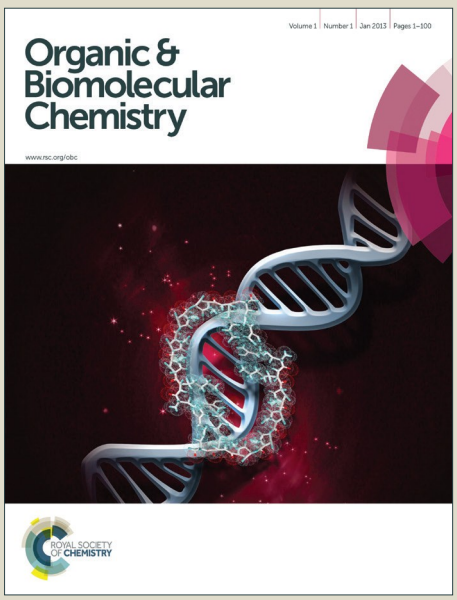
Organic & Biomolecular Chemistry

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

Organic & Biomolecular Chemistry

ARTICLE

Enhancement of the properties of a drug by mono-deuteriation: reduction of acid-catalysed formation of a gut-motilide enol ether from 8-*deuterio*-erythromycin B

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Pranab K. Bhadra[a], Abdolreza Hassanzadeh[a†], Biljana Arsic[a*], David G. Allison[a], Gareth A. Morris[b] and Jill Barber[a]

Erythromycin B is structurally very similar to erythromycin A, and also shares its clinically important antibacterial activity. Its potential advantage is that it is much more stable to acid. Both compounds are susceptible to 6-9-enol ether formation, involving loss of a proton from C-8. The enol ethers lack antibacterial activity and can give rise to unpleasant gut-motilide side-effects. Our previous work on degradation kinetics revealed that the formation of erythromycin B enol ether from erythromycin B is subject to a large deuterium isotope effect. We therefore synthesized 8-*d*-erythromycin B (in 87 % yield) in the hope that acid-catalysed enol ether formation would be reduced, relative to erythromycin B. In a range of microbiological and biochemical assays, deuteriation did not appear to compromise the efficacy of the drug. Degradation studies show, however, that incorporation of deuterium into erythromycin B reduces (though does not completely suppress) enol ether formation, opening the possibility of using a facile mono-deuteriation to reduce the gut motilide side-effects of the drug.

Introduction

Macrolide antibiotics represent a large class of therapeutically useful drugs that have been extensively used in the treatment of bacterial infections for more than six decades. Erythromycin A (**1**), the prototype drug[1] of the macrolide class, was discovered in 1952.[2] Although safe and effective, erythromycin is far from a perfect antibiotic.[3] Soon after its introduction, a number of formulations, chemically modified structures and acid stable salts derived from the parent drug erythromycin were developed, aiming to improve its activity against Gram-positive and Gram-negative organisms, reduce its associated gastrointestinal disturbances, optimize its pharmacokinetic features, and mask or alleviate the vile taste of the parent drug. [4-10]

One of the main drawbacks of erythromycin A (**1**) in clinical use is its poor pharmacokinetic profile, due to its acid instability. Under low pH conditions, resembling the gastric environment, the formation of erythromycin A 6,9-enol ether (**2**) and 6,9:9,12-spiroketal anhydroerythromycin A (**3**) occurs; these cyclized structures are much less active than erythromycin itself.[11,12] The former has been reported both to have 10 times greater gut motilide activity than the parent drug,[13,14] and possibly to promote bacterial resistance to erythromycin.[15] It is notable that the associated gastrointestinal disturbances are not dependent on the route of administration; they are dose-related and may be more common in children than in adults.[16] We would anticipate that erythromycin-based antibiotics with much reduced gut motilide activity would potentially be of considerable clinical interest.

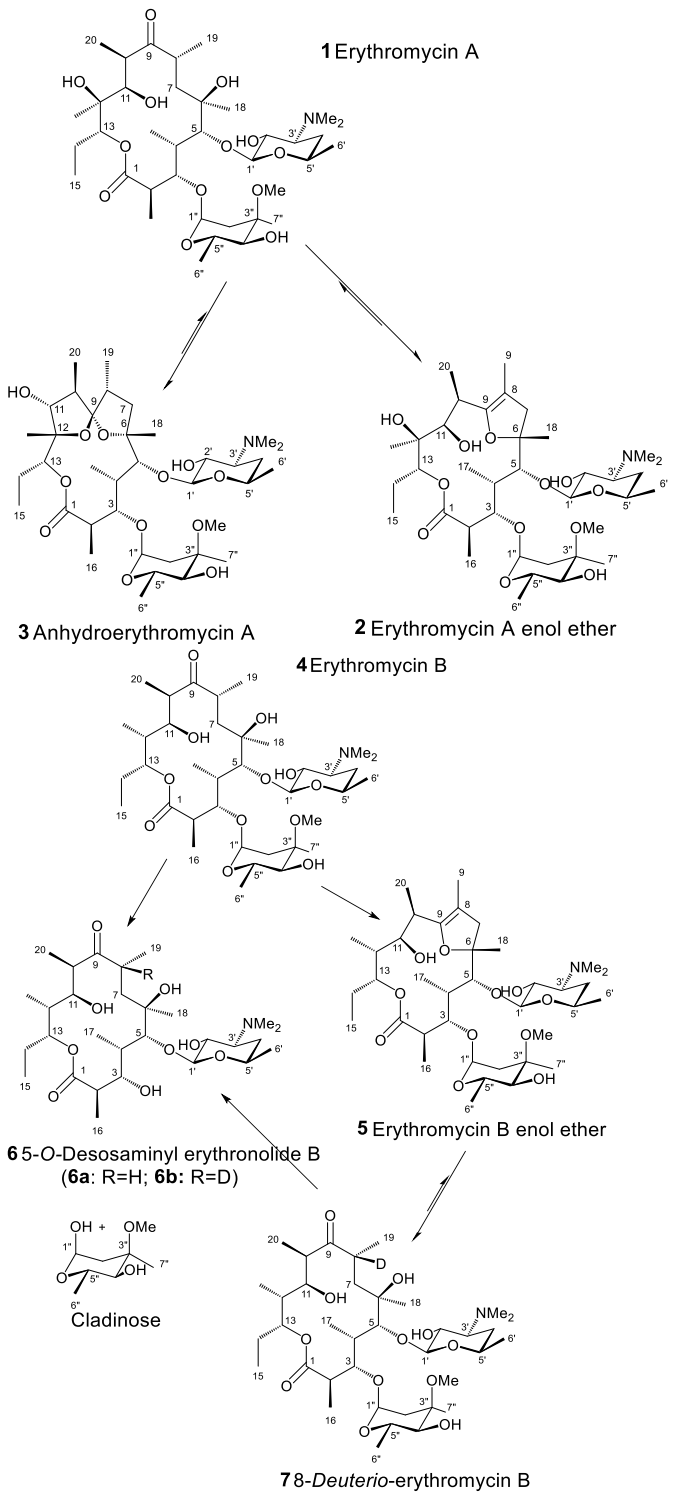
In previous reports[17-19] we have pointed to several advantages of erythromycin B (**4**) over erythromycin A (**1**). Erythromycin B is much more stable to acid than the clinical antibiotic erythromycin A; in this it resembles the more modern macrolides clarithromycin and azithromycin.[18] We have shown that it is possible to derivatize erythromycin B to form taste-free, insoluble pro-pro-drugs, suitable for testing as pediatric medicines.[19] The antibacterial properties of erythromycin B are similar to those of erythromycin A,[11, 20] and preliminary studies in rats suggest that its pharmacokinetic properties are superior[17].

[a] *Manchester Pharmacy School, University of Manchester, Oxford Road, Manchester, M13 9PT, UK, E-mail: ba432@ymail.com*
[b] *School of Chemistry, University of Manchester, Oxford Road, Manchester, M13 9PL, UK*
† Present address: Pharmaceutics Research Center, School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

In our study of the acid-catalysed degradation of erythromycin B and clarithromycin,[18] we observed an equilibration of erythromycin B (**4**) with its enol ether (**5**) in acidic aqueous solution. This equilibration was subject to a kinetic isotope effect of about 5 when deuterium was incorporated at position 8 of erythromycin B. Thus, after incorporation of deuterium into position 8 of erythromycin B, enol ether formation was effectively suppressed (Scheme 1). In view of the association of enol ethers with unpleasant gut-motilide activity, this result suggested that both 8-*d*-erythromycin B and 8-*d*-erythromycin A might be worthwhile therapeutic candidates.

Others have noted the incorporation of deuterium into position 8 of macrolides on treatment with acidic D_2O. Most importantly, Pariza and Freiberg[21] observed deuteriation in their 1994 work on erythromycin A degradation. They did not, however, report the isolation of 8-*d*-erythromycin A; rather, they isolated the degradation product, 8-*d*-anhydroerythromycin A, a compound of no therapeutic interest.

We decided therefore to attempt the preparation and evaluation of both 8-*deuterio*-erythromycin A and 8-*deuterio*-erythromycin B (**7**), recognizing that the facile acid-catalysed conversion of erythromycin A to anhydroerythromycin might preclude the isolation of realistic amounts of the 8-*deuterio*-derivative of **1**.



Scheme 1 Acid degradation pathways for erythromycins A and B in acidic aqueous solution. Deuterium becomes incorporated at C8 in the case of erythromycin B, but is not detected in the case of erythromycin A.

Results

Synthesis of 8-*d*-erythromycin B

The synthesis of 8-*d*-erythromycin B (**7**) was carried out in 2 steps (overall yield 87%) as shown in Scheme 1. Erythromycin B enol ether (**5**) was prepared from **4**, as described previously, [19] and then treated with acidic D_2O at room temperature. The apparent pH was optimized at 2.5; above this pH the reaction is slow, at lower pH there is significant loss of the cladinose sugar.[19]

It is in principle possible that a mixture of 8-*d*-erythromycin B (which has *R*-stereochemistry at C-8) and its 8-*S*-epimer could be obtained from the ring-opening reaction. Monte Carlo searches in Macromodel[22] using the MM2 force field and water solvation suggested that the two epimers were thermodynamically very similar (with global minima of -185.06 kJ mol^{-1} for erythromycin B and of -185.30 kJ mol^{-1} for 8-*epi*-(8*S*)-erythromycin B).

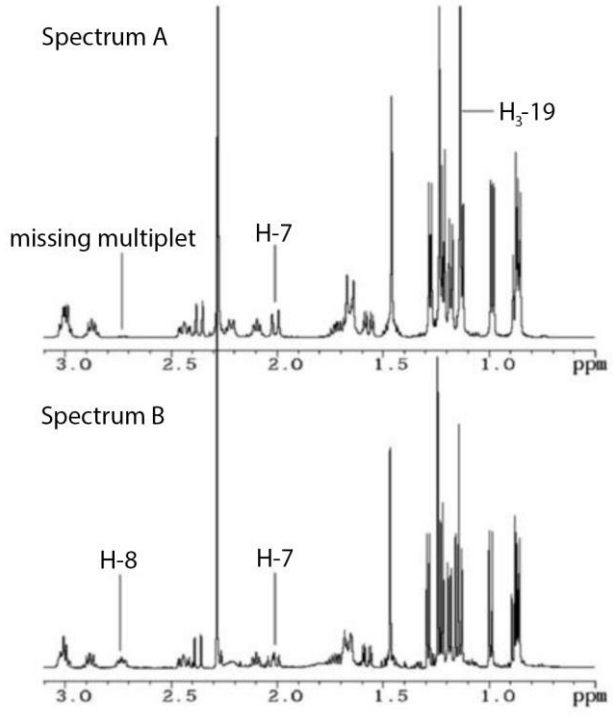


Fig. 1 500 MHz 1D ^1H spectra of erythromycin B (spectrum B) and 8-*d*-erythromycin B (spectrum A), showing the absence of H8 and a singlet due to H_3-19 in 8-*d*-erythromycin B.

Fig. 1 shows a comparison between the 1D-^1H spectra of erythromycin B (spectrum B) and 8-*d*-erythromycin B (spectrum A). In spectrum A, the signal at δ 2.74 (due to H-8) is absent and both the CH_3-19 doublet at δ 1.15 and the H-7 double doublet at δ 2.02 have collapsed, to a broad singlet and a doublet respectively, indicating successful and almost complete incorporation of deuterium at C-8.

Attempted synthesis of 8-*d*-erythromycin A

Erythromycin A is well known to equilibrate under aqueous acidic conditions with erythromycin A enol ether and, at the same time, to degrade to anhydroerythromycin A (Scheme 1).[18,23,24] The work of Vinckier *et al.* (1989)[25] suggests that there may be a window of opportunity at relatively high pH (pH 3.86) and low temperature (22 °C) to drive the ketone-enol ether equilibrium towards the ketone form. We therefore attempted to incorporate deuterium into C-8 of erythromycin A by treating erythromycin A enol ether (**2**) with relatively weakly acidic D_2O (pH 3-6) at room temperature. Erythromycin A enol ether 2'-ethyl succinate was also treated in the same way. In every experiment the principal product was the corresponding anhydride, and the ring-opened erythromycin could not be isolated. Although erythromycin A 2'-ethyl succinate is used as a pediatric pro-drug and is expected to be more acid-stable than **1**, our results[23] indicate that both have very similar lability to acid in solution and that any stabilization of erythromycin A 2'-ethyl succinate in pediatric suspension is a consequence of its relatively poor solubility.

Antibacterial activity of 8-*d*-erythromycin B

Deuteriation is a very conservative modification; nevertheless, it was important to confirm that the predicted significant improvement in metabolic properties was not accompanied by a significant decline in antibacterial activity. The antibacterial effect was not expected to change. The deuteriated form was synthesized with the aim of inhibiting the decomposition of erythromycin under acid conditions. Initially four Gram positive and four Gram negative organisms were challenged with 8-*d*-erythromycin B in a simple diffusion assay. Erythromycins A (**1**) and B (**4**) were used as positive controls. The results are shown in Table 1, and Figure 1S in the Electronic Supplementary Information (ESI) shows representative plates.

Microorganism		mm Inhibited Zone		
		Erythromycin A	Erythromycin B	8-*d*-Erythromycin B
G^+	*Micrococcus luteus*	10.5	12	12
	Staphylococcus aureus	8	10	10
	Staphylococcus epidermidis	7.5	8.0	9.0
	Bacillus cereus	6.5	6.0	6.0
G^-	*Klebsiella pneumoniae*	no inhibition	no inhibition	no inhibition
	Pseudomonas aeruginosa	no inhibition	no inhibition	no inhibition
	Citrobacter mulliniae	no inhibition	no inhibition	no inhibition
	Escherichia coli	no inhibition	no inhibition	no inhibition

Table 1 The susceptibility of various Gram +ve and Gram -ve organisms to erythromycins A, B and 8-*d*-erythromycin B in the well diffusion assay (single preliminary experiment)

The agar diffusion assay demonstrates, as expected, that Gram positive organisms, but not Gram negatives, are sensitive to all the erythromycins.

The well diffusion method tests of inhibition of bacterial growth were now followed by a determination of the

minimum bactericidal concentrations (MBCs) of **1**, **4** and **7** against four Gram positive organisms, using a serial dilution method adapted from published works.[26,27] These results are shown in Table 2.

Microorganism	MBC (μg ml^{-1})		
	Erythromycin A	Erythromycin B	8-*d*-Erythromycin B
Micrococcus luteus	1.4-2.8	2.8-5.7	2.8-5.7
Staphylococcus aureus	11.4-22.7	22.7-45.4	22.7-45.4
Staphylococcus epidermidis	2.8-5.7	1.4-2.8	2.8-5.7
Bacillus cereus	2.8-5.7	2.8-5.7	1.4-2.8

Table 2 Comparative MBCs of Erythromycin B, 8-*d*-erythromycin B and reference compound erythromycin A against four Gram-positive microorganisms

Tables 1 and 2 indicate that **1**, **4** and **7** show very similar *in vitro* antibacterial activity against a range of representative Gram positive bacteria. Indeed none of the small differences observed is statistically significant using the (duplicate) protocol employed here.

Transferred NOE experiments

Active macrolide antibiotics have been shown to take part in a weak interaction with bacterial ribosomes, which can be detected by NMR spectroscopy.[28,29] This interaction is additional to the strong interaction that leads directly to inhibition of ribosome function, but there is, nevertheless, considerable evidence that it is specific. The weak interaction may be detected by line-broadening measurements or, more informatively, by measuring transferred nuclear Overhauser effects (TRNOEs). The transferred NOE experiment provides a valuable tool for confirming receptor targets implicated in a biological assay for activity. However, its limitation is that it only works well for dissociation constants between 1 mM and 10 nM.[29]

The Transferred Nuclear Overhauser Effect Spectroscopy (TRNOESY) experiment was applied to **4** and **7** bound to bacterial ribosomes, in order to compare the bound conformations of 8-*d*-erythromycin B and erythromycin B. Fully deuteriated ribosomes were used to minimize spin diffusion. The protocol was tailored so as to minimize the contributions to the NOEs from weak non-specific binding of the ligand (drug); the drug (2.26 mM) was titrated with ribosomes until the line width of the methyl triplet signal at δ 0.87 doubled. At this point the ligand to ribosome concentration ratio was 3000:1. The experiment was performed at 500 MHz with the mixing time optimized at 100 ms.

Figure 2 shows representative regions of the two TRNOESY spectra. Qualitatively, the two spectra are almost identical, and very similar to those obtained for clarithromycin and azithromycin.[28] Strong 11-4 and 5-18 cross-peaks in both **4** and **7** (as well as 8-18 in **7**) indicate that both compounds predominantly adopt folded-out[30] conformations when bound to deuteriated ribosomes. The detailed conformational analysis of erythromycin B bound to bacterial ribosomes is described elsewhere.[31]

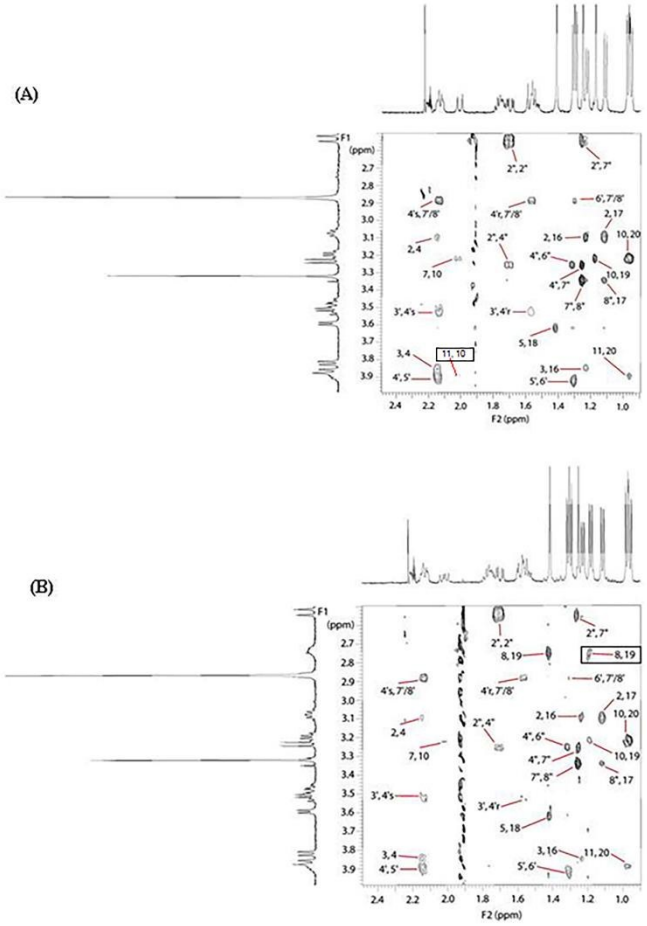


Fig. 2 500 MHz TRNOESY fingerprint region spectra of 8-*d*-erythromycin B (A) and erythromycin B (B) bound to the deuteriated *E. coli* ribosome in buffered D_2O at apparent pH 7.25. There are two small differences between the spectra. The 8,19-crosspeak in erythromycin B (spectrum B) is absent from the spectrum of 8-*d*-erythromycin B (spectrum A); and the loss of multiplicity in H10 results in the 10,11-crosspeak becoming visible in spectrum A, whereas in spectrum B it falls below the limit of detection.

Stability of 8-*d*-erythromycin B in acid

Having demonstrated that deuteriation does not adversely affect either antibacterial activity or ribosome binding, we now turned to the central hypothesis, that deuteriation of erythromycin B at C-8 would reduce the formation of erythromycin B enol ether (**5**) in aqueous, especially acidic, solution. Accordingly, a 4 mM sample of each compound was prepared in Britton-Robinson buffer (90 % H_2O-10 % D_2O, apparent pH 2.0). The WATERGATE pulse sequence[32] was used to suppress the strong H_2O signal and a sequential array of 1D-^{1}H NMR spectra was acquired. Figure 2S (A) in the ESI shows this array of spectra for erythromycin B (**4**). Three simultaneous results were expected: a decrease in the concentration of erythromycin B; accumulation of 5-*O*-desosaminyl erythronolide B (**6a**) through the loss of cladinose sugar; and the formation of erythromycin B enol ether (**5**). The 8"-OCH_3 region of the spectrum was well resolved and was used to monitor changes in the sample. The peak intensities measured for each signal were normalised with respect to TSP (the reference standard) to eliminate residual shimming

errors. The intensities were then plotted against time in order to determine the half-life and the rate constant (Figure 3A). It could be seen that the degradation of erythromycin B followed complex kinetics. This was attributed to the formation of erythromycin B enol ether (**5**) as an intermediate. These results are consistent with Mordi *et al.*, 2000.[18] The end products were confirmed by ESI-MS. Three distinct peaks were observed in the mass spectrum: 718 $[M+H]^+$ (52 %) representing **4**; 560 $[M+H]^+$ (100 %) representing **6a** and 177 $[M+H]^+$ (46 %) representing the cladinose sugar.

Figure 2S (B) illustrates the acid-catalysed degradation of 8-*d*-erythromycin B (**7**), showing the OCH_3-8" region. The array of acquisitions indicates degradation of 8-*d*-erythromycin B *via* loss of cladinose sugar.

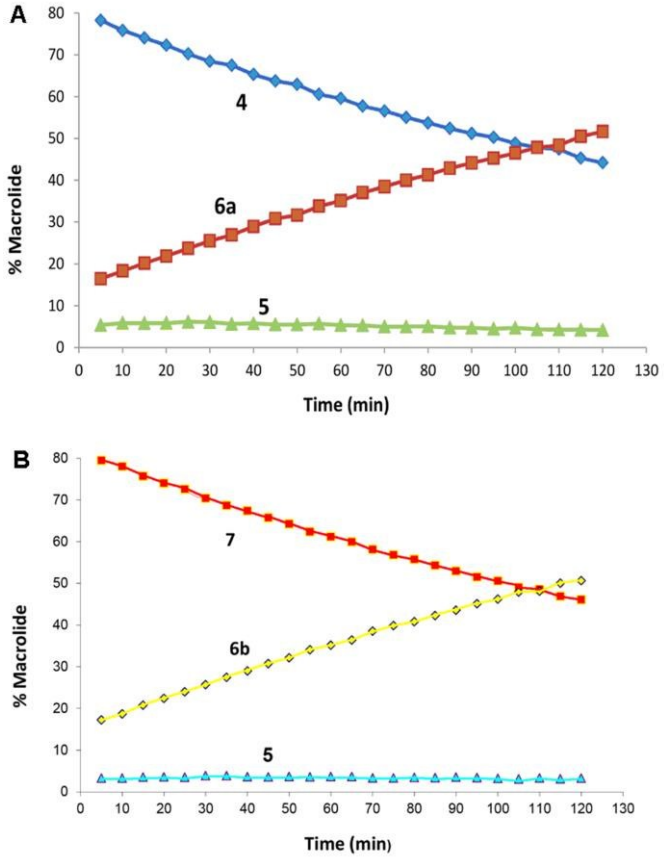


Fig. 3 Kinetic data for acid-catalysed degradation of (A) erythromycin B (**4**) and (B) 8-*d*-erythromycin B (**7**) in Britton-Robinson buffer (apparent pH 2.0; 37 °C) obtained by fitting the peak intensities (normalised with respect to the TSP signal).

In order to determine the half-life for the degradation of 8-*d*-erythromycin B (**7**) the peak intensities of the relevant signals (normalised with respect to TSP) were plotted against time (Figure 3B). The peak intensities were also utilized to calculate the proportion of enol ether formed in each case (Table 3).

Compound	Proportion of enol ether (%)		$t_{1/2}$ (min)	k (min^{-1})
	After 2 h	At the end of the experiment		
Erythromycin B	4.2	5.2	105	6.6×10^{-3}
8-*d*-erythromycin B	2.6	3.5	109	6.3×10^{-3}

Table 3 Proportion of enol ether (%) during the degradation of erythromycin B and 8-*d*-erythromycin B in aqueous acid at pH 2, and kinetic data obtained from fitting the peak intensities (normalised with respect to the TSP signal)

The enol ether formation in the latter was nearly halved compared to that in the case of erythromycin B. Halving of the enol ether formation might have a pronounced effect in reducing the gut motilide activity. With careful formulation, the compound has a lot of potential for improvement.

The results of the kinetic analysis indicated that erythromycin B and 8-*d*-erythromycin B degrade at the same rate in buffer, as expected. They both degrade *via* loss of cladinose sugar, which is consistent with the findings of Mordi *et al.*, 2000.[18] The results are summarised in Table 3.

Discussion

8-*d*-Erythromycin B has been successfully synthesized from erythromycin B in 87 % yield with no detectable racemization. Unsurprisingly, this compound behaved very similarly to erythromycin B in a range of microbiological and biochemical assays. Deuteriation does not appear to compromise the efficacy of the drug.

The objective in preparing this compound was to evaluate whether the formation of enol ether derivatives could be suppressed by deuteriation; we have shown that, indeed, the isotope effect is sufficient to reduce the accumulation of the potent gut motilide enol ether by about 50 %. Our previous work (Mordi *et al.*[18]) showed a much more dramatic suppression of enol ether formation in D_2O-based media, where enol ether concentration in acid peaked at 15 % but was almost completely suppressed by deuteriation at C-8. Clearly, solvent isotope effects contributed to that result.

The current experiments suggest that 8-*d*-erythromycin B might *in vivo* be a better drug than erythromycin B itself, especially for long-term use. Enol ether formation is suppressed, but not completely. We do not know the extent to which the gut motilide activity of the erythromycins is actually due to equilibration with their corresponding enol ethers, although pure enol ethers are reported to be 10 times more potent than erythromycins. Since the preparation of 8-*d*-erythromycin B is very straightforward, even benefits to small numbers of patients would make the drug worthwhile.

We were unable to adapt our synthesis of 8-*d*-erythromycin B to 8-*d*-erythromycin A. Our analysis of the kinetics of erythromycin A degradation[23] suggests that this is not surprising. The same analysis also shows that the benefits of deuteriating erythromycin A would be less than those of deuteriating erythromycin B. The isotope effect operating on enol ether formation in D_2O solution is only 2 for erythromycin A, against 5 for erythromycin B. Paradoxically, however, the benefits of suppressing enol ether formation from

erythromycin A would far exceed those from suppressing the corresponding erythromycin B reaction. When erythromycin B equilibrates with its enol ether in aqueous acid, the equilibrium lies on the side of the erythromycin; unfortunately, when the clinical antibiotic erythromycin A equilibrates, the equilibrium lies on the side of the enol ether.

8-*(S)*-Fluoroerythromycin, which was prepared biosynthetically, has enjoyed some success;[33] it was found to have better bioavailability and longer half-life, and to reach higher tissue concentrations, than erythromycin.[34,35] It is possible that a similar approach to the production of 8-*d*-erythromycin A would be successful.

In conclusion, the work described here demonstrates another advantage of erythromycin B over erythromycin A, and provides further impetus for a pre-clinical investigation of erythromycin B. Erythromycin B can be successfully deuteriated to give 8-*d*-erythromycin B, and this compound may have reduced side effects compared with erythromycin B itself.

Experimental Section

General procedures

All chemicals were purchased from Sigma Aldrich unless otherwise stated. The biological materials were purchased from Oxoid. Erythromycin B was a gift from Abbott Laboratories. ^{1}H NMR spectra were acquired using a Varian Unity 500 MHz Spectrometer operating at 500 MHz or Varian Inova 400 spectrometer operating at 400 MHz or Bruker AVANCE 300 spectrometer operating at 300 MHz. WILMAD 507-PP NMR tubes were used. Electrospray-ionization mass spectra (ESI-MS) were acquired on a Micromass Platform mass spectrometer and the data were analyzed using the program PLATFORM with the Masslynx data system. 10 µl of the sample were injected using a Hewlett Packard auto-sampler and the machine was operated at a cone value of 30 eV, at 80 °C. For identification purposes, all samples (0.2 mg) were prepared in acetonitrile (1 ml). For ultracentrifugation, a Beckman Coulter Ultracentrifuge (Optima MAX) using a MLA-80 fixed angle rotor equipped with Beckman 10 ml polycarbonate centrifuge tubes was used. A Beckman Coulter High-Speed Centrifuge (Avanti J-25I) using a JA 30.50 Ti fixed angle rotor supplied with 50 ml polyallomer centrifuge bottles was used for regular centrifugation. A MSE UK Micro Centrifuge was used on a small scale. For dialysis, regenerated cellulose membrane tubing with a molecular weight cutoff of 3500 Da (SPECTRA/POR) was used. The UV absorbance of isolated ribosomes was measured on a UVIKON 930 spectrometer (Kontron Instruments, UK). For microbiological work, a Unicam UV-visible spectrometer (Helios Delta, UK) was used to obtain the OD_{600} of the bacterial growth broth media.

Chemical Syntheses

Synthesis of Erythromycin B Enol Ether (5)

Erythromycin B enol ether was prepared as described in our previous work.[19]

Synthesis of 8-*d*-Erythromycin B (7)

Compound **5** (1.5 g, 2.14 mmol) was dissolved in 35 ml deuteriated Britton-Robinson buffer (0.04 M d_3-boric acid, 0.04 M orthophosphoric acid and 0.04 M d_3-acetic acid in D_2O adjusted to apparent pH 2.53 using 5 % NaOD) and was stirred for 2-2.5 h. 750 µl aliquots were withdrawn at regular intervals (every 15 min) and ^{1}H NMR spectra were acquired to monitor the progress of the reaction. The reaction was quenched by increasing the pH to 8.5 using 5 % NaOD solution. The solids were extracted with 3 x 25 ml dichloromethane and the combined organic layers were washed with brine, dried over anhydrous sodium sulphate (Na_2SO_4), and reduced to dryness *in vacuo*. The final product (**7**) was recrystallized from acetone. Yield 89.4%, mp 198-200 °C (from acetone). Anal. Calcd. for $C_{37}H_{66}DNO_{12}$: C, 61.79; H, 9.26; N, 1.94. Found: C, 61.75; H, 9.45; N 1.89, m/z (Electrospray) 719 $[M+H]^+$, Rf 0.32 (EtOAc: CH_3OH: NH_3 25%, 85:10:5). 1H NMR ($CDCl_3$, 500 MHz): δ 0.86 (CH_3-21, d, J = 7.1 Hz), δ 0.87 (CH_3-15, t, J = 7.3 Hz), δ 0.98 (CH_3-20, d, J = 6.8 Hz), δ 1.12 (CH_3-17, d, J = 7.3 Hz), δ 1.13 (CH_3-19, s), δ 1.18 (CH_3-16 , d, J = 7.3 Hz), δ 1.21 (CH_3-6', d, J = 6.0 Hz), δ 1.23 (CH_3-7", s), δ 1.27 (CH_3-6", d, J = 6.2 Hz), δ 1.56 (CH-7, dd, J = 15.2, 2.8 Hz), δ 2.0 (CH-7, dd, J = 15.0, 10.2 Hz), δ 2.28 ($N(CH_3)_2$-7',8', s), δ 2.89 (CH-2, dq, J = 9.1, 7.1 Hz), δ 3.0 (CH-10, dq , J = 6.8, 1.5 Hz), δ 3.21 (CH-2', dd, J = 10.3, 7.3 Hz), δ 3.31 (OCH_3-8", s), δ 3.49 (CH-5', m), δ 3.58 (CH-5, d, J = 7.5 Hz), δ 3.79 (CH-11, d, J = 10.0 Hz), δ 4.0 (CH-5", dq, J = 9.4, 6.0 Hz), δ 4.06 (CH-3, dd, J = 9.0, 1.5 Hz), δ 4.42 (CH-1', d, J = 7.3 Hz), δ 4.88 (CH-1", d, J = 4.7 Hz), δ 5.33 (CH-13, dd, J = 9.4, 4.4 Hz).

Synthesis of Erythromycin A Enol Ether (2)

Compound **2** was prepared from erythromycin A by adaptation of the literature method.[36] Yield 80 %, mp 133-138 °C (lit. 135-140 °C).

Attempted Synthesis of 8-*d*-Erythromycin A

A solution of **2** (2 g, 2.79 mmol) in 100 ml deuteriated phosphate buffer (apparent pH 6.02) was stirred at room temperature and the progress of the reaction was monitored by ^{1}H NMR spectroscopy. 750 µl aliquots were withdrawn at regular intervals (every 15 min) and ^{1}H NMR spectra were acquired. As no apparent change was observed in the spectrum after the reaction had been left overnight, the pH was reduced to 3.05 until a noticeable change was observed in the NMR spectrum. After 5.5 h, 80 % of the starting material had disappeared. The reaction was continued until no starting compound was seen and then quenched using 5 % NaOD. The solids were extracted using 3 x 25 ml dichloromethane; the organic layers were combined, washed with brine and finally dried over anhydrous Na_2SO_4. The organic layer was reduced to dryness *in vacuo*. The product was a mixture of several components and resisted all attempts at recrystallization. The major species in the crude product was found to be **3** (anhydroerythromycin A).

Acid-catalysed degradation of erythromycin B (4), 8-*d*-erythromycin B (7)

A 4 mM sample of **4**, **7** was prepared in protiated Britton-Robinson buffer, pH 2.0, containing 10 % D_2O. The time course consisted of an array of 24 1D ^{1}H spectra acquired on a Varian

Inova 400 MHz spectrometer at 37 °C, using the WATERGATE pulse sequence for suppression of the water signal. Each experiment consisted of 24 spectra of 64 transients and each spectrum took 5 min to acquire. The spectra were processed with reference deconvolution using the FIDDLE (free induction decay deconvolution for lineshape enhancement) algorithm[37] for line shape correction with a Gaussian time constant of 0.15 s, using the TSP signal as the reference, followed by cubic spline baseline correction.

Isolation of deuteriated ribosomes

The protocol was adapted from a literature source[38] and was performed in a cold room or on an ice box. 10.45 g of deuteriated cells from *E. coli* MRE600, as received from Dr. Kalju Vanatalu, were thawed and suspended in 21 ml deuteriated Tico association buffer[39] (20 mM HEPES-KOH adjusted to pH 7.6 at 0 °C, 6 mM magnesium acetate, 30 mM ammonium acetate, and 4 mM β-mercaptoethanol in D_2O base). The mixture was centrifuged at 12,096 g (10,000 rpm), 4 °C, for 10 min in an Avanti J-25I Centrifuge using a JA 30.50 Ti rotor. The pellet (8.85 g) was mixed with twice its weight of Alcoa A-305 (17.7 g), the cells were ground for 2 min in a mortar, and the cell paste homogenized with 1.5 ml deuteriated Tico association buffer. To remove Alcoa, the cell paste was subjected to low-speed centrifugation at 12,096 g, 4 °C, for 20 min. The supernatant was further centrifuged at 14,516 g (12,000 rpm), 4 °C, for 8 min and finally at 13,000 rpm, 4 °C, for 6 min using a Micro Centrifuge, to remove cell debris and the remaining Alcoa. The resulting "S-30 supernatant"[38] was centrifuged at 31,990 g (25,000 rpm), 4 °C, for 22 h using an Optima MAX Ultracentrifuge with MLA-80 rotor. The ribosomal pellet was carefully separated from the supernatant and dissolved in 150 µl deuteriated Tico dissociation buffer (20 mM HEPES-KOH adjusted to pH 7.6 at 0 °C, 1 mM magnesium acetate, 300 mM ammonium acetate, and 4 mM β-mercaptoethanol in D_2O). The solution of ribosomal particles was dialyzed against deuteriated Tico association buffer, using dialysis membrane tubing MWCO 3500 (2 x 30 min). The ribosomes were then pelleted at 13,000 rpm, 4 °C, for 6 min using a Micro Centrifuge. The isolated ribosomes were aliquoted and snap frozen in liquid nitrogen and stored at -80 °C. The concentration of the ribosomes was measured using the following literature relationship [38]

1 A_{260} unit = 24 pmol ml^{-1} of 70S ribosomes

1240 pmol ribosomes were isolated in a volume of 128.5 µl buffer.

TRNOESY measurements

2.26 mM solutions of **4** and **7** in 50 mM deuteriated potassium phosphate buffer (apparent pH 7.0), containing 6 mM $MgCl_2$ and 30 mM NH_4Cl to facilitate ribosome binding, were titrated with deuteriated ribosomes at 25 °C until in the corresponding 1D ^1H spectra the linewidth of the methyl triplet signal at δ 0.87 doubled. At this point the concentrations of the drug and ribosome were found to be 2 mM and 0.67 µM, respectively. The TRNOESY experiment was carried out at 500 MHz with a mixing time optimized at 100 ms.

Antimicrobial evaluation study

Bacterial strains and growth conditions

The Gram-positive organisms *Staphylococcus aureus* NCTC 6528, *Staphylococcus epidermidis*, *Bacillus cereus* and *Micrococcus luteus*, and the Gram-negative bacteria *Pseudomonas aeruginosa* PA01, *Citrobacter murliniae*, *Escherichia coli* AG 100, and *Klebsiella pneumoniae* NCTC 4352 were obtained from the in-house culture collection of the Biofilm Research Group, Manchester Pharmacy School. Unless otherwise stated, all cultures were grown in LB broth (10 g L^{-1} tryptone, 10 g L^{-1} yeast extract and 10 g L^{-1} sodium chloride, pH 7.4 at 30 °C, and maintained on LB agar (LB broth plus 1 % bacteriological agar) at 4 °C.

Antibacterial samples

All antibacterial stock solutions were prepared on the same day to avoid any loss of potency. For the antibacterial sensitivity test, the stock solutions were prepared to a concentration of 50 µg ml^{-1} by dissolving 0.005 g of **1**, **4** and **7** in 5 ml of ethanol (99 %) followed by the addition of distilled water to make the volume up to 100 ml. As a negative control, a solution of 5% ethanol (99 %) was used. To determine the MBCs, the stock solutions were prepared to a concentration of 200 µg ml^{-1} by dissolving 0.2 g of **1**, **4** and **7** in 1 ml acetone followed by addition of distilled water to make the volume up to 100 ml. A solution of 1 % acetone was used as a negative control. The stock solutions were filter sterilized using 0.2 µm Millipore filters and were dispensed into sterile Eppendorf tubes and stored at –20 °C. Frozen samples were thawed as required on the day of experiment and any unused drug was discarded.

Well diffusion assay

The well diffusion assay described by Alcid and Seligman (1973)[40] was used for the *in vitro* test of bacterial sensitivity to **1**, **4** and **7**. 20 ml fresh LB broth was inoculated with 500 µl of an overnight culture of the test microorganisms at 30 °C. When the OD_{600} of the bacterial suspensions reached 0.5, 100 µl of each suspension was transferred to LB agar and the entire surface of the plate covered with the inoculum using a sterile spreader. The plates were then left for 5 min to dry. Four 5 mm wells were aseptically cut into in each plate; three of those were filled with 25 µl of the antibacterial samples of **1**, **4** and **7** and the fourth well was filled with 25 µl 5 % ethanol (99 %) as a negative control. The plates were incubated at 30 °C overnight (18 h), then the zone of inhibition was measured (mm) from the edge of the well to the edge of the zone of clearing.

MBC determination assay

A broth macrodilution procedure, adapted from Stratton and Cooksey (1991),[41] was carried out to measure the Minimum Bactericidal Concentrations of **1**, **4** and **7** against the four sensitive Gram-positive microorganisms. Firstly, 500 µl of an overnight culture of each of the test microorganisms was inoculated at 30 °C into 20 ml fresh LB broth. The first tube of the dilution series was prepared by diluting 500 µl of a 200 µg ml^{-1} solution of the test antibiotic with 500 µl fresh double strength LB broth, to make the final concentration of antibiotic in the first dilution tube of 100 µg ml^{-1}. This was then repeated with the next tube to produce ten two-fold serial dilutions of

the test antibiotics, the most dilute having an antibiotic concentration of 0.16 μg ml^{-1}. Each tube was then inoculated with 50 μl bacterial suspension from a mid-log-phase culture, (OD_{600} = 0.5 unit). An aqueous solution of 1 % acetone was used as a negative control. After overnight incubation at 30 °C, the tubes were agitated and about 8 μl from each tube was streaked onto antibiotic-free LB agar plates. The plates were incubated at 30 °C overnight, then, the MBC endpoint calculation was performed using the method described by Pearson et al. (1980).[27]

Conclusion

An efficient synthesis of 8-*d*-erythromycin B was achieved, and its antibacterial activity and ribosome binding activity were shown to be comparable with the parent erythromycin B. NMR time courses showed that, under conditions of acid catalysis, formation of erythromycin B enol ether was slower for 8-*d*-erythromycin B than for erythromycin B. This is predicted to lead to a reduction in gut motilide activity.

Acknowledgements

A.H. would like to thank the Ministry of Health and Medical Education of Iran for a fellowship. We thank Abbott Laboratories, Chicago, USA for a gift of erythromycin B.

References

1 G. Katzung Bertram, Basic & clinical pharmacology, 8th ed., New York, McGraw-Hill, 2001, 64.
2 J. M. McGuire, R. L. Bunch, R. C. Anderson, H. E. Boaz, E. H. Flynn, H. M. Powell and J. W. Smith, *Antibiot. Chemother.*, 1952, **2**, 281-283.
3 A. D. Buss and R. D. Waigh, Burger's medicinal chemistry and drug discovery, Wolff, M.E. (ed.), 5th edition, New York, John Wiley & Sons, 1995, Vol. 1, 1009-1012.
4 L. D. Sabath, V. Lorian, D. Gerstein, P. B. Loder and M. Finland, *Appl. Microb.*, 1968, **16**, 1288-1292.
5 G. Griesgraber, M. J. Kramer, R. L. Elliott, A. M. Nilius, P. J. Ewing, P. M. Raney, M.-H. Bui, R. K. Flamm, D. T. W. Chu, J. J. Plattner and Y. S. Or, *J. Med. Chem.*, 1998, **41**, 1660-1670.
6 A. A. Sinkula, *J. Pharm. Sci.*, 1974, **63**, 842-848.
7 A. Bryskier and M. T. Labro, *Presse med.*, 1994, **23**, 1762-1766.
8 D. R. P. Guay, L. E. Gustavson, K. J. Devcich, J. Zhang, G. Cao and C. A. Olson, *Clin. Ther.*, 2001, **23**, 566-577.
9 D. T. W. Chu, *Expert Opin. Investig. Drugs*, 1995, **4**, 65-94.
10 N. Bahal and M. C. Nahata, *Ann. Pharmacother.*, 1992, **26**, 46-55.
11 I. O. Kibwage, J. Hoogmartens, E. Roets, H. Vanderhaeghe, L. Verbist, M. Dubost, C. Pascal, P. Petitjean, and G. Levol, *Antimicrob. Agents. Chemother.*, 1985, **28**, 630-633.
12 H. A. Kirst, *Prog. Med.Chem.*, 1993, **30**, 57-88.
13 T. Sunazuka, K. Tsuzuki, S. Marui, H. Toyoda, S. Omura, N. Inatomi and Z. Itoh, *Chem. Pharm. Bull.*, 1989, **37**, 2701-2709.
14 K. Tsuzuki, T. Sunazuka, S. Marui, H. Toyoda, S. Omura, N. Inatomi and Z. Itoh, *Chem. Pharm. Bull.*, 1989, **37**, 2687-2700.
15 J. Majer, *Antimicrob. Agents Chemother.*, 1981, **19**, 628-633.
16 W. H. Martindale, Martindale: the extra pharmacopoeia, 33rd edition, London, Pharmaceutical Press, 2002, 201.
17 M. N. Mordi, V. Boote, R. Hodgkiss, A. Mistry, J. Barber, unpublished data.
18 M. N. Mordi, M. D. Pelta, V. Boote, G. A. Morris, J. Barber, *J. Med. Chem.*, 2000, **43**, 467-474.
19 P. K. Bhadra, G. A. Morris and J. Barber, *J. Med. Chem.*, 2005, **48**, 3878-3884.
20 W. E. Grundy, E. F. Alford, C. J. Rickher, R. R. Bower, E. J. Rdzok and J. C. Sylvester, *Antibiot. Chemother.*, 1955, **V**, 212-217.
21 R. J. Pariza and L. A. Freiberg, *Pure & Appl. Chem.*, 1994, **66** (10/11), 2365-2368.
22 M. Saunders, K. N. Houk, W. D. Wu, W. C. Still, M. Lipton, G. Chang and W. C. Guida, *J. Am. Chem. Soc.*, 1990, **112**, 1419-1427.
23 A. Hassanzadeh, P. Gorry, G. A. Morris and J. Barber, *J. Med. Chem.*, 2006, **49**, 6334-6342.
24 A. Hassanzadeh, J. Barber, G. A. Morris, P. A. Gorry, *J. Phys. Chem.*, 2007, **111**, 10098-10104.
25 C. Vinckier, R. Hauchecorne, T. Cachet, G. Van den Mooter and J. Hoogmartens, *Int. J. Pharm.*,1989, **55**, 67-76.
26 J. P. Anhalt, L. D. Sabath and A. L. Barry, In "Manual of clinical microbiology" by: E. H. Lennette, A. Balows, W. J. Hausler, Jr. and J. P. Truant, (ed.), 3rd ed. American Society for Microbiology, Wshington, D. C, 1980.
27 R. D. Pearson, R. T. Steigbigel, H. T. Davis and S. W. Chapman, *Antimicrob. Agents Chemother.*, 1980, **18**, 699-708.
28 R. J. Brennan, A. Awan, J. Barber, E. Hunt, K. L. Kennedy and S. Sadegholnejat, *J. Chem. Soc., Chem. Commun.*, 1994, **13**, 1615-17.
29 M. P. Williamson, The transferred NOE, in: Modern magnetic resonance, by: G. A. Webb (ed.), Springer, 2006.
30 A. Awan, R. J. Brennan, A. C. Regan and J. Barber, *J. Chem. Soc. Perkin Trans 2*, 2000, **8**, 1645-1652.
31 P. T. Tyson, Mode of action of macrolide antibiotics, PhD thesis, University of Manchester, 2000.
32 M. Liu, X.-A. Mao, C. Ye, H. Huang, J. K. Nicholson and J. C. Lindon, *J. Magn. Reson.*, 1998, **132**, 125-129.
33 D. Saverino, E. A. Debbia', A. Pesce, A. M. Lepore and G. C. Scfaho, *J. Antimicrob. Chemother.* 1992, **30**, 261-272.
34 G. Benoni, L. Cuzzolin, R. Leone, U. Consolo, G. Ferronato, C. Bertrand, V. Puchetti, M. E. Fracasso, *Antimicrob. Agents Chemother.*, 1988, **32**, 1875-1878.
35 M. T. Fera, M. Giannone, S. Pallio, A. Tortora, G. Blandino, M. Carbone, *Int. J. Antimicrob. Ag.*, 2001, **17**, 151-154.
36 P. Alam, P. C. Buxton, J. A. Parkinson and J. Barber, *J. Chem. Soc. Perkin Trans. 2*, 1995, 1163-1167.
37 G. A. Morris, H. Barjat and T. J. Horne, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1997, **31**, 197-257.
38 K. H. Nierhaus, Reconstitution of ribosomes, In: Ribosomes and Protein Synthesis: A Practical Approach by: G. Spedding (ed.), Oxford University Press, New York, 1990.
39 C. L. Hanson, P. Fucini, L. L. Ilag, K. H. Nierhaus and C. V. Robinson, *J. Biol. Chem.*, 2003, **278**, 1259-1267.
40 D. V. Alcid and S. J. Seligman, *Antimicrob. Agents Chemother.*, 1973, **3**, 559-561.
41 C. W. Stratton and R. C. Cooksey, Susceptibility tests, in: Manual of clinical microbiology, by: A. Balows, W. J. Hausler, Jr., K. L. Hermann, H. D. Isenberg and H. J. Shadomy (eds.), 5th ed. American Society for Microbiology, Washington, D. C., 1991.

