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# Erythromycin B: conformational analysis and antibacterial activity

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## SUPPLEMENTARY DATA

**Fig. 1**: 500 MHz $^{1}$H NMR spectrum of erythromycin B in buffered $D_2O$

**Table 1:** Full assignments of $^{1}$H and $^{13}$C spectra of erythromycin B in $CDCl_3$ and phosphate buffered $D_2O$

### Assignment strategy for the $^{1}$H and $^{13}$C NMR erythromycin B in buffered $D_2O$

**Fig. 2**: 500 MHz gradient HMQC spectrum of erythromycin B in aqueous phosphate buffer at apparent pH 7.4.

**Table 2:** 500 MHz ROESY connectivity table of erythromycin B in phosphate buffered $D_2O$ at apparent pH 7.4; mixing time is 250 ms. 500 MHz TRNOESY connectivity table of erythromycin B bound to deuteriated ribosomes at apparent pH 7.4; mixing time 75 ms.

**Table 3:** Folded-out, folded-in and 8-*endo*-folded-out conformers of erythromycin B: full structural details.

**Fig. 3:** The 500 MHz TRNOESY NMR spectrum of erythromycin B bound to 50S cores from *E. coli* ribosomes treated with 2M LiCl in buffered $D_2O$ at apparent pH 7.4.

### Determination of MICs for erythromycins A and B and clarithromycin against various micro-organisms

**Table 4**: Relationship between OD, CFU and time to reach mid-log phase for different micro-organisms

**Fig. 4 A**: Growth curves of *Staphylococcus aureus* ACC 601065 in increasing concentrations of erythromycin B over 24 h. **B**: Growth inhibition of *Staphylococcus aureus* ACC 601065 in increasing concentrations of erythromycin B.

**Fig. 5:** Length of the lag phase (h) of *Staphylococcus aureus* ACC 610065 grown in the presence of increasing concentrations of erythromycin B. The arrow shows the calculated MIC for erythromycin B against this organism.

**Table 5:** Comparison of the calculated MICs by the specific growth rate and length of lag phase methods for erythromycins A and B, clarithromycin againt *S. aureus, S. pyogenes* and *H. influenzae.*

### Experimental – NMR spectra

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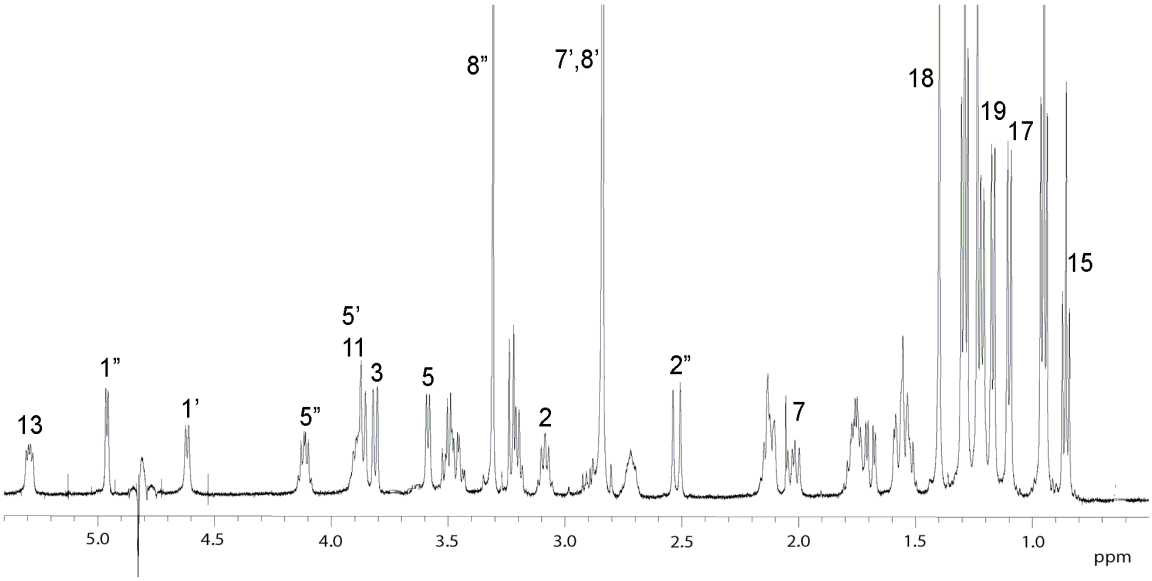


Fig. 1 500 MHz $^{1}$H NMR spectrum of erythromycin B in buffered $D_2O$

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# SUPPLEMENTARY DATA

**Table 1.** Full assignments of $^{1}H$ and $^{13}C$ spectra of erythromycin B in $CDCl_3$ and phosphate buffered $D_2O$.

| Position | $D_2O$ | | | | | $CDCl_3$ | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $\delta_H$ | | $J_{H-H}$ | $\delta_C$ | HMBC connectivities (H→C) | $\delta_H$ | | $J_{H-H}$ | $\delta_C$ | HMBC connectivities (H→C) |
| 1 | | | | 177.35 | | | | | 176.50 | |
| 2 | 3.08 | bq | 7.5[a] | 47.23 | 1, 3 | 2.89 | bquint | 7.5[a] | 45.00 | 3, 4, 16 |
| 3 | 3.81 | d | 9.5 | 87.52 | 1",17 | 4.08 | dd | 8.98, 1.10 | 80.50 | 1", 2, 4, 5, 16 ,17 |
| 4 | 2.15 | M | | 32.03 | 5, 6, 7 | 2.10 | quint | 7.14[a] | 39.75 | 5, 6, 17 |
| 5 | 3.59 | d | 6.5 | 87.03 | 3, 6, 18, 1', 7'/8' | 3.59 | d | 7.51 | 83.80 | 1", 2, 3, 4, 6, 7, 18 |
| 6 | | | | 77.81 | | | | | 75.60 | |
| 7 *proR* | 1.57 | dd | 15.0, 4.0 | 40.00 | | 1.67 | bdd | 14.65, 2.70 | 38.10 | 5, 18 |
| 7 *proS* | 2.02 | dd | 15.0, 9.5 | | 6 | 2.03 | dd | 14.83, 10.81 | | 5, 6, 18, 19 |
| 8 | 2.72 | m | | 47.01 | | 2.75 | ddq | 10.62, 6.78, 2.75 | 44.80 | 7, 19 |
| 9 | | | | 222.13 | | | | | 220.50 | |
| 10 | 3.20 | bq | 6.8 | 42.01 | 9, 20 | 3.00 | q | 10.12, 6.95 | 39.25 | 20, 11 |
| 11 | 3.86 | d | 10.5 | 72.63 | 20 | 3.81 | d | 10.07 | 69.60 | 12, 13, 20, 21 |
| 12 | 1.75 | m | | 42.42 | 11, 13 | 1.67 | m | | 39.90 | 11, 21 |
| 13 | 5.29 | dd | 9.0, 5.0 | 79.11 | | 5.35 | dd | 4.76, 9.52 | 75.20 | 11, 12, 14, 15, 21 |
| 14 *proR* | 1.79 | m | | 28.25 | 13, 15 | 1.72 | m | | 25.55 | 12, 13, 15 |
| 14 *proS* | 1.52 | m | | | 15 | 1.48 | m | | | 12, 15 |
| 15 | 0.86 | t | 7.5 | 12.36 | 13, 14 | 0.89 | t | 7.23 | 10.25 | 13, 14 |
| 16 | 1.22 | d | 7.0 | 17.38 | 1, 2, 3 | 1.20 | d | 7.14 | 15.80 | 2, 3 |
| 17 | 1.10 | d | 7.5 | 11.69 | 3, 4, 5 | 1.14 | d | 7.32 | 9.70 | 2, 3, 4, 5 |
| 18 | 1.40 | s | | 28.66 | 5, 6, 7 | 1.46 | s | | 27.30 | 5, 6, 7 |
| 19 | 1.17 | d | 7.5 | 20.37 | 7, 8, 9 | 1.16 | d | 6.78 | 18.20 | 7 |
| 20 | 0.96 | d | 6.8 | 11.20 | 11, 12 | 1.00 | d | 6.96 | 9.80 | 10, 11 |
| 21 | 0.95 | d | 7.0 | 11.37 | 11, 12, 13 | 0.87 | d | 7.14 | 9.60 | 11, 12 |
| 1' | 4.60 | d | 6.8[a] | 104.42 | | 4.42 | d | 7.33 | 103.50 | 5, 3', 5' |
| 2' | 3.52 | dd | 11.25, 6.8 | 71.86 | 1', 3' | 3.20 | dd | 7.33, 10.25 | 71.00 | 1', 3' |
| 3' | 3.45 | dt | 11.25, 4.2 | 97.71 | | 2.45 | ddd | 3.66, 10.43 | 65.70 | 2', 4', 7'/8' |
| 4' *proR* | 1.53 | m | | 31.65 | 5' | 1.26[b] | m | | 28.90 | 2', 3', 5' |
| 4' *proS* | 2.15 | m | | | | 1.73 | m | | | 2', 3' |
| 5' | 3.90 | m | | 70.42 | | 3.50 | m | | 69.00 | 3' |
| 6' | 1.29 | d | 6.0 | 22.85 | 4', 5' | 1.23 | d | 6.04 | 21.00 | 3', 6' |
| 7'/8' | 2.84 | s | | 41.62 | 2', 3', 4' | 2.28 | s | | 40.30 | 3' |
| 1" | 4.96 | d | 5.0 | 99.65 | | 4.89 | d | 4.76 | 96.80 | 3, 3", 5" |
| 2" *proR* | 1.79 | dd | 15.5, 5.0 | 37.19 | | 2.39 | d | 15.20 | 35.10 | 1", 3", 4", 7" |
| 2" *proS* | 2.53 | d | 15.5 | | 1", 3", 4" | 1.59 | dd | 15.19, 4.94 | | 1", 3", 7" |
| 3" | | | | 79.19 | | | | | 73.00 | |
| 4" | 3.23 | d | 9.5 | 79.69 | 5", 6" | 3.03 | d | 9.15 | 78.30 | 5", 6", 7", 8" |
| 5" | 4.14 | dq | 9.5, 6.5 | 68.48 | | 4.03 | dq | 9.15, 6.23 | 65.90 | 3", 4", 6" |
| 6" | 1.30 | d | 6.5 | 20.45 | 4", 5" | 1.29 | d | 6.23 | 18.50 | 4", 5" |
| 7" | 1.23 | s | | 22.97 | 2", 3", 4" | 1.24 | s | | 21.50 | 1", 2", 3", 4", 5" |
| 8" | 3.31 | s | | 51.82 | 3" | 3.32 | s | | 49.50 | 3" |

[a] obscured

## Assignment strategy for the $^{1}H$ and $^{13}C$ NMR erythromycin B in buffered $D_2O$

In the one-dimensional $^{1}H$ spectrum, the 3H-singlet at δ 3.31 and the 6H-singlet at δ 2.84 were assigned by inspection to $H_3$8" and H7'8' respectively. The 3H triplet at δ 0.86 was assigned by inspection to $H_3$15. The TOCSY and DQF-COSY spectra were used to assign the spin group 15-14-

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13; $H_2$14 were provisionally distinguished by analogy with the spectrum of erythromycin A[1] and these assignments were later confirmed using the connectivity data derived from the ROESY spectrum. The doublet at δ 4.96 formed part of a spin group with no methyl group and was therefore assigned to H1", allowing assignment of the spin group H1"-$H_2$2". The doublet at δ 4.60 formed part of the longest spin group in the TOCSY spectrum, and was assigned to H1'; the TOCSY and DQF-COSY spectra allowed assignment of the 1'-2'-3'-4'-5'-6' spin group. Again the H4' signals were distinguished provionally by analogy to erythromycin A. The remaining isolated high frequency signal, a double quartet at δ 4.14, was assigned by inspection to H5". The TOCSY and DQF-COSY spectra permitted the assignement of H4" and H6".

The remaining low frequency methyl groups (doublets at δ 0.95, 0.96, 1.10, 1.17 and 1.22) were used as starting points for the rest of the assignments. These are due, non-specifically, to $H_3$16, $H_3$17, $H_3$19, $H_3$20 and $H_3$21. The TOCSY and DQF-COSY spectra showed a six-membered spin-group, assigned to $H_3$16-H2-H3-H4(H5)-$H_3$17, connecting δ1.10 ($H_3$17) and δ1.22 ($H_3$16). Very weak coupling between H3 and H4 is characteristic of this spin group, reflecting the constrained 90° torsion angle H3-C3-C4-H4 seen in several 14-membered ring macrolides. The methyl group at δ1.17 was assigned to $H_3$19, on the basis of its coupling a methylene group ($H_2$7) and the DQF-COSY and TOCSY spectra were used to assign the $H_3$19- $H_2$7-H8 spin group. The remaining methyl groups might be expected to appear in the same spin group; instead δ0.95 coupled to δ1.78 and δ3.86, wheras δ0.96 coupled to δ3.20. δ1.78 is consistent with H12 but not with H10, so $H_3$21-H12-H11 forms one spin group and $H_3$20-H10 another, reflecting another constrained 90° torsion angle in the molecule.

The gHMQC spectrum was now used to assign all the carbon signals corresponding to the proton signals assigned above, and the quaternary carbons C6 and C3" were distinguished using the gHMBC spectrum.

Figure 2 shows the gHMQC spectrum of erythromycin B in aqueous solution.

[1] J Barber, *JI Gyi*, L Lian, GA Morris, *DA Pye* and JK Sutherland. *J. Chem. Soc. Perkin Trans. 2*, 1991, 1489-1494.

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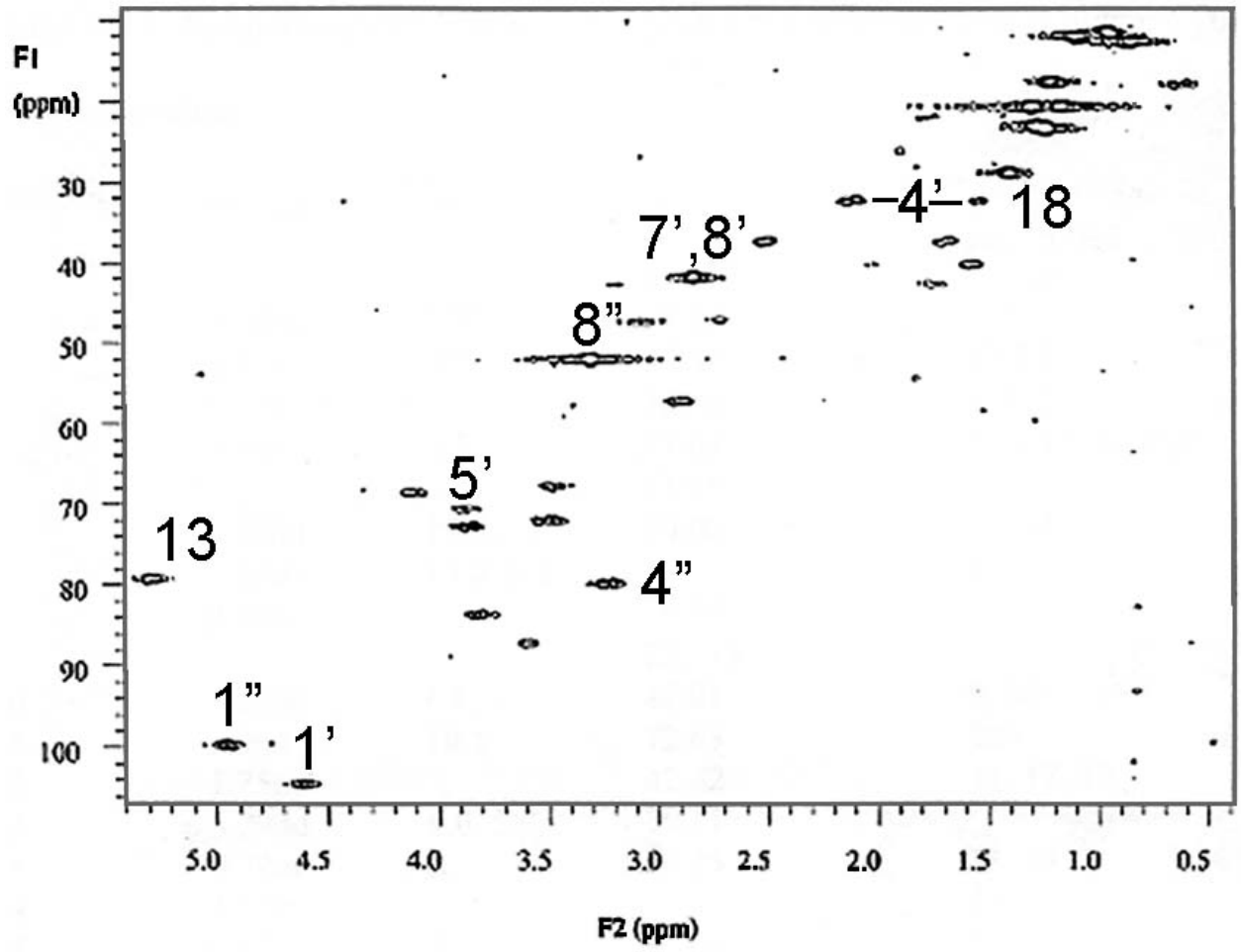


**Fig. 2**. 500 MHz gradient HMQC spectrum of erythromycin B in aqueous phosphate buffer at apparent pH 7.4. Selected signals are indicated.

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| | 2 | 3 | 4 | 5 | 7s | 7r | 8 | 10 | 11 | 12 | 13 | 14r | 14s | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 1' | 2' | 3' | 4'r | 4's | 5' | 6' | 7'/8' | 1" | 2"r | 2"s | 4" | 5" | 6" | 7" | 8" |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | ● | | S | | | | | | | | | | | | M | M | | | | | | | | | | | | | | | | | | | | |
| 3 | **S** | ● | M | M | | | | | | | | | | | S | | | | | | | | | | | | | | M | | | | | | | |
| 4 | M | M | ● | V | | | | | M | | | | | | | M | | | | | | | | | | | | | | | | | | | | |
| 5 | | M | V | ● | | | | | | | | | | | | V | M | | | | M | | | | | | | | | | | | M | V | | |
| 7s | | | | | ● | L | | M | S | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 7r | | | | | L | ● | | | | | | | | | | | S | S | | | | | | | | | | | | | | | | | | |
| 8 | | | | | **V** | **M** | ● | | S | | | | | | | | M | M | | | | | | | | | | | | | | | | | | |
| 10 | | | | | M | **M** | | ● | M | | | | | | | | | M | L | | | | | | | | | | | | | | | | | |
| 11 | | | M | | M | **S** | S | M | ● | **V** | S | | | | | | | | | S | | | | | | | | | | | | | | | | |
| 12 | | | | | | | | | S | ● | | | | | | | | | | L | | | | | | | | | | | | | | | | |
| 13 | | | | | | | | | M | | ● | M | S | S | | | | | | | | | | | | | | | | | | | | | | |
| 14r | | | | | | | | | | | M | ● | L | M | | | | | | | | | | | | | | | | | | | | | | |
| 14s | | | | | | | | | | | S | L | ● | M | | | | | | | | | | | | | | | | | | | | | | |
| 15 | | | | | | | | | | | M | M | S | ● | | | | | | | | | | | | | | | | | | | | | | |
| 16 | M | S | | | | | | | | | | | | | ● | | | | | | | | | | | | | | M | | | | | | | |
| 17 | M | **V** | M | V | | | | | | | | | | | | ● | | | | | S | | | | | | | | | | | | | | | S |
| 18 | | | **V** | M | | V | M | | | | | | | | | | ● | | | | | | | | | | | | | | | | | | | |
| 19 | | | | | | S | M | M | | | | | | | | S | | ● | V | | | | | | | | | | | | | | | | | |
| 20 | | | | | | | | L | | | | | | | | | | V | ● | | | | | | | | | | | | | | | | | |
| 21 | | | | | | | | | M | L | **S** | **L** | **S** | | | | | | | ● | | | | | | | | | | | | | | | | |
| 1' | | | | M | | | | | | | | | | | | M | | | | | ● | | | | | M | | | | | | | M | | | S |
| 2' | | | | | | | | | | | | | | | | | | | | | | ● | | | S | | | M | | | | | | | | |
| 3' | | | | | | | | | | | | | | | | | | | | | **M** | | ● | | V | S | | M | | | | | | | | |
| 4'r | | | | | | | | | | | | | | | | | | | | | | | **S** | ● | L | | S | M | | | | | | | | |
| 4's | | | | | | | | | | | | | | | | | | | | | | S | S | L | ● | | S | M | | | | | | | | |
| 5' | | | | **V** | | | | | | | | | | | | | | | | | M | | M | | **M** | ● | M | | | | | | M | | | |
| 6' | | | | **V** | | | | | | | | | | | | | | | | | | | | M | S | M | ● | | | | | | | | | |
| 7'/8' | | | | | | | | | | | | | | | | | | | | | | M | M | M | M | | | ● | | | | | | | | |
| 1" | | M | | | | | | | | | | | | | M | | | | | | | | | | | | | | ● | M | S | | | | | |
| 2"r | | | | | | | | | | | | | | | | | | | | | | | | | | | | | M | ● | L | M | | | S | |
| 2"s | | | | | | | | | | | | | | | | | | | | | | | | | | | | | M | L | ● | | | | S | M |
| 4" | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | M | **V** | ● | | M | M | |
| 5" | | | | M | | | | | | | | | | | | | | | | | M | | | | | M | | | | | | **V** | ● | M | | |
| 6" | | | | V | | | | | | | | | | | | | | | | | **V** | | | | | | | | | | | M | M | ● | | |
| 7" | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | S | S | M | | | ● | M |
| 8" | | | | | | | | | | | | | | | | S | | | | | S | | | | | | | | | | M | | | | M | ● |
| | 2 | 3 | 4 | 5 | 7s | 7r | 8 | 10 | 11 | 12 | 13 | 14r | 14s | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 1' | 2' | 3' | 4'r | 4's | 5' | 6' | 7'/8' | 1" | 2"r | 2"s | 4" | 5" | 6" | 7" | 8" |

**Table 2. Top (above diagonal)** 500 MHz ROESY connectivity table of erythromycin B in phosphate buffered $D_2O$ at apparent pH 7.4; mixing time is 250 ms. **Bottom (below diagonal)** 500 MHz TRNOESY connectivity table of erythromycin B bound to deuteriated ribosomes in phosphate buffered $D_2O$ at apparent pH 7.4; mixing time 75 ms. (H-7r: δ 1.57; H-7s: δ 2.02; H-14s: δ 1.52; H-14r: δ 1.79; H-4'r: δ 1.53; H-4's: δ 2.15; H-2"r: δ 1.79; H-2"s: δ 2.53. The symbols r and s are used as abbreviations for *proR* and *proS* in this table.). Significant differences between the ROESY and TRNOESY spectra are indicated in red.

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| | **Erythromycin B folded-out conformation** | **Erythromycin B folded-in conformation** | **Erythromycin B 8-*endo*-folded-out conformation** |
|---|---|---|---|
| **Energy** | 149.3 kJ $mol^{-1}$ | 144.4 kJ $mol^{-1}$ | 158.2 kJ $mol^{-1}$ |
| **Tosion angle (degrees)** | | | |
| H2-H3 | 169.3 | 103.7 | 171.0 |
| H3-H4 | -74.3 | -63.2 | -85.3 |
| H4-H5 | 140.9 | 115.3 | 155.6 |
| H7$_{proS}$-H8 | 173.3 | 55.9 | 98.4 |
| C3-H1" | 39.7 | 39.0 | 39.5 |
| C5-H1' | 44.7 | 53.4 | 54.6 |
| **Internuclear distance (Å)** | | | |
| H2-H3 | 3.0 | 2.8 | 3.0 |
| H2-H4 | 2.8 | 2.3 | 2.7 |
| H2-C17 | 2.8 | 2.8 | 2.9 |
| H3-H4 | 2.6 | 2.4 | 2.7 |
| H3-H5 | 2.4 | 2.4 | 2.5 |
| H3-H8 | 5.7 | 2.3 | 4.9 |
| H3-H11 | 3.7 | 2.3 | 4.2 |
| H3-C16 | 2.9 | 3.4 | 2.9 |
| H3-C17 | 3.5 | 3.5 | 3.5 |
| H3-H1" | 2.5 | 2.6 | 2.4 |
| H4-H5 | 3.0 | 2.8 | 3.0 |
| H4-H11 | 2.5 | 3.2 | 2.7 |
| H4-C18 | 4.1 | 2.7 | 4.1 |
| H4-C16 | 4.2 | 3.9 | 4.1 |
| H4-C21 | 3.5 | 4.5 | 3.5 |
| H5-C17 | 3.1 | 3.3 | 3.0 |
| H5-C18 | 2.6 | 3.5 | 2.2 |
| H5-H1' | 2.4 | 2.4 | 2.4 |
| H7$_{proS}$-H8 | 3.1 | 2.5 | 2.7 |
| H7$_{proS}$-C18 | 3.5 | 2.7 | 3.4 |
| H7$_{proS}$-C19 | 2.8 | 3.5 | 3.4 |
| H7$_{proR}$-H8 | 2.5 | 3.0 | 3.0 |
| H7$_{proR}$-C18 | 2.8 | 2.7 | 2.7 |
| H7$_{proR}$-C19 | 2.6 | 2.9 | 2.6 |
| H8-H10 | 3.7 | 3.4 | 3.5 |
| H8-H11 | 4.2 | 2.3 | 2.3 |
| H8-C18 | 2.8 | 4.3 | 3.3 |
| H8-C20 | 4.4 | 4.3 | 4.5 |

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| | | | |
|---|---|---|---|
| H10-H11 | 2.5 | 2.5 | 2.5 |
| H10-H12 | 3.2 | 3.3 | 3.3 |
| H10-C21 | 2.6 | 2.7 | 2.6 |
| H11-H12 | 3.0 | 3.0 | 3.0 |
| H11-C20 | 3.4 | 3.5 | 3.5 |
| H11-C21 | 2.9 | 2.8 | 2.9 |
| H11-H13 | 2.8 | 2.9 | 2.6 |
| H12-H13 | 2.5 | 2.4 | 2.6 |
| H12-C20 | 3.0 | 2.9 | 2.9 |
| H12-H14$_{proR}$ | 2.4 | 2.5 | 2.4 |
| H13-C15 | 2.7 | 2.7 | 2.7 |
| C15-C16 | 4.8 | 5.2 | 4.5 |
| C16-C17 | 4.4 | 3.5 | 4.5 |
| C16-H1" | 2.6 | 2.9 | 2.7 |
| C17-C18 | 6.3 | 3.6 | 5.1 |
| C17-H1' | 3.0 | 3.6 | 3.1 |
| C17-H5" | 4.3 | 4.8 | 3.7 |
| C17-C8" | 4.1 | 4.6 | 4.1 |
| C18-C19 | 4.4 | 4.5 | 3.4 |
| H1'-H5" | 4.3 | 4.8 | 3.7 |
| H1'-H8" | 3.6 | 3.1 | 4.9 |
| H3'-H5" | 4.0 | 3.1 | 4.3 |
| H5'-H5" | 2.6 | 2.5 | 3.2 |
| C7'/8'-C8" | 6.0 | 5.1 | 8.3 |

**Table 3.** Folded-out, folded-in and 8-*endo*-folded-out conformers of erythromycin B: full structural details.

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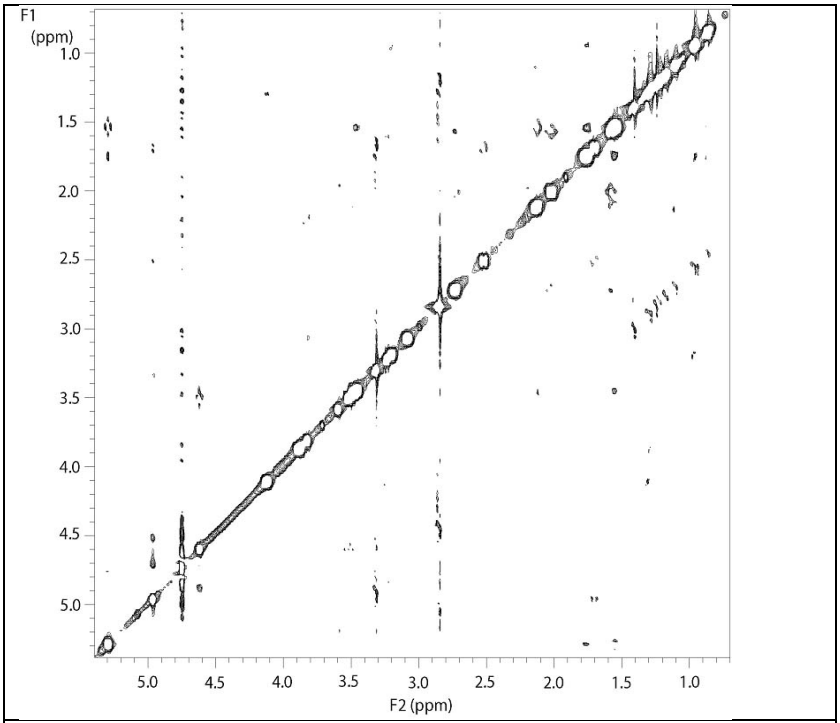


**Fig. 2.** The 500 MHz TRNOESY NMR spectrum of erythromycin B bound to 50S core particles from *E. coli* ribosomes treated with 2M LiCl in buffered $D_2O$ at apparent pH 7.4.

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## Determination of MICs for erythromycins A and B and clarithromycin against various micro-organisms

### Bacterial strains

The microorganisms used in this study were the Gram-positive *Staphylococcus aureus* ACC 601065 and *Streptococcus pyogenes*, and the Gram-negative *Haemophilus influenzae* ATC 12699 and *Bulkhoderia cepacia*. *S. pyogenes* and *B. cepacia* were isolates from the laboratory collection of Dr David Allison of the School of Pharmacy and Pharmaceutical Sciences, University of Manchester.

### Media

Lauria-Bertani (LB) agar and broth were used for culture maintenance and (MIC) studies for *S. aureus*, *S. pyogenes* and *B. cepacia*. For *H. influenzae*, several broth and agar media were tested. Haemophilus Test Medium (HTM) [2] [3] gave the most satisfactory results.

### Bacterial culture maintenance

Stock cultures were prepared by growing an overnight culture (16-18 h) of the microorganisms in appropriate broth and adding sterile glycerol to a final concentration of 15%. The cultures were dispensed into individual eppendorf tubes and stored at -20°C and –80°C. Weekly stock cultures were maintained on appropriate agar plates at 4°C for *S. aureus* and *S. pyogenes*, and at room temperature (18°C) for *B. cepacia* and *H. influenzae.* Stock cultures were checked for contamination before use.

### Stock solutions of antibiotics

Erythromycin A, erythromycin B and clarithromycin in their free-base form were gifts from Dr Warren Mann (Abbott Laboratories, UK), and were used without further purification. Stock solutions of erythromycin A, erythromycin B, clarithromycin were prepared by dissolving 0.01 g of drug in 5 ml ethanol (99%). Distilled water (245 ml) was added to achieve a final concentration of 40 μg $ml^{-1}$. The solutions were filter sterilised using a 0.22 μm membrane filter (Millipore, Bedford, MA, USA), dispensed into sterile eppendorf tubes, and stored at –20°C.

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[2] Scriver SR, Low DE, Simor AE, Toye B, Mcgeer A, Jaeger R & Canadian Haemophilus Study Group (1992) Broth microdilution testing of *Haemophilus influenzae* with Haemophilus Test Medium versus Lysed Horse Blood Broth. *J. Clin. Microbiol.* **30**, 2284-2289.

[3] Jorgensen JH, Redding JS, Maher LA & Howel AW (1987) Improved medium for antimicrobial susceptibility testing of *Haemophilus influenzae*. *J. Clin. Microbiol.*, **25**, 2105-2113.

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Acid-treated erythromycin A, erythromycin B and clarithromycin solutions were prepared by dissolving 0.01 g of drug in 5 ml ethanol (99%) to produce stock solutions of 0.002 g $ml^{-1}$. For each drug, an aliquot (0.5 ml) of each stock solutions was then transferred into a 50 ml Erlenmeyer flask and 12.25 ml of 0.1% HCl added to achieve a pH of 1.8. These solutions were incubated at 37°C and shaken at 50 rpm for 40 min using a G24 environmental orbital shaker (New Brunswick Scientific, NJ, USA) in order to simulate the environment of the stomach. . The degradation reactions were quenched with 0.01 M NaOH (12.25 ml). The final concentration of the degradation mixture was 40 µg $ml^{-1}$ and the pH was 6.8 (at 25°C). These solutions were filter sterilised, transferred into sterile eppendorf tubes and stored at –20°C.

For MIC experiments, fresh antibiotic samples were thawed as needed and used the same day. Any unused drug was discarded at the end of the day.

**Optical density, viable cell count and growth curves**

The starting cell density of a culture for MIC measurements was 5 x $10^5$ CFU $ml^{-1}$. Thus the relationship between optical density and colony forming units (CFU) was required. For each strain, an early stationary phase culture was diluted in the same broth to several different concentrations. The optical density was measured at 470 nm (Uvikon 930 spectrophotometer, Kontron instruments, Milano, Italy). The corresponding viable cell counting was performed by serial dilution of cell suspensions in sterile saline solution (0.85% w $v^{-1}$), spreading 0.1 ml aliquots on an agar plates and counting bacterial colonies after 24 hours at 37 °C . The viable cell count (CFU $ml^{-1}$) was calculated and plotted against optical density.

| **Micro-organism** | **Time to reach mid-log phase (h)** | **average OD (470 nm)** | **average CFU $ml^{-1}$** |
|---|---|---|---|
| *S. aureus* | 4 | 3.0 | 3 x $10^9$ |
| *S. pyogenes* | 6 | 0.6 | 4.8 x $10^8$ |
| *H. influenzae* | 6 | 1.2 | 8.6 x $10^8$ |
| *B. cepacia* | 4.5 | 3.2 | 4 x $10^9$ |

**Table 4:** Relationship between OD, CFU and time to reach mid-log phase for different micro-organisms

**Seeded broth**

For each micro-organism, a mid-log phase culture was serially diluted in the appropriate broth to achieve a final concentration of 10 x $10^5$ CFU $ml^{-1}$ (twice the concentration required in the MIC test).

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**Determination of MIC by the broth microdilution method - National Committee for Clinical Laboratory Standards guidelines (NCCLS, 1993)**

A pilot experiment was carried out to establish a reasonable range of drug concentrations needed for the inhibition of microbial activity. All the macrolides used in this study were inactive against *B. cepacia* (MICs more than 64 μg $ml^{-1}$), which was not studied further.

Antibiotics were prepared at twice the desired final concentration, and 100 μl of each antibacterial solution was placed in a well of a plastic, sterile 96-well tray (Falcon, Becton Dickinson Labware, NJ, USA). A typical working concentration was 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 2.0 and 4.0 μg $ml^{-1}$ for untreated drug solutions or 0.2, 0.4, 0.6, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 (or 15.0) μg $ml^{-1}$ for acid-treated drug solutions. An aliquot of 100 μl of seeded broth was then introduced into each appropriate well, so that the final concentration in each well was 5 x $10^5$ CFU $ml^{-1}$ (range of 3 to 7 x $10^5$ CFU $ml^{-1}$). MICs were determined by using Anthos Plate Reader (Model HTIII, Labtec Instruments, Austria). The 96-well plate reader was programmed to take readings of the well contents at the wavelength of 492 nm (the closest available filter to 470 nm) for every 1 h over 24 h. The plate was agitated hourly allowing aeration. The temperature was maintained either at 30°C or 37°C, depending on the microbe tested. Deviations from linearity of OD measurement were observed above OD of about 0.9 [4] The strength of the culture broth was therefore adjusted so that the a final OD did not exceed 0.9 after 24 h incubation . [5] This was achievable by using half-strength of LB and HTM broths.

The MIC is the lowest concentration of antimicrobial agent that completely inhibits growth of the organism. Two methods were used to assist data interpretation. Firstly the specific mid-log phase growth rate for each drug concentration was estimated as below:

$$\mu = \frac{\ln(\text{final absorbance of mid-log phase} / \text{initial absorbance of mid-log phase})}{\text{Time}}$$

The specific growth rate (μ) from each growth curve was represented as a percentage of the mid-exponential phase growth rate of the control (no antibiotic). The percentage of growth rate was plotted against the antibiotic concentration to aid the determination of the MIC. Typical results are shown in Fig 3.

[4] Maclehose HG (1998) Microbial resistance towards biocides. Ph.D. thesis, The University of Manchester.
[5] Lawrence JV & Maier S (1976) The antibacterial activity of non-ionic surface active agents. *Microbios.* 1, 97-101.

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**Length of the lag phase**

The length of the lag phase of bacterial growth was determined from the growth curve and plotted against antibiotic concentration. An exponential trend line was fitted to the data and extrapolated to 24 h. The intercept on the x-axis (antibiotic concentration, μg $ml^{-1}$) was determined and was taken as the MIC value for that particular microorganism. Figure 4 shows the length of lag phase of growth for *S. aureus* as a function of increasing concentrations of erythromycin B over 24 h. The intercept on the X-axis after 24 h incubation period is about 1.4 μg $ml^{-1}$ which was taken as the MIC value.

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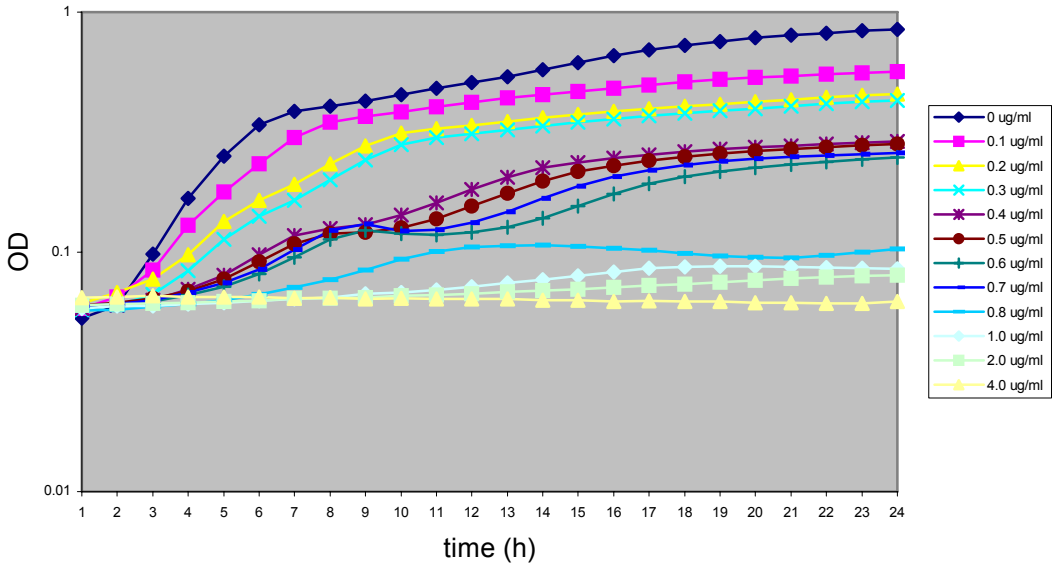
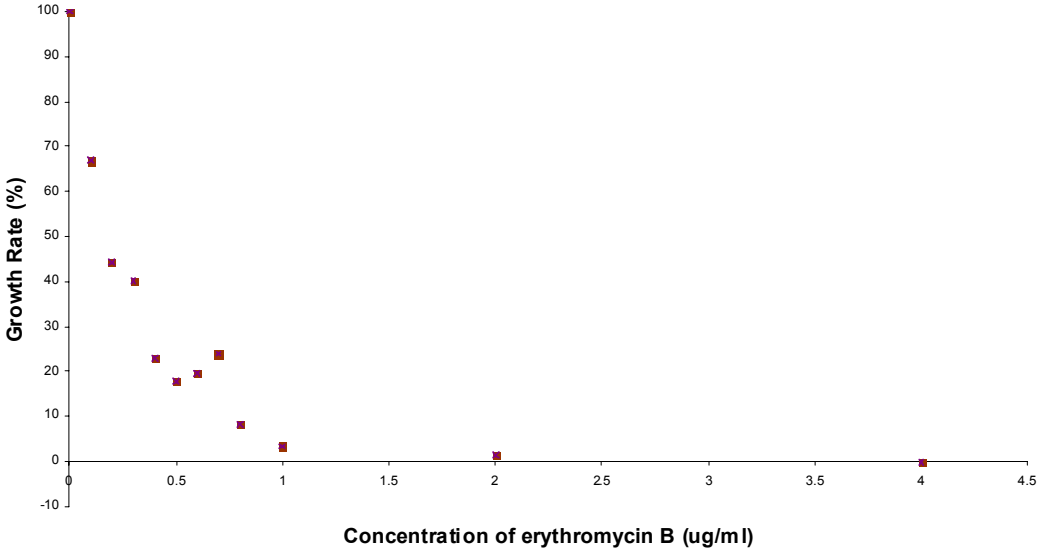



A




B

**Fig. 3 A:** Growth curves of *Staphylococcus aureus* ACC 601065 at increasing concentrations of erythromycin B over 24 h. **B:** Growth inhibition of *Staphylococcus aureus* ACC 601065 at increasing concentrations of erythromycin B. The MIC lies between the highest concentrations tested in which growth occurred (1 µg $ml^{-1}$) and the lowest concentration in which no growth occurred (2 µg $ml^{-1}$).

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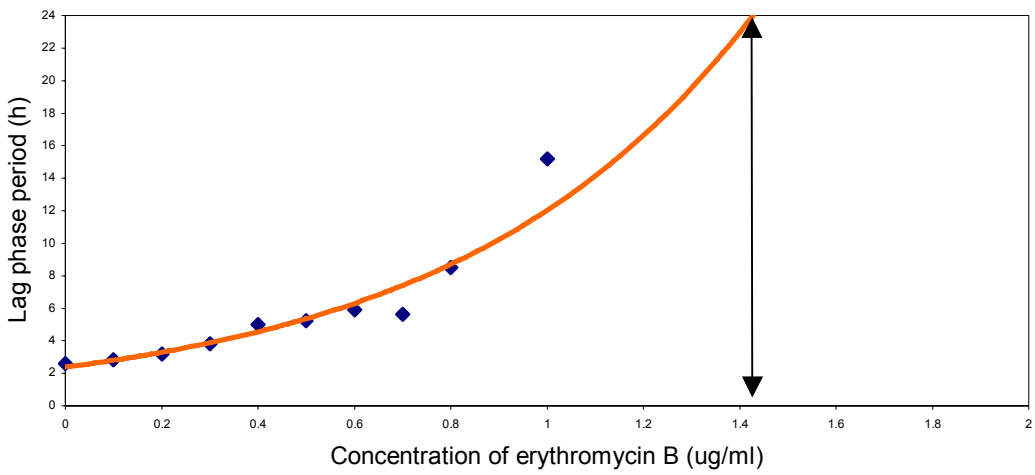


**Fig. 4** Length of the lag phase (h) of *Staphylococcus aureus* ACC 610065 grown in the presence of increasing concentrations of erythromycin B. The arrow shows the calculated MIC for erythromycin B against this organism.

| Organism | *S. aureus* MIC (µg ml$^{-1}$) | | *S. pyogenes* MIC (µg ml$^{-1}$) | | *H. influenzae* MIC (µg ml$^{-1}$) | |
|---|---|---|---|---|---|---|
| Drug | Untreated | Pretreated | Untreated | Pretreated | Untreated | Pretreated |
| Erythromycin A SGR method | 0.8-1.0 | >8 | 0.1-0.2 | 0.8-1.0 | 2.0-4.0 | >4 |
| Erythromycin A LLP method | 0.9 | >8 | 0.2 | 1 | 2.2 | >4 |
| Erythromycin B SGR method | 0.6-0.8 | 1.0-2.0 | 0.1-0.2 | 0.2-0.3 | 2.0-4.0 | 2-4 |
| Erythromycin B SGR method | 0.7 | 1.4 | 0.2 | 0.3 | 2.3 | 3.6 |
| Clarithromycin SGR method | 0.5-0.6 | 0.8-1.0 | 0.1-0.2 | 0.2-0.3 | 0.5-0.6 | 1-2 |
| Clarithromycin LLP method | 0.6 | 0.9 | 0.2 | 0.3 | 0.6 | 1.8 |

**Table 5:** Comparison of the calculated MICs by the specific growth rate and length of lag phase methods for erythromycins A and B and clarithromycin againt ***S. aureus, S. pyogenes*** and ***H. influenzae..*** Very close agreement is seen for the two methods

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# Experimental

### Assignments of the $^{1}H$ and $^{13}C$ NMR spectra of erythromycin B in phosphate buffered $D_2O$

Solutions of 4-10 mM erythromycin B in 50 mM phosphate buffered $D_2O$ (pH 7.4) were used to acquire spectra. $^{13}C$ one dimensional spectra were not successful using saturated solutions of the drug (~ 12-15 mM). Spectra were acquired at ambient temperature (20 ± 2 °C) or at 30 °C.

The one-dimension $^{1}H$ spectrum was acquired using 128 scans over 32768 data points, and was processed using a Gaussian window function with negative linebroadening.

The DQF-COSY spectrum was acquired using a data matrix of 4096 x 512 complex points. The spectrum was processed using a Gaussian window function and zero-filling in $F_1$. The tnTOCSY spectrum was acquired using a 4096 x 1028 data matrix and a mixing time of 60 ms and processed using a Gaussian window function. Both the HMBC and HMQC spectra were acquired using pulsed-field gradients. Spectral widths of 18 500 Hz in $F_1$ were used in both spectra in order to allow folding back of upfield carbon signals in the gHMBC spectrum. The spectra were accumulated over 4096 x 512 data points, and were processed using a pseudo-echo function[6] with zero filling in $F_1$. The gHMQC spectrum was processed using either a pseudo-echo function to optimize line shape or a Gaussian function to optimize S/N.

### Assignments of the $^{1}H$ and $^{13}C$ NMR spectra of erythromycin B in $CDCl_3$ solution

Solutions of 60 mg $ml^{-1}$ erythromycin B in $CDCl_3$ were used throughout the experiment and spectra run as described above.

The ROESY (mixing time 250 ms), TOCSY (mixing time 60 ms) and DQF-COSY spectra were acquired over data matrices of 2048 x 1024 complex points and processed using Gaussian window functions.

[6] A. Bax, R. Freeman, G. A. Morris. A simple method for suppressing dispersion-mode contributions in NMR spectra - the pseudo echo. *J. Magn, Reson*. **1981**, *43*, 333-338.