Determination of the stereochemistry of anhydroerythromycin A, the principal degradation product of the antibiotic erythromycin A†

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Received 4th January 2006, Accepted 25th January 2006
First published as an Advance Article on the web 15th February 2006
DOI: 10.1039/b518182h

Anhydroerythromycin A arises from the acid-catalysed degradation of erythromycin A both *in vitro* and *in vivo*. It has negligible antibacterial activity, but inhibits drug oxidation in the liver, and is responsible for unwanted drug–drug interactions. Its structure has 18 chiral centres common with erythromycin A, but C-9 (the spiro carbon) is also chiral in anhydroerythromycin and its stereochemistry has not previously been reported; both 9*R*- and 9*S*-anhydroerythromycin A are plausible structures. An understanding of the chirality at C-9 was expected to throw light on the mechanism of acid-catalysed degradation of erythromycin A, a subject that has been debated in the literature over several decades. We now report a determination of the three-dimensional structure of anhydroerythromycin A, including the stereochemistry at C-9, by NMR and molecular modelling. In parallel, the relative stereochemistry of anhydroerythromycin A 2′-acetate was determined by X-ray crystallography. Both compounds were shown to have 9*R* stereochemistry, and anhydroerythromycin A exhibited considerable conformational flexibility in solution.

Introduction

Anhydroerythromycin A (**1**) is the principal product of the acid-catalysed degradation of the antibiotic erythromycin A (**2**), both *in vitro* and in the body. Erythromycin A is sensitive to acid below pH 6.9, and despite enteric coating (for adults) or formulation as esters (for children), formation of **1**, *in vivo*, is an appreciable problem.[1,2] Compound **1** has been reported to be a potent drug oxidation inhibitor[2] through the inhibition of steroid 6 β-hydroxylase activity; this can result in overdosing of drugs such as theophylline, when they are administered concurrently with erythromycin.

The kinetics and pathway of the acid-catalysed degradation of erythromycin A have been studied extensively[3,4] and the consensus is that the drug degrades as shown in Scheme 1. Surprisingly, however, the stereochemistry of **1** at C9, though it is usually drawn as *S*, has not been determined. In fact, both 9*R*- and 9*S*-anhydroerythromycin A are plausible structures.

It is known that erythromycin A exists in aqueous solution as a mixture of the 9-ketone (**2a**) and the 9,12-hemiacetal (**2b**). Compound **2b** is a possible intermediate in the formation of **1** from **2**, as is compound **2c**, which has been observed in DMSO solution.[5] We would expect that knowledge of the chirality of C9 of **1** would throw light on the mechanism of the degradation of erythromycin A.

We were therefore interested to determine the stereochemistry of **1** at C-9.

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† Electronic supplementary information (ESI) available: complete ^{1}H and ^{13}C assignments of compounds **1** and **4**. See DOI: 10.1039/b518182h

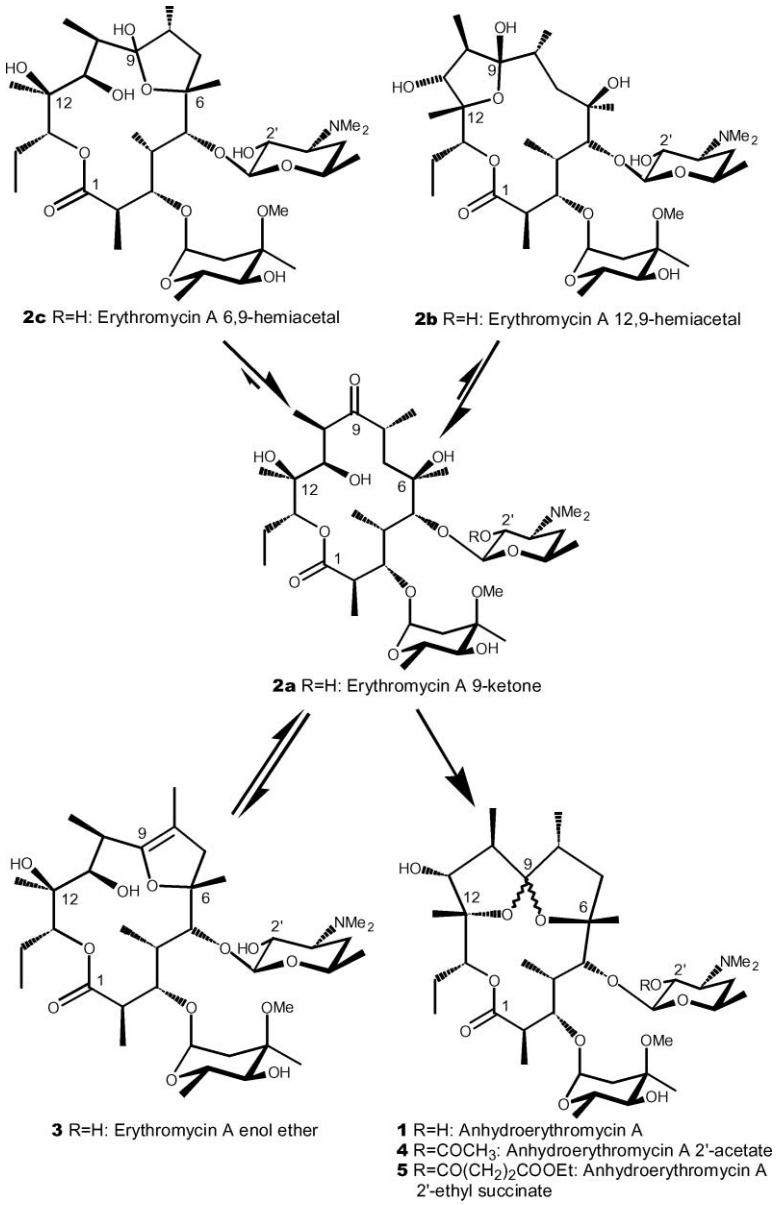
Results

The formation of **1** from **2b** or **2c** would be expected to proceed *via* the 12,9-cyclized or 6,9-cyclized oxonium ions respectively. Scheme 2 shows these two planar oxonium ions as generated by Macromodel.[6] In the virtual 6,9-cyclized oxonium ion (Scheme 2, structure **A**) the 12-**O**H lies 4.80 Å from C-9 and attack of the hydroxy group at C-9 would be from above leading to 9*S* stereochemistry. In the virtual 12,9-cyclized oxonium ion (Scheme 2, structure **B**), the 6-**O**H lies 4.44 Å from C-9 and attack of the hydroxy group at C-6 would again be from above, leading to 9*R* stereochemistry.

Anhydroerythromycin A was prepared by a published procedure[7] through the treatment of erythromycin A with aqueous hydrochloric acid, in 81% yield. Its stereochemistry was investigated by molecular modelling, NMR spectroscopy and by X-ray crystallography. Erythromycin A has 18 chiral centres, whose absolute stereochemistry is known. All our experiments were strictly determinations of relative stereochemistry; the absolute stereochemistry was deduced from the structure of erythromycin A.

Unconstrained molecular modelling

Initially, 9*R*-anhydroerythromycin A (**1*R***) and 9*S*-anhydroerythromycin A (**1*S***) were created using Macromodel version 6.5 and subjected to local minimization. The resulting structures were then submitted to unconstrained Monte Carlo search routines both *in vacuo* and in water, using the MM2 and AMBER force fields, both of which are suitable for molecules of this size. Searches were halted when a global minimum had been found at least 30 times or 10 000 steps had been completed and good convergence had been obtained. The results are shown in Table 1.



Scheme 1 Acid-catalysed degradation of erythromycin A.

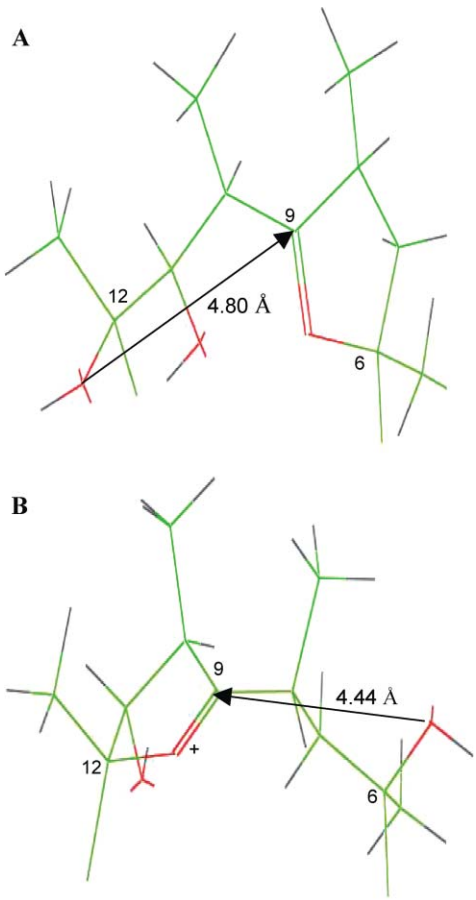
Table 1 Energies of the global minima of Monte Carlo conformational searches of 9*R*-anhydroerythromycin A (**1*R***) and 9*S*-anhydroerythromycin A (**1*S***) *in vacuo* and in water. (Note that energies in different rows are not directly comparable; only comparisons within a row are valid)

Energy of global minimum/kJ mol^{-1}	**1*S***	**1*R***
in vacuo (MM2)	−90.58	−95.67
In water (MM2)	−263.61	−285.14
In water (AMBER)	21.78	0.1

ROESY NMR analysis

Compound **1** was now dissolved in D_2O-based buffer at apparent pH 7, and a ROESY NMR spectrum acquired. The ROESY experiment is generally preferred to the NOESY experiment for erythromycin derivatives because NOEs are close to zero and may be positive or negative, whereas in the corresponding rotating frame experiment, ROEs are positive and quantifiable. The resulting spectrum is shown in the Supplementary Material, as is the Table of Connectivities derived from analysis of this spectrum. There were 128 cross-peaks in the ROESY spectrum, leading to 64 constraints. Of these, 25 were obligatory, that is they were between protons such as H2 and H16, which will always be close together, irrespective of the conformation or stereochemistry.

The NMR data were compared with the global minima obtained by modelling **1*R*** and **1*S***. Discrepancies between the NMR data and the modelled structures are shown in Table 2. It can be seen that the MM2 global minimum conformation of **1*R*** largely satisfies the ROESY data, with just six cross-peaks not indicated by this structure. For **1*S***, there are 14 signals in the ROESY spectrum that could not be accounted for. Similarly, when the AMBER force field was used, just two cross-peaks were not satisfied by **1*R***, whereas for **1*S*** there were 11 constraints not satisfied. Attempts were made to generate structures that satisfied all the constraints by carrying out restrained Monte Carlo searches. However, only



Scheme 2 Plausible intermediate oxonium ions in the acid-catalysed conversion of **2** to **1**.

very high energy structures (>150 kJ mol^{-1} in MM2) were found, and some internuclear distances still remained outside the required range. It is clear that anhydroerythromycin A does not exist as a single conformer in aqueous solution.

Attention was now turned to the higher energy structures found by the unconstrained Monte Carlo searches. When the MM2 force field was used, a second structure with energy −269.90 kJ mol^{-1} (*i.e.* 16.24 kJ mol^{-1} above the global minimum) satisfied the remaining six constraints. The AMBER force field, however, gave a structure less than 1 kJ mol^{-1} above the global minimum (structure 2), which satisfied the two constraints not satisfied by the AMBER global minimum. It seems likely that the calculations using the AMBER force field better represent the conformations of anhydroerythromycin A in aqueous solution. These two structures are shown in Fig. 1 and their key structural details are summarized in Table 2.

In parallel with the NMR and modelling analyses, we attempted to determine the crystal structure of compound **1**. Unfortunately, however, even after slow crystallization from five different solvent systems, no large crystals of **1** were obtained. We attempted to introduce a heavy atom by preparing anhydroerythromycin A 2′-tribromoacetate, but the preparation failed, yielding only an intractable oil. Two esters, anhydroerythromycin A 2′-acetate (**4**) and anhydroerythromycin A 2′-ethyl succinate (**5**), were, however, synthesized successfully. The full characterization of **5** is described elsewhere.[8] Compound **4** was fully characterized by

Table 2 Key structural details of compound **1** resulted from molecular modelling or were derived from the crystal structure. Energy has been measured in water. When the isomer does not satisfy the ROESY data, the internuclear distance is given in bold type

			Internuclear distances/Å [a]												Torsion angles/° [b]					
Compound	Force field	Energy/ kJ mol^{-1}	2–4	2–10	3–18	4–10	4–1′	5–17	5–18	5–5″	7s–20	8–18	11–19	14r–21	17–8″	2′–20	C5–C6	C8–C9	C9–C10	C10–C11	C11–C12	C1–C2
1*S* global minimum	MM2	−263.61	**3.36**	**7.79**	**5.14**	**5.95**	**4.38**	**3.40**	**3.40**	**5.53**	**5.34**	**4.40**	**5.33**	**4.33**	**4.65**	**7.39**	137	156	−108	−107	−152	107
1*R* global minimum	MM2	−285.14	2.23	**5.70**	**2.53**	**3.99**	**4.10**	3.06	2.65	2.59	**4.64**	2.94	**5.26**	3.31	4.08	**6.83**	−43	−113	46	−89	−142	−26
1*R* structure 14	MM2	−269.90	2.56	2.40	2.79	**4.39**	2.26	2.49	2.77	**5.06**	3.01	2.91	3.68	3.33	4.41	3.30	−68	−74	−104	−152	−87	20
1*R* crystal-derived	MM2	−254.74	2.30	**3.00**	2.87	2.40	**4.24**	2.86	2.43	2.55	2.99	2.79	3.29	3.50	3.93	**5.96**	1	−77	96	−164	−80	−1
1*S* global minimum[c]	AMBER	21.78	**2.94**	**7.45**	**4.47**	**5.12**	**4.31**	3.21	2.71	2.29	**5.37**	**4.37**	**5.14**	**4.28**	4.34	**8.48**	58	140	−110	−106	−152	145
1*R* global minimum	AMBER	0.1	2.17	2.82	2.92	2.21	**4.32**	2.88	2.45	2.38	2.99	2.86	3.58	3.14	4.10	**5.64**	0	−79	84	−155	−83	−7
1*R* structure 2	AMBER	1.0	2.57	2.31	2.84	**4.32**	2.40	2.49	2.80	**5.11**	2.98	**5.22**	3.70	3.12	4.40	3.04	−65	−74	−111	−152	−84	21

[a] Internuclear distances are H–H for methane and methylene carbons, H–C or C–C where one or both partners are methyl groups. [b] Torsion angles were measured consistently: H5–C5–C6–C18; H8–C8–C9–O6,9; O12,9–C9–C10–H10; H10–C10–C11–H11; H11–C11–C12–O12,9; O13,1-C1–C2–H2. These are abbreviated to the central bond in the table header. [c] In addition, the constraint H17–H5″ is not satisfied in this structure.

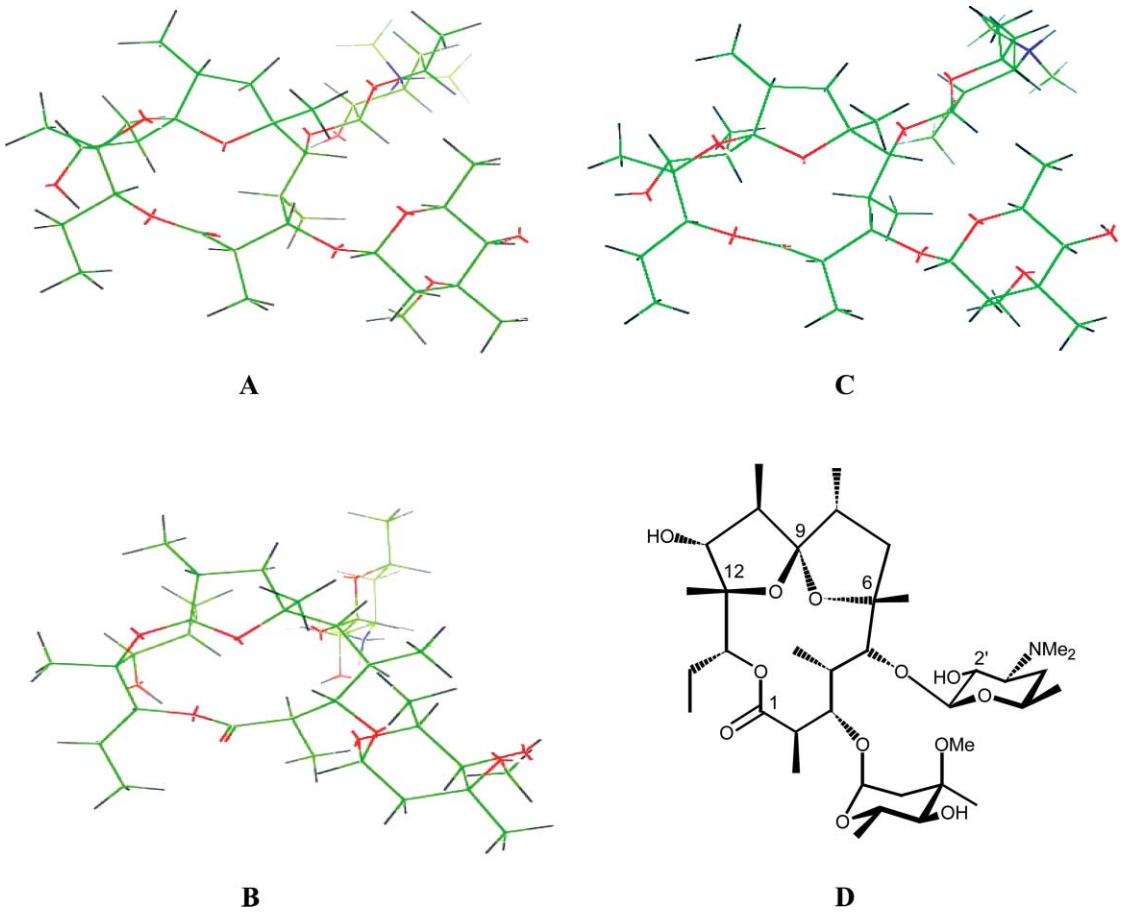


Fig. 1 9*R*-Anhydroerythromycin A in AMBER: structure with the global minimum energy (**A**), structure 2 (**B**) and structure with the correct C9-stereochemistry (**D**). 9*R*-Anhydroerythromycin A 2′-acetate: crystal-derived structure (**C**).

two-dimensional NMR spectroscopy and complete ^{1}H and ^{13}C assignments are given in the ESI.† Although the ethyl succinate ester (**5**) did not yield good crystals, crystallization of the acetate ester (**4**) from acetone was successful and crystallographic data for this compound were obtained as described in the Experimental section. The X-ray crystal structure for anhydroerythromycin A 2′-acetate shows *R* stereochemistry at C-9. Table 2 also shows the structural torsion angles and key internuclear distances of the global minimum for anhydroerythromycin A obtained through modelling analysis, compared with those of anhydroerythromycin A 2′-acetate obtained *via* X-ray crystallographic analysis.

Fig. 1 shows the lowest energy structure of anhydroerythromycin A derived from the NMR and AMBER modelling analysis, compared with the structure of anhydroerythromycin A 2′-acetate derived from X-ray crystallographic analysis. Both the crystal structure and the global minimum from modelling the ROESY data clearly have the *R*-configuration at C-9 and the differences between them are small (see Table 2 for details); since the comparison is between two slightly different molecules (**1** and **4**) small differences would be expected. In anhydroerythromycin A, unlike erythromycin A itself, there is considerable flexibility about the glycosidic linkages. Thus, in solution, **1** is quite flexible and able to adopt a range of conformations, not identical with the crystal structure. Fig. 1B shows structure 2 from the AMBER modelling calculation. This is rather different from the crystal structure, especially in the orientation of the desosamine sugar, but appears to be significantly populated in aqueous solution.

Discussion

We have shown by NMR spectroscopy and molecular modelling that anhydroerythromycin A (**1**) adopts the 9*R* stereochemistry and not that suggested by the Chemical Abstracts Service. This conclusion was found independently by X-ray crystallographic analysis of the 2′-acetate ester of **1**. This suggests that erythromycin A 12,9-hemiacetal (**2b**), which exists in equilibrium with erythromycin A ketone (**2a**), is an intermediate in the formation of **1**. The mechanism of the degradation of erythromycin in acid has been the subject of continuing debate in the literature because of the importance of this antibiotic in clinical use.[3,4,9] The intermediacy of **2b** has not previously been suggested.

The results also indicate that erythromycin A anhydride shows considerable flexibility in solution. No single conformer, whether derived from crystallography or from modelling, satisfied all the ROESY NMR constraints. In this case the AMBER force field gave slightly different results from the MM2 force field and the AMBER results were more consistent with the experimental crystallographic data, suggesting that they more accurately represent the conformational space occupied by **1** in aqueous solution. We have recently suggested a systematic approach for the derivation

of macrolide conformers from NMR data.[10] If this approach is to be extended to flexible molecules and to commercial modelling packages with built-in force fields, it is desirable to use two or more appropriate force fields for detailed conformational analysis.

Experimental

Syntheses

Synthesis of anhydroerythromycin A (1). Anhydroerythromycin A was prepared by a minor adaptation of the procedure used by Stephens and Conine.[7] To a solution of **2** (1 g) in water (30 ml), concentrated HCl, diluted with 3 parts water, was gradually added until the pH reached 1.7. The resulting mixture was stirred until the solid dissolved. The pH was again adjusted to 1.7 and the reaction was allowed to proceed at room temperature for 20 min. The mixture was then neutralized with saturated sodium bicarbonate solution (to pH 6.5–7). The resulting mixture was extracted with chloroform (3 × 50 ml). The combined organic layers were washed with saturated sodium bicarbonate solution (150 ml) and finally with water (150 ml). The organic layer was dried over anhydrous sodium sulfate and the solvent removed *in vacuo*. Finally, anhydroerythromycin A (**1**) was recrystallized from dichloromethane to give white crystals (0.79 g, 81%). The structure of **1** was confirmed by NMR spectroscopy.[11] Mp 140–149 °C (from dichloromethane) (lit.,[12] 142–150 °C); *m/z* (electrospray) 716 $[M + H]^+$.

Preparation of anhyroerythromycin A 2′-acetate (4). Anhydroerythromycin A 2′-acetate was prepared in an analogous way to **1** from erythromycin A 2′-acetate (1 g), which was synthesized by the literature method given in ref. 13. It was finally recrystallized from acetone as white crystals (0.68 g, 70%). The full ^{1}H and ^{13}C assignments of **4** are given in the ESI.† Mp 165.5–174.5 °C (from acetone); (Found: C, 60.70; H, 9.03; N, 1.65. Calc. for $C_{39}H_{67}NO_{13}\cdot 0.5H_2O$: C, 61.08; H, 8.94; N, 1.83%).

Preparation of anhyroerythromycin A 2′-ethyl succinate (5). Anhydroerythromycin A 2′-ethyl succinate was prepared by an adaptation of the published procedure for the preparation of 2′-esters of erythromycin.[13] To a solution of anhydroerythromycin A (1 g) in acetone (10 ml, previously dried over anhydrous sodium sulfate) dried sodium bicarbonate (0.533 g) was added. To the reaction mixture a solution of ethyl succinyl chloride (0.23 ml) previously dissolved in dried acetone (2 ml) was added dropwise. The reaction was stirred overnight at room temperature. The volume of the reaction mixture was then reduced to 5 ml *in vacuo*. Sodium phosphate buffer (0.2 M, pH 6.5, approx 15 ml) was added to a final pH of above 6 and the mixture was stirred for 10 min. The white residue was filtered off and dissolved in chloroform (30 ml). The organic layer was washed with water (30 ml), dried over anhydrous sodium sulfate and reduced to dryness *in vacuo*. Compound **5** was recrystallized from a small volume of dichloromethane–hexane with difficulty (0.77 g, 65%). Its structure was confirmed by NMR spectroscopy. Mp 92–102 °C (from dichloromethane–hexane); *m/z* (electrospray) 844 $[M + H]^+$; (Found: C, 60.89; H, 8.85; N, 1.52. Calc. for $C_{43}H_{73}NO_{15}$: C, 61.19; H, 8.72; N, 1.66%).

Attempted preparation of anhydroerythromycin A 2′-tribromoacetate. We attempted to prepare anhydroerythromycin A 2′-tribromoacetate in the same way as **5**. The reaction yielded a thick intractable tar which failed to crystallize.

Molecular modelling

Unconstrained Monte Carlo searches on 9*R*- and 9*S*-anhydroerythromycin A. 9*R*- and 9*S*-anhydroerythromycin A were constructed from erythromycin A on a Silicon Graphics Iris 4D Indigo workstation using Macromodel 6.5 software.[6] The structures were minimized using the Truncated Newton Conjugate Gradient (TNCG) method in order to obtain local minima.

The Monte Carlo Multiple Minimum (MCMM) conformational search was used to find the global minima.[14] The TNCG method was used as the minimization procedure. A water solvation option using the GB/SA model (Still *et al.*, 1990)[15] was used for calculations in water. The search was set for 10 000 structures to be minimized and all structures within 50 kJ mol^{-1} energy range were stored. Online manuals describing the Monte Carlo protocols in details are available at http://www.chem.arizona.edu/facilities/cgf/mm6/batchmin/bmintoc.htm and http://www.psgvb.com/MacroModel/primer/prmrindx.htm.

Fully constrained Monte Carlo searches of 9*R*- and 9*S*-anhydroerythromycin A. A second constrained Monte Carlo conformational search *in vacuo* was carried out using constraints derived from ROESY experiment. The cross-peaks from the ROESY spectrum were given the following constraints, based on known internuclear distances within the molecule: very small (3.5 ± 1.5 Å), small (2.5 ± 1.0 Å), medium (2.3 ± 0.5 Å) or large (2.0 ± 0.4 Å).

Monte Carlo search of 9*R*-anhydroerythromycin A constrained between H10 and H4. This was carried out in the same way, with the single H10–H4 (small) constraint.

NMR studies

ROESY NMR of anhydroerythromycin A. The ROESY spectrum was obtained using 2 mM solution of **1** in deuteriated sodium phosphate buffer (50 mM, apparent pH 7.0). The spectrum was acquired at 25 °C using data matrices of 2048 × 2048 complex points and a mixing time of 0.5 ms. The spectrum was processed using a Gaussian window function with zero-filling in *F*1. All negative ROESY cross-peaks were plotted.

Crystallography

Crystal data for 4. $C_{39}H_{67}NO_{13}\cdot 0.5H_2O$, $M = 766.94$, monoclinic, $a = 28.764(3)$, $b = 12.3834(13)$, $c = 11.8736(13)$ Å, $\beta = 104.728(2)°$, $U = 4090.4(8)$ Å^3, space group $C2$ (no. 5), $Z = 4$, μ(MoKα) = 0.093 mm^{-1}, 16 413 reflections measured, 4428 unique reflections ($R_{int} = 0.0464$), 4096 reflections with $I > 2.00\sigma(I)$. The structure was solved by direct methods and refined using full matrix least squares on F^2 using all the data. The asymmetric unit contains a molecule of **4** together with half a molecule of water, which is located on a two-fold axis; its hydrogen atom was found by difference Fourier methods and refined isotropically, the other water hydrogen atom being generated by rotation about the two-fold axis. All the remaining hydrogen atoms were included in calculated positions. The final $R_1 = 0.0314$, $wR_2 = 0.0722$ [for $I > 2\sigma(I)$]; $R_1 = 0.0343$, $wR_2 = 0.0731$ (all data).

CCDC reference number 286533. For crystallographic data in CIF or other electronic format see DOI: 10.1039/b518182h.

Acknowledgements

A. H. thanks the Ministry of Health and Medical Education of Iran for a fellowship. We would like to thank Dr Richard Bryce for his generous support on Molecular Modelling. We thank the Royal Society for a Small Grant for computer equipment for modelling.

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