and erythromycin A (**3**) ketone in D_2O buffered solution and TRNOEs in presence of *E. coli* ribosomes



peptide-protein, and lectin-oligosaccharide, the binding is sufficiently weak to allow Transferred Nuclear Overhauser Effect. It is hence reasonable to suggest that TRNOE NMR experiments could describe binding sites present on ribosomes important in the inhibition process of macrolide antibiotics. TRNOE NMR experiments constitute probably the only technique available to study in solution the conformation of bound ligands in rapid exchange with a macromolecule.

3.2 *Structural characteristics of these ligands*

The medically important macrolide antibiotic roxithromycin (**1**) is composed of an erythronolide ring substituted with desosamine and cladinose sugar units, the specificity of this molecule lying in its 9-oxime chain (the tautomeric equilibrium observed for **3** is not possible for 9-oximo derivatives [40] such as roxithromycin). The compound **1** exists in solution in one predominant conformation [41,42]. The TRNOE NMR experiments (Fig. 2) suggests that **1** binds in its receptor site in essentially the same conformation than the major free one in aqueous solution.

The TRNOE NMR experiments at 150 ms demonstrates the spatial proximity of 6-Me to 16-H, 17-H, 18-H and 19-OMe (Table 1). The oxime chain is held towards 6-Me and finally, the far NOEs observed at 5′-Me, 1″-H, 5″-H and 5″-Me from 19-OMe show the presence of the end of the oxime chain close to the joining part of the two sugars, cladinose and desosamine [Fig. 4(a)].

Two desosamine-lactone NOEs [5-H]1′-H and [4-Me]1′-H were observed as well as three TRNOEs indicative of inter-sugar interactions [5″-H]5′-H, [5″-Me]1′-H, [3″-OMe]1′-H (Table 1). These TRNOEs showing the spatial proximity of 1′-H to 5-H, 4-Me, 5″-H, 5″-Me and 3″-OMe are exactly those expected if the two sugar rings are oriented approximately perpendicularly to the macrocycle lactone ring with 5′-Me and 5″-Me pointing up. One TRNOE is not observed in the corresponding **1** bound structure but expected on the basis of the D_2O **1** solution structure, [3″-OMe]3′-$N(Me)_2$. The lack of this NOE indicated that in bound conformation the distance between these two groups is greater than 4 Å, while the observation of TRNOEs [5″-H]5′-H and [3″-OMe]1′-H may be due to the relatively small distance involved in the bound **1** structure. The sugar-lactone and inter-sugars TRNOEs represent an unambiguous determination of the orientation of the sugar rings in **1** bound structure. They confirm that the two α faces of the cladinose and the desosamine rings are opposite one another.

There is other experimental evidence to support this assumption, [3″-OMe]4-Me which shows the spatial proximity of these two methyl groups and [5″-Me]6-Me which is indicative of the weak distance between these two methyl groups and only found in the **1** bound structure (Table 1). A comparison of the free and bound structures for **1** shows (Table 1), except for the NOEs previously indicated, interactions between protons [11-H]7ax-H, [2-Me]13-H, [16-H]8-H, [16-H]10-Me, [16-H]11-H, [4-Me]3′-H, [4-Me]5″-H, in **1** bound to ribosome for which no corresponding solution structure exists. In the ribosome complex, the relative rigidity of the sugar units and the oxime chain is evident from the constraint of the different observed NOEs. It results in a globular form for the whole **1** bound molecule.

Roxithromycin (**1**) and erythromycin (**3**) structures were largely described in previous publications [31,41–47]

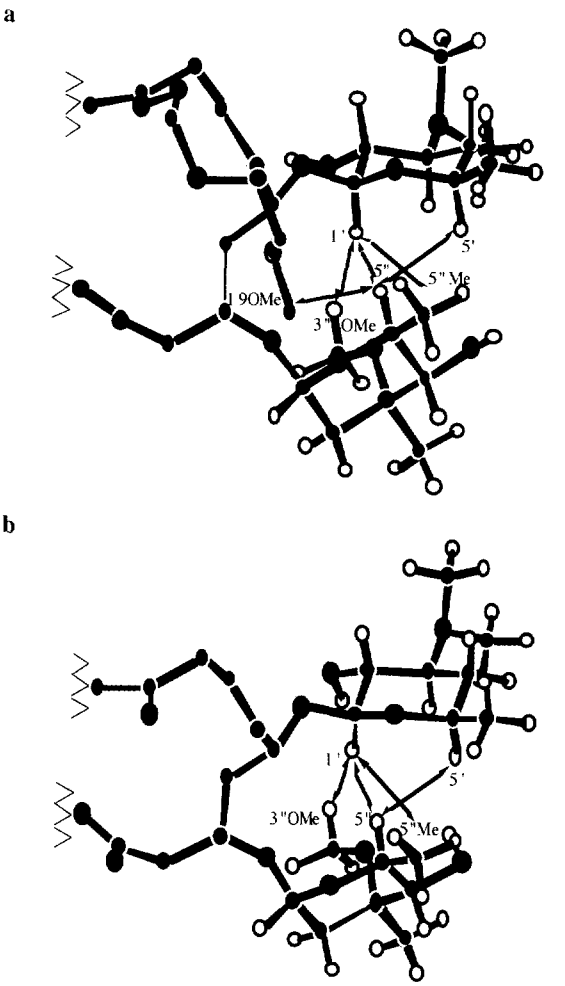


Fig. 4. Some inter-sugars NOEs characteristic of the **a** sugar conformation are observed for (a) roxithromycin (**1**) and (b) erythromycin A (**3**). In particular, no cross-peak 5″-5′ has been observed in the NOESY of free erythromycin A ketone in solution. This cross-peak appears strongly in transferred NOESY spectra suggesting that the ribosome may be able to 'freeze' the particular **a** conformation of the drug. One TRNOE for roxithromycin (**1**) indicates the position of the end of the oxime chain close to the joining part of the two sugars.

by the mean of NMR in solution and molecular dynamics. Here we analyse the conformations in the weakly bound states and compare them to the previously studied conformational averaging in solution.

Before discussing the structures representing the bound state of the drugs from TRNOESY data, it is worth reviewing the most prominent features of the structures in solution such as excellent models for their dominant bound conformer.

As it would be helpful, the notation for conformer designation is defined in Table 2 (i.e. define '**A1**, **A2**, **A3**, **B1**, **B2**, **a**, **b**, **c**, **d**, **e**'). The different positions of sugar moieties '**a**, **b**, **c**, **d**, **e**' are shown in Fig. 5 and their characteristics are reported in Table 2. The major conformations of the lactone ring were termed '**A** (A1, A2, A3) and **B** (B1, B2)', and this macrocycle flexibility induced five different orientations '**a**, **b**, **c**, **d** and **e**' for the desosamine sugar [31,41,42]. Conformations '**A**' and '**B**' differ in many ways but the major change is the inward folding of the C(3) fragment in '**B**'. Conformer '**a**' exhibits an orientation of the desosamine nearly perpendicular to the macrocycle whereas the two units are in the same plane in conformations '**c** and **e**'. For conformation '**b**', the cladinose unit rises above the macrocycle. Conformation '**d**' exhibits a turned-back cladinose.

A preliminary study on roxithromycin (**1**) has been published [41,42], the solution conformation of roxithromycin and its motional properties have been determined by NMR spectroscopy. In D_2O roxithromycin derivatives (**1** and **2**) solution, the oxime chain reduces the degree of freedom of the macrocyclic lactone ring which corresponds to conformation '**A**' with the two conformations '**a** and **c**' of the sugar rings [Fig. 5(a) and (b)]. The desosamine sugar was found to be essentially perpendicular to the macrocycle ('**a**' conformer) and both sugar groups are parallel to reduce the steric energy. In D_2O erythromycin A (ketone form) solution, the macrocycle is mainly found in '**A**' conformation with sugar rings in the '**a** and **b**' orientations [Fig. 5(c)] {the **3** ketone structure '**A**' is in conformational equilibrium with '**B**' conformation}, while erythralosamine derivatives (**4** and hemiketal **3**) always present the '**B**' macrocycle with two conformations '**b** and **c**' of sugar rings [Fig. 5(d)]. Steric parameters favour planar '**c**' conformer for **1** and **2** owing to the oxime chain, while stacking sugar attractions stabilize the perpendicular '**a**' conformer. Similarly, the hindered macrocycle of hemiketal **3** and **4** favours '**b**' conformers in which the desosamine moiety is tilted up.

The observed TRNOEs (Table 1) are compared to the spatial proximity values computed from the low-energy conformers [31] with sugars in '**a**, **b**, **c**, **d**, **e**' orientations (Table 3). The bound structures resulting from experimental TRNOESY values are discussed with regard to these conformers. The ^1H NMR chemical shifts in D_2O solution for **1** and **3** are listed in Table 4.

The lactone ring of bound roxithromycin is found to be relatively rigid in '**A1**' conformation with desosamine and cladinose sugar in '**a**' conformation. TRNOEs [4-H]2-H, [4-H]11-H and [2-Me]15-Me (Table 1) indicate clearly that in presence of ribosome, the corresponding erythronolide ring remains in '**A1**' conformation similar as the major one in D_2O solution. In the same way, all inter sugars and sugar-macrocycle TRNOEs [1′-H]4Me, [1′-H]3″OMe, [1′-H]5″H and [5′-H]5″H, imply that the orientation of the sugar rings with respect to one another is the same '**a**' conformation in the bound state [Fig. 6(c)] as in the free major one [Fig. 6(a)].

The chemical shifts of the *N,N*-dimethyl amino protons will depend on the state of ionization of that group [42]. In presence of ribosomes, the signal at 2.86 ppm,

Table 2
Notation for conformer designations defined as **A**, **B**, **a**, **b**, **c**, **d**, and **e**

	Macrocycle conformations				
Torsion angles	**A1**	**A2**	**A3**	**B1**	**B2**
H2-H3	160	177	165	105–115	145
H3-C1	40	46	48	3	32
H4-H5	135	100	140	145–165	165
H5-Me6	43	55	43	55	−8
H11-Me12	−73	−76	−63	−76	−58
H13-C1	−8	124	−20	10	3
	Sugar conformations				
Torsion angles	**a**	**b**	**c**	**d**	**e**[a]
ψ_1	15–20	±5;−15	−30;±10	15	20
ψ_2	35–45	50–60	−30;10	55	160
ψ_3	25	35	20	−22	a
ψ_4	45	45	40	−3	a

[a]Conformation **e** is only generated by MD for the roxithromycin metabolite without cladinose sugar.

corresponds to the resonance of the *N,N*-dimethyl amino group in the protonated form (Table 4). The stacking of cladinose and desosamine rings could be due to the existence of an intramolecular hydrogen bond between sugars involving the 3′-$NH^+(Me)_2$ equatorial of the desosamine sugar, in hetero-nuclear pairs such as nitrogen and oxygen. The great majority of hydrogen bonds involve NH ··· O interactions, for example, as in peptides [48] while fewer examples of strong OH···N bondings are known [49]. The presence of protonated roxithromycin (**1**) {or erythromycin A (**3**)} at physiological pH with ribosomes might have consequences on

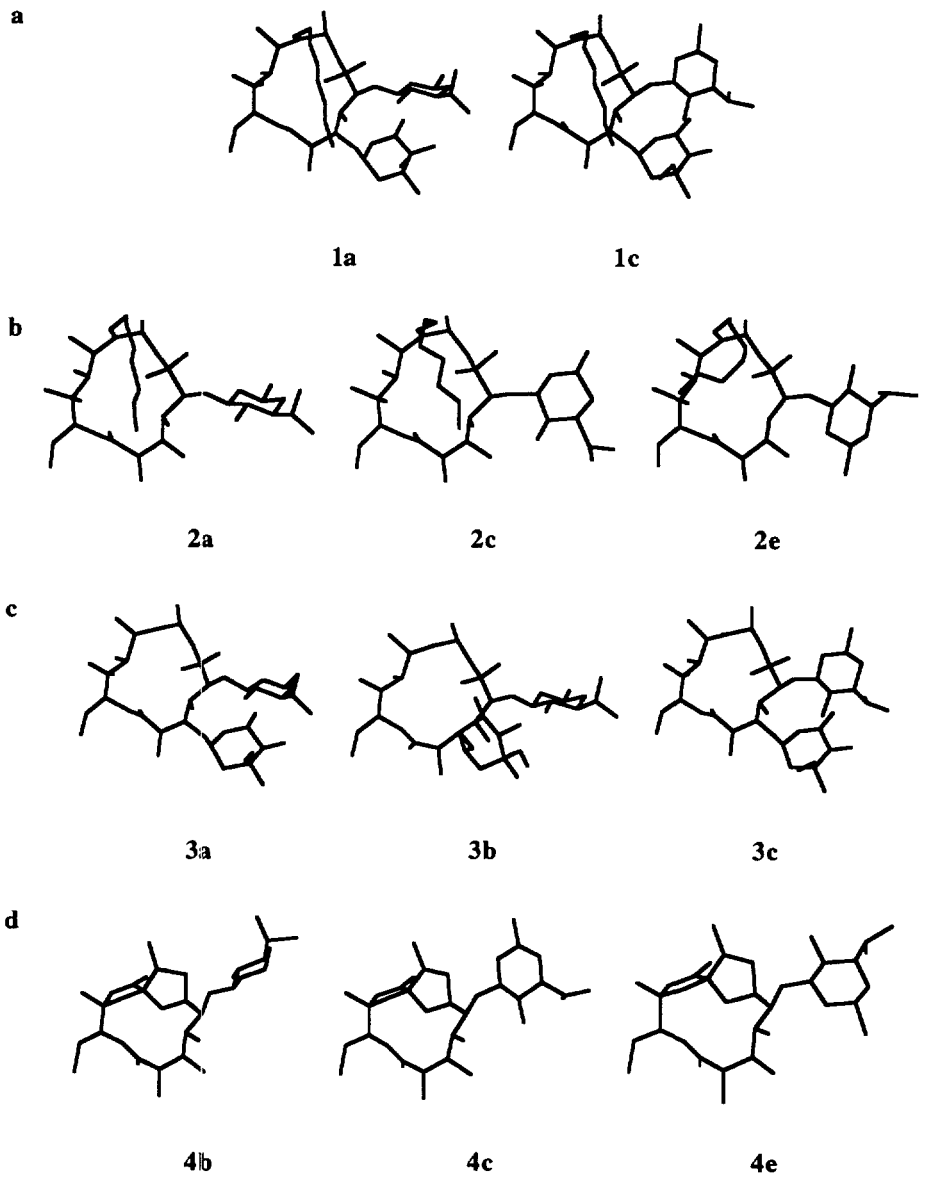


Fig. 5. (a) The two **a** and **c** conformers of roxithromycin (**1**) with perpendicular and coplanar orientation of the desosamine unit. (b) The **a** conformer of RU39001 (**2**) shows an orientation of the desosamine nearly perpendicular to the macrocycle whereas the two units are in the same plane in conformations **c** and **e**. (c) The three **a**, **b** and **c** conformers of erythromycin A (**3**). Conformer **a** exhibits an orientation of the desosamine nearly perpendicular to the macrocycle whereas the two units are in the same plane in conformations **c**. For conformation **b**, the cladinose unit lifts up above the macrocycle. Conformation **d** exhibits a turned-back cladinose. (d) The **b** conformer of erythralosamine (**4**) shows a perpendicular desosamine sugar which is rejected toward 8-Me. The **c** and **e** conformers exhibit **a** desosamine sugar coplanar to the erythronolide but with 180° rotation of the angle Ψ_2 (glycosidic bond, Ψ_2 = H1′-C1′-O-C5) one to another.

Table 3
Summary of distances inter-protons (2–3.5 Å) from minimized structures derived from molecular dynamics experiments

Macrocycle	A1	A2	A3	B1	B2
4-2	p	p	a	p	p
4-2Me	a	a	a	p	a
5–4	p	p	p	a	a
5-4Me	a	a	a	p	p
6Me-3	a	a	a	a	p
7a-4Me	a	p	a	a	a
10Me-8Me	p	p	a	p	p
10Me-4Me	a	a	a	a	p
10Me-4	a	a	a	a	p
10Me-7a	a	a	a	a	p
11-4	p	p	p	a	a
11-3	a	a	a	p	a
11-10Me	a	a	a	a	p
13-12Me	a	a	a	a	p
15Me-2Me	p	a	a	a	a
Sugar macrocycle	**a**	**b**	**c**	**d**	**e**
1′-5	p	p	p	p	a
1′-4Me	p	p	a	p	p
1′-6Me	a	a	p	a	a
2′-5	a	a	p	a	p
2′-4Me	a	a	p	a	a
2′-6Me	a	p	p	a	a
2′-7a	a	p	a	a	a
2′-10Me	a	p	a	a	a
5′-4Me	a	a	a	a	p
5′-6Me	a	a	p	a	a
5′Me-3	a	a	a	a	p
5′Me-5	a	a	a	p	a
5′Me-6Me	p	a	p	a	a
5′Me-7e	a	p	a	a	a
1″-3	p	p	p	p	a
1″-2Me	p	p	p	a	a
1″-5	a	a	a	p	a
2″-2Me	p	a	p	p	a
3″OMe-2Me	a	a	a	p	a
3″OMe-4Me	p	p	p	a	a
5″-5	p	p	p	a	a
5″-4Me	a	a	a	p	a
5″-6Me	a	p	a	a	a
5″Me-6Me	p	a	p	a	a
Inter-sugars	**a**	**b**	**c**	**d**	**e**
3″OMe-1′	p	p	a	a	a
3″OMe-3′	p	a	a	a	a
3″OMe-5′	a	p	a	a	a
3″OMe-N(Me)2	p	a	a	a	a
5″-1′	p	a	p	a	a
5″-5′	p	a	a	a	a
5″Me-1′	a	a	p	p	a
5″Me-3′	a	a	p	p	a
5″Me-5′	p	a	a	p	a

p: present; a: absent.

their relative access to the ribosomes, but the protonated or deprotonated form of the *N,N*-dimethyl amino group in the bound conformations was not established [18]. A pH dependence of macrolide-ribosomes complex formation seems difficult to study by NMR spectroscopy.

The ribosomal bound conformation of erythromycin A (**3**) has been identified from TRNOEs as the ketone

Table 4
^{1}H NMR chemical shifts (δ in ppm) for **1** and **3** in D_2O buffered solution

Position	1	3	
		Major (keto)	Minor
2	3.07	3.09	2.89
2-Me	1.23	1.23	1.29
3	3.84	3.84	3.95
4	2.03	2.04	2.15
4-Me	1.07	1.09	1.09
5	3.52	3.58	3.57
6-Me	1.45	1.40	1.26
7-ax	1.65	1.96	1.84
7-eq	1.65	1.62	1.41
8	3.65	2.77	2.13
8-Me	1.14	1.20	1.13
10	2.94	3.32	2.27
10-Me	1.19	1.13	1.15
11	3.68	3.84	4.04
12-Me	1.23	1.23	1.43
13	5.14	5.09	4.85
14-ax	1.57	1.58	1.81
14-eq	1.86	1.85	1.90
15-Me	0.86	0.85	0.89
16	5.20	—	—
16A	5.22	—	—
16B	5.18	—	—
17	3.84	—	—
18	3.64	—	—
18A	3.65	—	—
18B	3.63	—	—
19-OMe	3.40	—	—
1′	4.59	4.62	4.58
2′	3.51	3.52	3.59
3′	3.38	3.46	3.45
3′-N(Me)$_2$	2.77	2.84	2.84
4′-ax	1.54	1.55	1.62
4′-eq	2.09	2.12	2.14
5′	3.87	3.88	3.85
5′-Me	1.29	1.30	1.33
1″	4.95	4.94	4.87
2″-ax	1.69	1.70	1.70
2″-eq	2.53	2.52	2.48
3″-Me	1.25	1.25	1.26
3″-OMe	3.32	3.32	3.31
4″	3.24	3.24	3.24
5″	4.14	4.12	4.19
5″-Me	1.31	1.31	1.29

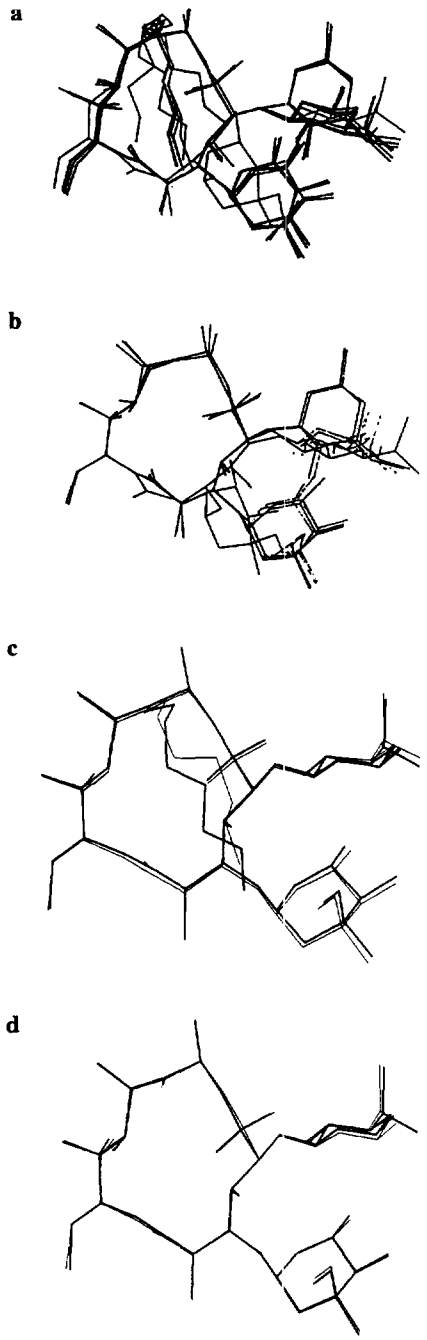


Fig. 6. Conformational averaging in aqueous solution for the protonated structures **A1a**, **A3a**, **A1c**, and **B1b** of (a) roxithromycin (**1**) and (b) erythromycin (**3**). Superimposition of **A1a** conformation generated by MD and TRNOESY derived structures, weakly bound to bacterial ribosomes for (c) roxithromycin (**1**) and (d) erythromycin A (**3**). Quality of superimposition of all the atoms was evaluated by mean square (rms) deviation between the atoms constituting the bound conformer in bold and the corresponding atoms in the **A1a** conformation, for **1** (rms, 0.3709) and **3** (rms, 0.1548).

form (Fig. 3). This structure is blocked in a specific and privileged conformation in weak interaction with *E. coli* ribosomes [Fig. 4(b)]. Particularly, NOEs [5′-H]5″-H, [1′-H]3″-OMe, which are nondetectable in free erythromycin A (**3**) in D_2O solution appear strongly with ribosomes (Table 1), indicating that the two sugars cladinose and desosamine are facing each other in the bound conformation. At the same time, NOEs [2-H]15-Me and [2-Me]15-Me indicate that the ethyl group of the macrocycle is directed essentially toward the 2-Me group (Table 1). TRNOE analysis established the similarity between the bound state conformation [Fig. 6(d)] of this active compound and '**A1a**' conformation taking part in the free state [Fig. 6(b)].

Molecular modelling was carried out for the bound structures to bacterial ribosome at physiological apparent pH 7.6. TRNOESY data were applied to roxithromycin (**1**) and erythromycin (**3**) protonated molecules and the following constraints were used: 3 ± 1 Å. From previous detailed NMR and MD analysis [31], reasonable conformational averaging of **1** and **3** protonated in solution had been postulated [Fig. 6(a) and (b)]. When TRNOESY data were applied to roxithromycin and erythromycin molecules, they gave rise after minimization to the low energy structures shown in Fig. 6(c) and (d). The structures representing the bound state of drugs show great similarity with '**A1a**' conformation. Only the position of the roxithromycin oxime chain shows some variation relative to the free state, but in both cases the chain is repelled above the macrocyclic lactone ring towards the carbone C(3). It is interesting to note that the **A1a** conformation participated slightly in solution for the major metabolite **2** and not at all in **4** solution.

TRNOESY experiment is well adapted to determine the conformation of bound ligands in rapid exchange with a macromolecule but this experiment could provide another information from differential line broadening. In general, a correlation between line broadening and involvement in binding is expected. Line broadening in NMR spectra may result from two distinct processes: rapid equilibrium between states with different chemical shifts, and slowing of molecular motion. Finally, a model of roxithromycin (**1**) showing the hydrogen atom giving the most extensively broadened resonances is constructed [Fig. 7(a)] using van der Waals' surfaces. The model is based on the minimised structure derived from molecular dynamics experiments [31] and will be compared to the one obtained for erythromycin A (**3**) [Fig. 7(b)]. Fig. 7 suggests that roxithromycin (**1**) bind to ribosomes at an identical 'surface' of the drug molecule to erythromycin A (**3**), involving the C(13)–C(5) lactone region of the aglycon and both sugar rings essentially the cladinose one. The 'surface' deduced from the variation in line broadening would be indicative of the relatively rigid part of the molecule.

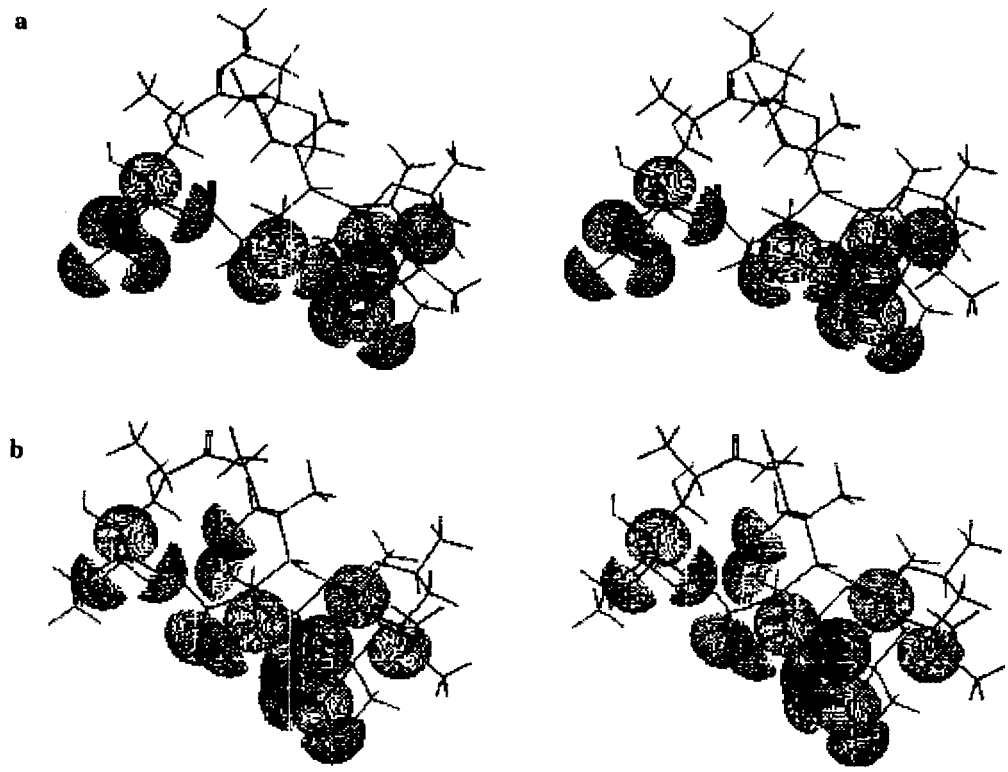


Fig. 7. Stereoviews of minimized structures from transferred NOE's data (bound to ribosomes) (a) of roxithromyin (**1**) and (b) of erythromycin A (**3**). The van der Waals' surfaces are shown to indicate the atoms whose signals are most broadened (12-Me, 15-Me, 4-Me, 2″eq-H, 3″-OMe, 4″eq-H, 1′-H). The broadening observed may reflect proximity to a binding surface.

4. Conclusion

In conclusion, TRNOE NMR experiments were proved to be efficient to locate and detect the weak interactions, Mg^{2+} independent, due to ribosomal activity in order to provide a relationship between **A1a** conformation in interaction and biological activity. From these experiments, **A1a** conformation appears to be the plausible bound structure relative to this weak specific binding to the bacterial ribosome and the broadening observed in the C(13)–O(14)–C(2)–C(4) region may reflect proximity to a binding surface. The results may be potentially useful for the design of new macrolide derivatives possessing improved properties for medical applications.

5. Experimental

5.1 Ribosome preparation

The Ribosomes are prepared at *Roussel Uclaf* as described [50] by tangential ultrafiltration technique and the ribosomal purity is given by one unity A_{260} (absorption at 260 nm) which corresponds to 24 pmol ml^{-1} of 70S ribosomes. Microdialysis on Millipore membrane filters MF-type with 0.025 μm pore size was carried out beforehand to remove Tris impurities present during ribosome isolation. A concentration of 0.8 μM of *E. coli* MRE 600 strain 70S ribosomes is appropriate because it give a twofold increase of the line width of roxithromycin (**1**). The 50S ribosomal subunit is isolated essentially as described [51] by saccharose gradient technique.

5.2 NMR spectroscopy

The antibiotic samples have been dissolved in an aqueous NaD_2PO_4/Na_2DPO_4 buffer (0.05 M), with KCl (0.2 M) at physiological apparent pH 7.6 (pD = pH + 0.4, uncorrected here) and it was possible to attain concentrations of 4 mM for ^1H experiments. The high K^+ concentration (200 mM) is used in this study for a better stability of ribosomes. Despite it is much high as compared with the physiological condition, it does not play a role in the macrolide-ribosome interaction. We obtained the same result with KCl 0.1 M than 0.2 M. A crystal of $TSPD_4$, 3-(trimethylsilyl)[2,2,3,3-d_4]

propionic acid, sodium salt, was used as internal reference for the proton shifts. The errors on the chemical shifts are 0.01 ppm for ^1H.

The experiments were run at 500 MHz for ^1H, at 293 K, on Bruker AMX 500 Spectrometers equipped with a Silicon Graphics workstation. A presaturation of the solvent was used for all the 1-D and 2-D ^1H experiments. The 2-D phase-sensitive using States-TPPI method ^1H TRNOESY experiments in D_2O buffered solution were performed using a mixing time of 150 ms. FIDs were acquired (32 scans) over 5555 Hz into a 2K data block for 256 incremental values of the evolution time and a relaxation delay of 2 s. Water suppression was performed by a low power transmitter pulse of presaturation (70 dB) during relaxation delay and mixing time. One half-sinusoid (5% truncated) shape homospoil gradients of 10 G/cm were used during mixing time.

5.3 *Molecular modelling*

The calculations were run on a Silicon-Graphics computer using the Biosym software 'INSIGHT II' and 'DISCOVER' with the CVFF Forcefield from Dauber-Osguthorpe and Hagler [52].

We have run experiments starting from three different starting conformations (Aa, Ac, and Bb) of the two compounds (roxithromycin and erythromycin), TRNOESY data were applied and the following constraints were used: 3 ± 1 Å. The dynamics were run for 100 ps (constant temperature), the trajectory was sampled by minimizing and storing the structure every picosecond.

Firstly, the Aa, Ac, and Bb structures were built from the crystallographic coordinates of the solid **1** and **3** as a starting point. Their atomic potentials and charges were recalculated using the built-in algorithm of the program [53] and the structures were modified into protonated molecules as they are bound to the bacterial ribosome at physiological pH 7.6. In order to take account of the polarization state of these molecules and to check for eventual structural effects due to protonation of the dimethyl-amino group (at pH 7.6), the charges were recalculated [54] using the DISCOVER algorithm, with a total charge of +1. All calculations were performed with implicitly taking account for the solvent in using in the description of the coulombic interaction a distance-dependant dielectric constant ($\varepsilon = 4r$). The Aa, Ac and Bb structures were generated from a preliminary exploration of the conformational space. After energy minimization and an equilibration period of 6 ps, we performed a 50 ps MD run at 300 K with a periodic temperature jumps to 600 K to supply the system with energy (to pass conformational barriers). The 50 ps trajectory is sampled every picosecond and the remaining structure are then minimized by molecular mechanics and stored. The final conformers found with lowest energies were then further minimized to a gradient less than 0.01 kcal/mol to obtain their energies at higher accuracy.

Secondly, the three minimized conformations (Aa, Ac, and Bb) were then used as starting structures of both macrolides for a 100 ps MD run and TRNOESY data were applied. We can observed that, whatever the protocol, the conformation 'Aa' was unique (100%). Upon the beginning, the Aa conformation is generated from these experiments.

The lowest energy-minimized constrained structure extracted from simulations were compared with the free 'Aa' major ligand for both compounds **1** and **3**, using a superimposition procedure. Conformational similarities were evaluated by calculating the RMS of deviation between heavy atoms for each possible pair of the different structures. The results represent the bound structures whose small RMS deviations (< 0.5 Å) suggested that they may belong to the same conformational 'Aa' family.

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