Specific, Weak Binding of Erythromycin A (Ketone) and Chloramphenicol to 50S Subunits of *E. coli* Ribosomes: A ^{1}H NMR Study

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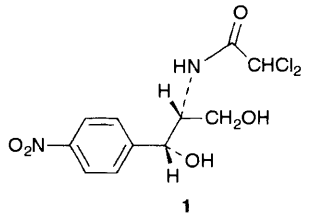
Chloramphenicol and erythromycin A ketone are shown by ^{1}H NMR spectroscopy to bind to 50S subunits of bacterial ribosomes in a fast exchange process.

The important antibiotics chloramphenicol **1** and erythromycin A **2** both act by inhibiting bacterial protein synthesis. Both drugs have been shown to bind to bacterial ribosomes, specifically to 50S subunits, with dissociation constants of 2×10^{-6} mol dm^{-3} (chloramphenicol) and about 10^{-8} mol dm^{-3} (erythromycin A).[1] Such tightly bound complexes would be expected to give rise to slow exchange on the NMR time-scale, yet Tritton[2] was able to demonstrate differential line broadening in the ^{1}H spectrum of a large excess of chloramphenicol on addition of *E. coli* ribosomes. This differential line broadening is indicative of a fast exchange process suggesting weak binding ($K_d > 10^{-5}$ mol dm^{-3}) of chloramphenicol to



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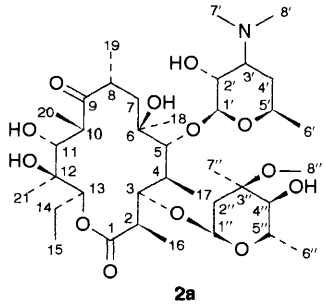
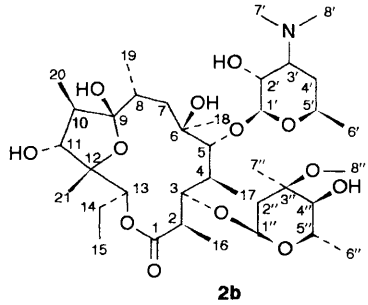


2a



2b

ribosomes. More recently we have demonstrated a similar weak (fast exchange) binding of erythromycin A to ribosomes.[3] In aqueous solution erythromycin A exists as two slowly interconverting isomers,[4] and only the 9-ketone form **2a** binds. Taken together these experiments indicate that each of these drugs is able to bind to ribosomes at two distinct sites, a high affinity site on the 50S subunit and also a lower affinity site. The relatively weak binding at this latter site may in both cases be monitored by NMR spectroscopy.

In the present study we sought to determine whether the low affinity binding sites for erythromycin A and chloramphenicol are associated with a particular subunit and, if so, which. Weak binding of chloramphenicol (K_d *ca.* 10^{-4} mol dm^{-3}) by 30S subunits has previously been indicated by equilibrium dialysis,[5] but there is no comparable observation in the case of erythromycin A. In addition to determining the location of the low affinity binding sites we also sought to identify which parts of the drug molecules are responsible for these weak interactions.

Pure 70S *E. coli* ribosomes and 30S and 50S subunits[6] were prepared essentially as described. 50S 'core' particles were prepared by incubating 50S subunits with 1.3 mol dm^{-3} LiCl solution to remove outer proteins.[6] 600 MHz ^{1}H NMR spectra of each drug alone (2 mmol dm^{-3} chloramphenicol, 4 mmol dm^{-3} erythromycin A) and in the presence of 12 μmol dm^{-3} ribosomes, 10 μmol dm^{-3} 50S subunits, 10 μmol dm^{-3} 30S subunits and 19.5 μmol dm^{-3} 'cores' in deuteriated buffer (50 mmol dm^{-3} sodium phosphate, 200 mmol dm^{-3} KCl, pH 7.6) were run. The spectra were processed without weighting and the line-width at half height of each separated signal measured.

Fig. 1 shows the upfield region of the ^{1}H NMR spectra of (*A*) erythromycin, (*B*) erythromycin plus 30S subunits, and (*C*) erythromycin plus 50S subunits. It can be seen that the addition of 30S subunits to the drug results only in a slight broadening of all the lines in the spectrum. Spectrum 1*B* is almost indistinguishable from the control spectrum (erythromycin plus cores, not shown here, but see ref. 3). Each line is

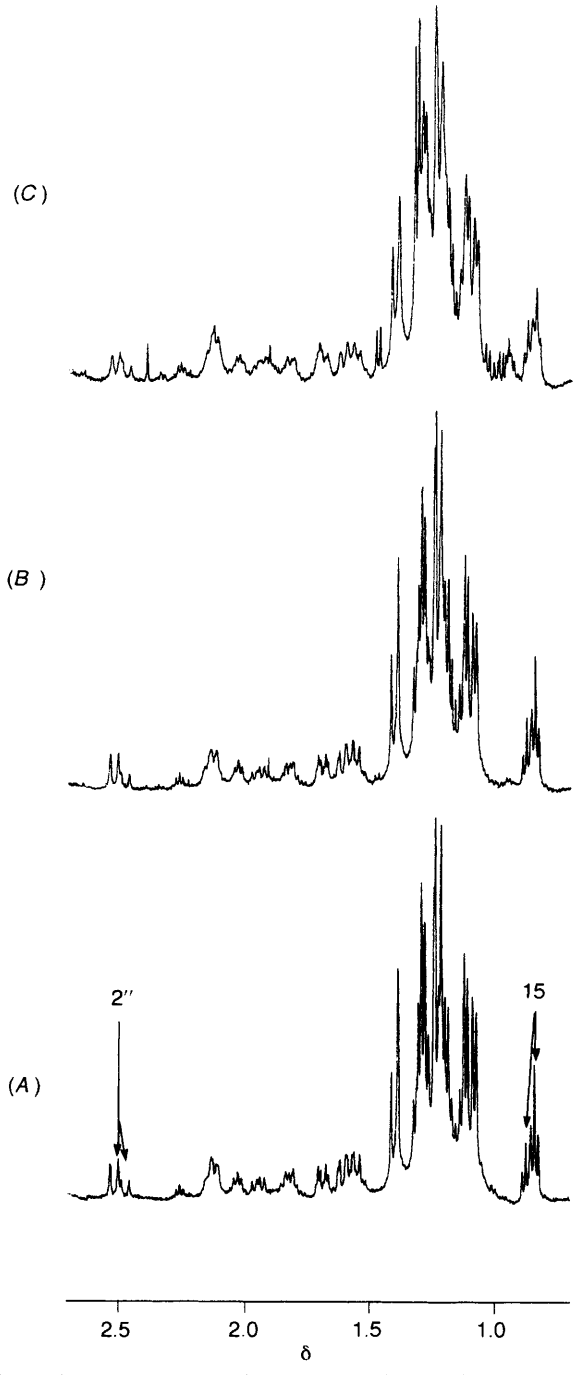


Fig. 1 ^{1}H NMR spectra (upfield region) of (*A*) 4 mmol dm^{-3} erythromycin A at pH 7.6; (*B*) 4 mmol dm^{-3} erythromycin A in the presence of 10 μmol dm^{-3} 30S ribosomal subunits; (*C*) 4 mmol dm^{-3} erythromycin A in the presence of 10 μmol dm^{-3} 50S ribosomal subunits

broadened by an average factor of 1.5 at half height with a standard deviation of only 0.4, with no significant difference between the major and minor isomers. This indicates that the only interactions between erythromycin and 30S subunits are non-specific, and typical of weak interactions between small molecules and macromolecular complexes. In spectrum 1*C*, however, selective line broadening in resonances due to the major isomer is observed (see H-2'' and H_3-15 especially). The spectrum closely resembles (though is not quite identical to) that of erythromycin in the presence of whole 70S ribosomes.[3] Extensive line broadening occurs only in the spectrum of the major isomer, and particularly affects resonances associated with the sugar rings and the region of the macrolide around the lactone function.

Fig. 2 shows the downfield region of the ^1H NMR spectra of (*A*) chloramphenicol, (*B*) chloramphenicol plus 30S subunits, and (*C*) chloramphenicol plus 50S subunits. Again, the line broadening caused by 70S ribosomes[2] is associated entirely with the 50S subunits. Spectrum 2*B* closely resembles that of the control (not shown).

In a further experiment 70S ribosomes (12 μmol dm^{-3}) were added to a mixture of chloramphenicol (2 mol dm^{-3}) and erythromycin A (2 mol dm^{-3}). The spectra of both drugs broadened in the usual way; there was no evidence of competition between the drugs.

Finally, a model of erythromycin A ketone showing the hydrogen atoms giving the most extensively broadened resonances was constructed (Fig. 3). The model is based on the crystal structure of erythromycin A 9-[*O*-(2,5-dioxahexyl) oxime] hydrate[7] and minimised using QUANTA/CHARMm software. Line broadening in NMR spectra may result from two distinct processes: rapid equilibrium between states with different chemical shifts, and slowing of molecular motion. It is difficult to distinguish between these but, in general, a correlation between line broadening and involvement in binding is expected. Those parts of the molecule which give rise to the most line broadening, for example, the cladinose ring and H_3-21 are expected therefore to be those most closely involved in binding. Data from repeated experiments using 70S ribosomes (see Table 1) were used in assessing which lines were most extensively broadened and the hydrogen atoms giving rise to those lines are indicated using van der Waals' surfaces on the model. The model suggests that erythromycin binds to ribosomes at a distinct surface involving both sugar rings and the 2-3-4 region of the macrolide ring. These are among the parts of the molecule identified by Everett and Tyler as having restricted mobility in solution.[8] The two sugar rings have also been reported to be necessary for the bacteriostatic action of the drug.[2]

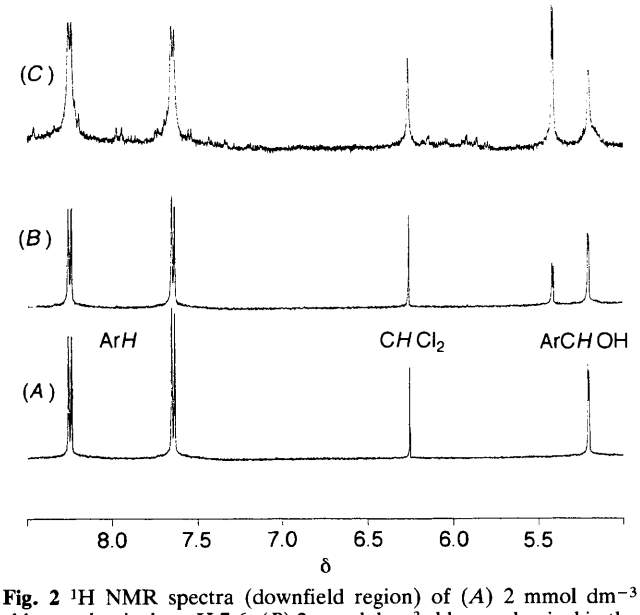


Fig. 2 ^1H NMR spectra (downfield region) of (*A*) 2 mmol dm^{-3} chloramphenicol at pH 7.6; (*B*) 2 mmol dm^{-3} chloramphenicol in the presence of 10 μmol dm^{-3} 30S ribosomal subunits; (*C*) 2 mmol dm^{-3} chloramphenicol at pH 7.6 in the presence of 10 μmol dm^{-3} 50S ribosomal subunits. The doublet at δ 5.4 in *B* and *C* is an impurity in the ribosomal subunit preparation.

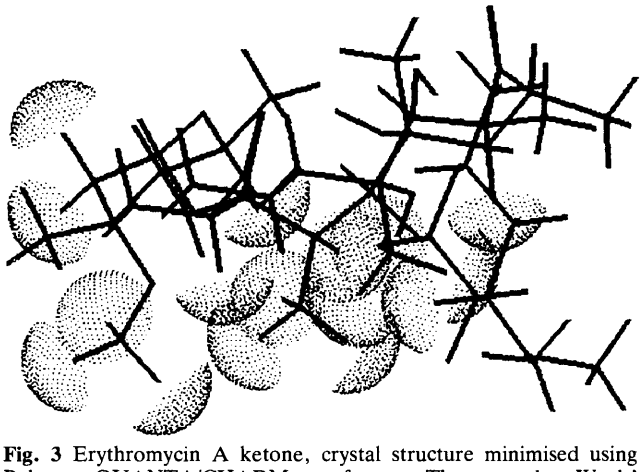


Fig. 3 Erythromycin A ketone, crystal structure minimised using Polygen QUANTA/CHARMm software. The van der Waals' surfaces are shown to indicate the atoms whose signals are most extensively broadened. Methylene resonances are expected to broaden more extensively than methyl or methine signals, so the broadening observed in both H-2″ signals (shown on the outside of the surface here) may reflect proximity to, rather than participation in, a binding surface.

Table 1 Linewidths at half height (Hz) in the ^1H NMR spectrum of erythromycin A at pH 7.6 alone and in the presence of ribosomal components (keto isomer only, selected resonances)[a]

		Linewidth 7.6				
Position	δ	Alone	Plus 19 μmol dm^{-3} cores	Plus 12.5 μmol dm^{-3} 70S ribosomes	Plus 10 μmol dm^{-3} 50S subunits	Plus 10 μmol dm^{-3} 30S subunits
1″	4.91	2.75	4.4	5.45	11.5	4.6
2″eq	2.46	2.75	4.5	*12.5*	7.15	4.3
2″ax	1.68	2.5	4.5	*10.2*	9.9	5.45
5″	4.15	2.5	5.0	*12.25*	5.8	4.25
7″	1.23	2.0	3.75	8.6	10.15	4.9
8″	3.32	1.5	2.75	8.5	6.2	—
3'	2.43	2.0	5.0	*11.9*	—	—
6'	1.29	3.6	—	—	11.95	4.25
2	3.07	3.6	6.9	*12.5*	6.05	5.1
4	2.03	3.75	6.25	*11.9*	11.0	5.8
7ax	1.61	3.75	4.9	*12.5*	—	—
13	5.03	4.1	6.5	8.75	8.2	5.7
14*ax*	1.55	6.1	—	—	12.2	7.2
15	0.84	1.9	3.75	7.0	9.55	3.5
17	1.08	3.6	4.0	*9.4*	9.5	5.24
18	1.37	2.4	5.0	7.4	9.55	3.9
19	1.19	3.5	—	—	7.3	4.0
20	1.12	2.25	5.0	8.6	10.3	3.75
21	1.21	2.25	4.9	*13.1*	16.8	6.0

[a] The most extensively broadened lines (Fig. 3) are given in italics.

Our previous experiments and those of Tritton have demonstrated some surprising correlations between binding and the inhibition of protein synthesis by erythromycin and chloramphenicol. For example, the most extensive line broadening in the chloramphenicol–ribosomes spectrum relates to the parts of the molecule which cannot be modified radically without loss of activity, and line broadening in the erythromycin–ribosomes spectrum increases with pH, as does the activity of the drug. We have now shown that low affinity binding sites for both drugs, like the high affinity sites, are associated with the 50S subunit; both drugs inhibit processes which occur on that subunit. In addition, the binding surface now indicated on the erythromycin molecule involves regions which are required for its antibacterial activity. (Preliminary data indicate that the binding surfaces of both drugs may be extended somewhat when 50S subunits replace 70S ribosomes. The difficulties of preparing active isolated subunits free of NMR-active impurities have so far precluded the detailed quantitative analysis required to clarify this point). We have not been able to mimic the non-reciprocal competition of erythromycin with chloramphenicol on the ribosome, but in general the properties we observe for the low affinity (NMR detected) binding correlate with activity of the drugs and with what is known of the high affinity binding sites for these drugs on 70S ribosomes. It is tempting to conclude that there are low affinity pre-inhibition binding sites for both erythromycin and chloramphenicol on ribosomal 50S subunits, but, in view of the large excess of drug used in these studies, such a model must allow for both the high affinity sites and the 'pre-inhibition' sites being occupied simultaneously. It should, however, be mentioned that at this stage there is little direct evidence connecting the high affinity binding sites to inhibition. It is intuitively unlikely, but not impossible, that the NMR experiments described here monitor the binding sites responsible for the inhibition of protein biosynthesis.

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