Weak Binding of Erythromycin Analogues to Bacterial Ribosomes: A ¹H NMR Study

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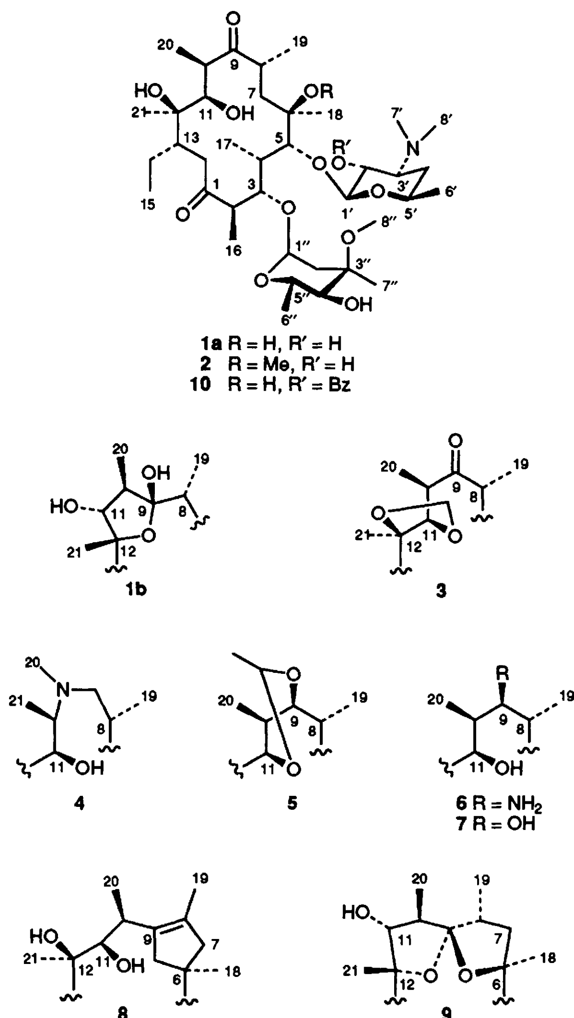
Weak binding of a number of erythromycin analogues to bacterial ribosomes is monitored by ¹H NMR methods; transferred NOE data are shown to correlate with antibacterial activity.

The important macrolide antibiotic erythromycin A acts by binding to the bacterial ribosome, so inhibiting protein biosynthesis. When measured by equilibrium dialysis, this binding has been found to be rather tight, $K_d = 10^{-7}$–10^{-9} mol dm^{-3}.[1-3] We have demonstrated, however, that in aqueous solution, erythromycin A exists as a mixture of two compounds, the 9-ketone **1a** and the 12,9-hemiacetal **1b**,[4] and that the ketone (only) is able to take part in a weak binding interaction with bacterial ribosomes.[5] This interaction has been monitored by ¹H NMR and is a fast exchange process, indicating a dissociation constant of 10^{-3}–10^{-5} mol dm^{-3}.

Like the tight binding, this relatively weak binding is associated exclusively with the 50S subunit of the ribosome.[6] It is possible, therefore, that erythromycin A binds to the bacterial ribosome in a two stage process, the first stage being the weak interaction monitored by NMR.

In the present study we sought to test this postulate by NMR investigations of the binding of a number of active and inactive analogues of erythromycin A to *E. coli* ribosomes. Nine analogues in total have been tested. Of these, the relatively new clinical antibiotic clarithromycin **2**[7,8] and erythromycin A 11,12-methylene acetal **3**[9] are significantly more active than erythromycin. The novel clinical antibiotic azithromycin **4**,[10] the 9,11- cyclic acetal of (9*S*)-9-dihydroerythromycin A **5**[11] and (9*S*)-erythromycylamine A **6**[12] have similar activities to the parent drug. (9*S*)-9-Dihydroerythromycin A **7**[13] is significantly less active and the remaining compounds, erythromycin A enol ether **8**,[14] anhydroerythromycin A **9**[15] and 2′-benzoylerythromycin A **10**[16] are essentially inactive.



Deuteriated *E. coli* ribosomes were prepared as previously described[17] (deuteriation reduces the possibility of spin diffusion in transferred NOE experiments). Ribosomal 'cores' were prepared by incubating ribosomes with 2.0 mol dm^{-3} LiCl, in order to remove outer proteins.[18] (Cores were used in place of whole ribosomes in control experiments; they lack proteins L15 and L16 which are required for erythromycin binding). 600 MHz Spectra were run of each drug alone, in the presence of 0.8 μmol dm^{-3} ribosomes (a concentration known to give a ≥ twofold increase in linewidth of erythromycin A) and (separately) in the presence of 1.6 μmol dm^{-3} cores. The concentration of each drug was 4 mmol dm^{-3} apart from compound **10**, the concentration of which was 0.5 mmol dm^{-3}. In this case the concentration of ribosomes was adjusted appropriately. The buffer in each case was 50 mmol dm^{-3} sodium phosphate in D_2O, apparent pH 7.6. The spectra were processed without weighting and the linewidths at half height of selected, separated signals measured. Fig. 1 shows the effect of ribosomes on the characteristic low frequency triplet of erythromycin A and the analogues **2–4** and **8**. Table 1 summarises the line broadening data using separated signals that can be reliably assigned for all of the compounds.

It was clear from these data that the active analogues **2** and **4–6** bound weakly to bacterial ribosomes in a similar way to erythromycin A ketone, as indicated by selective line broadening in the ¹H NMR spectra. The inactive compound **10** also gave rise to selective line broadening which was not exhibited

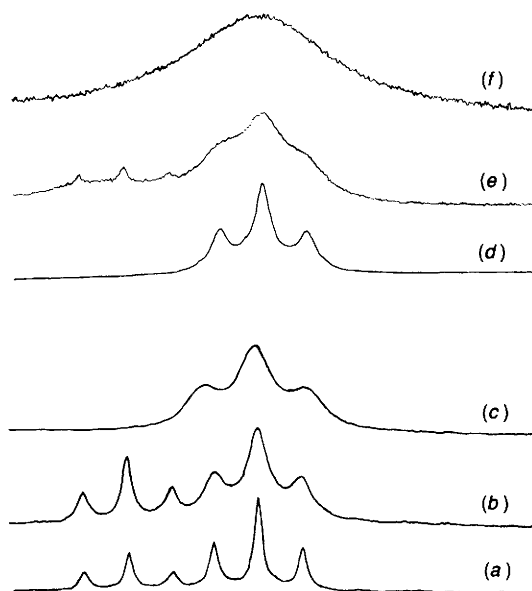


Fig. 1 High frequency triplet (15-H_3) of 600 MHz ¹H NMR spectra of erythromycin A and analogues (4 mmol dm^{-3}) in 50 mmol dm^{-3} buffer. (*a*) **1** alone, (*b*)–(*f*) **1**, **4**, **2**, **3**, **8** respectively in the presence of 0.8 μmol dm^{-3} ribosomes.

in the control. The inactive compound **9** and the weakly active compound **7** showed very little line broadening above that seen in the control. Compounds **3** and **8** were, however, anomalous. The inactive compound **8** showed extreme line broadening in the presence of ribosomes (see Fig. 1), but significant line broadening also occurred in the control spectrum (Table 1). The active compound **3** was similar, showing extreme line broadening in the presence of ribosomes and slightly more line broadening in the control spectrum than the other analogues.

In addition to line broadening measurements, we have also investigated the binding of erythromycin A to ribosomes, taking advantage of the phenomenon of the transferred NOE.[19] When the NOESY experiment is performed on a ligand–macromolecule complex, with the ligand in large excess, the crosspeaks which dominate the spectrum are not those due to the free ligand, but those due to the bound complex, transferred to the free ligand.[17,20] The NOEs are negative and appear when relatively short mixing times are used. The erythromycin A–ribosome transferred NOESY (TRNOESY) spectrum showed over 100 cross peaks, all but seven of these were due to the 9-ketone **1a**. This experiment can therefore be used as another indicator of weak binding.

The TRNOESY experiments were carried out using the same samples as the line-broadening measurements. The spectra were acquired at 600 MHz using a data matrix of 2048 × 256 points and processed with zero filling in F_1. The mixing times were optimised empirically and were in the range 75–150 ms. The compounds **2** and **4–6** which resembled erythromycin A in their line broadening behaviour, also gave rise to TRNOESY spectra with around 100 cross peaks. This is a very strong indication of weak binding (additional information, particularly full assignments, is required for the analysis of the conformational information contained in these spectra. At this stage they were used qualitatively). As expected, compounds **7** and **9**, in the presence of ribosomes, gave essentially blank TRNOESY spectra.

Compound **10** gave rise to a very weak TRNOESY spectrum with only about 20 crosspeaks above the level of the noise. A control spectrum (with ribosomal cores replacing whole ribosomes) contained a similar number of crosspeaks, suggesting that these signals arise from unbound or non-specifically bound **10**. This interpretation was confirmed by the NOESY spectrum of the drug alone, which, when run under the same conditions, was also essentially identical to the spectrum run in the presence of ribosomes. The TRNOESY spectra of the anomalous compounds **3** and **8** in the presence of ribosomes are shown in Fig. 2. The active compound **3** gave rise to a good TRNOESY spectrum resembling (qualitatively) those due to erythromycin A and the other active compounds. The inactive analogue **8**, however, gave a weak TRNOESY spectrum, entirely dominated by spin diffusion [Fig. 2(*b*)]. This indicates that **8** does not bind to a single site on the ribosome, allowing NOE build-up, but has a high degree of mobility on binding, *i.e.* binding is non-specific. Compound **8** gave a very similar TRNOESY spectrum in the presence of

Table 1 Linewidths at half height of selected signals in the ^{1}H NMR spectra of the erythromycin A analogues **2–10** alone, in the presence of 0.8 μmol dm^{-3} deuteriated ribosomes and (separately) in the presence of 1.6 μmol dm^{-3} cores

		Linewidth at half peak height/Hz		
Analogue	Position	Alone	+ Ribosomes	+ Cores
2	15	2.0	6.2	3.2
	2″	3.2	9.4	5.4
	8″	1.6	4.2	2.7
3	15	2.0	12.9	3.8
	2″	3.0	13.2	6.7
	8″	1.7	9.2	5.5
4	15	1.9	8.0	3.1
	2″	3.1	9.8	6.3
	8″	1.5	5.9	2.5
5	15	2.2	5.5	2.6
	2″	3.2	8.7	4.1
	8″	1.7	3.9	2.0
6	15	2.2	7.5	2.6
	2″	3.5	10.5	4.8
	8″	1.9	5.9	2.3
7	15	1.8	3.3	2.5
	2″	3.2	7.3	5.4
	8″	1.6	3.1	2.3
8	15	2.0	14.3	6.4
	2″	3.1	*a*	7.5
	8″	2.1	27.4	5.5
9	15	2.0	3.4	2.3
	2″	*b*	*b*	*b*
	8″	1.5	2.7	1.7
10	15	2.5	13.5	2.5
	2″	3.9	6.2	4.9
	8″	1.3	3.4	1.4

[a] Resonance too broadened to measure. [b] Unambiguous assignment of resonance not available.

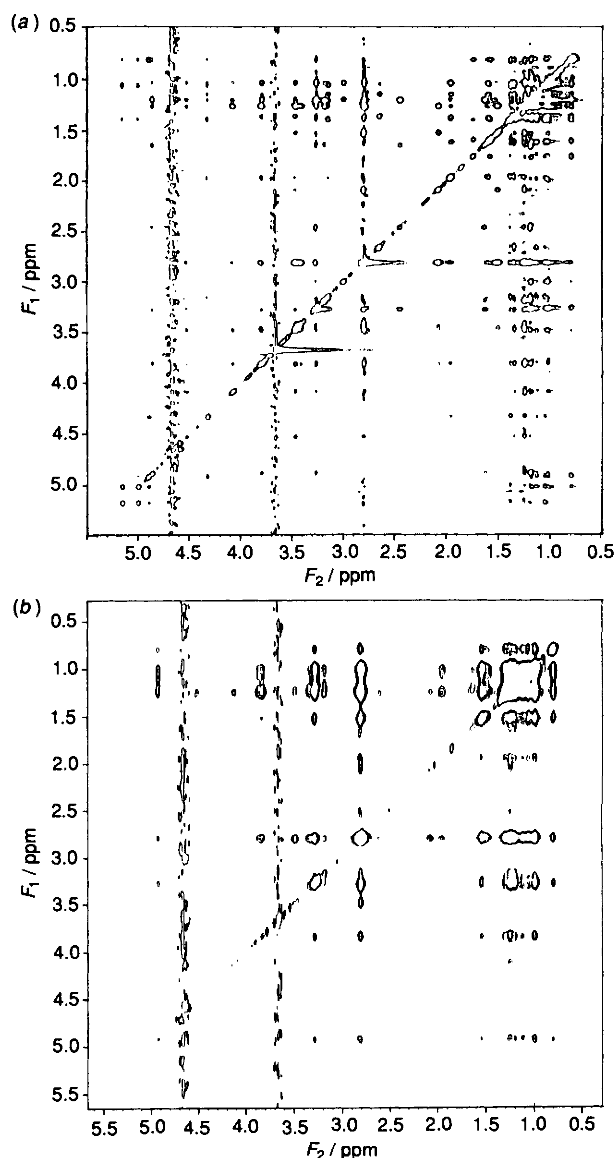


Fig. 2 Transferred NOESY spectra of 4 mmol dm^{-3} solutions of (*a*) erythromycin A 11,12-methylene acetal **3**, (*b*) erythromycin A enol ether **8**, both in the presence of 0.8 μmol dm^{-3} ribosomes.

ribosomal cores, whereas the control spectrum for **3** was almost blank.

In conclusion, although some of the line broadening data, especially those due to compound **10**, are difficult to interpret, there is an absolute correlation between TRNOESY data and antibacterial activity for the compounds tested. In the cases for which it has been tested, compounds **6**,[21] **9**[22] and **10**[22] this correlation extends to tight binding to ribosomes; compound **6** binds, **9** and **10** do not. These results provide further strong evidence that the observed weak binding between erythromycin and ribosomes is physiologically significant, forming the first stage of a two stage inhibitory interaction.

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