The Structure of Erythromycin Enol Ether as a Model for Its Activity as a Motilide

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Erythromycin enol ether is a potent mimic of the peptide hormone motilin. To understand its biological activity, its three-dimensional structure in CD_2Cl_2 was determined from constrained molecular mechanics using constraints derived from NMR spectra. The structure of the enol ether is well defined by 10 structures that minimize the energy and satisfy the NMR data. We infer the molecular basis for its activity as a motilide from a comparison of its structure with that of motilin. The macrolide ring of the enol ether is a β-turn mimic of the peptide. Furthermore, a superposition of the structures of the enol ether and motilin shows a striking overlap of the sugar rings attached to the macrolide ring with essential aromatic side chains in the peptide.

Introduction

Erythromycin A is the most commonly used and extensively studied of the macrolide antibiotics.[1] It is effective in the treatment of infection by Gram-positive bacteria and along with its derivatives has been the subject of numerous structural studies.[2] These include our determination of the three-dimensional, aqueous-phase structure of 6-*O*-methylerythromycin A, an antibiotic marketed under the name biaxin.[3] Erythromycin has several shortcomings. It decomposes under acidic conditions such as those present in the stomach to form erythromycin enol ether, also known as 8,9-anhydroerythromycin A 6,9 hemiketal (Figure 1).[4] This breakdown has two consequences. First, the enol ether does not retain the antibiotic activity of the parent erythromycin A.[1] Second, the enol ether is the agent of gastrointestinal (GI) distress, a significant side effect with the use of erythromycin A.[1] Studies of the biochemical basis for the GI distress led to the discovery that erythromycin enol ether is a motilide, a mimic of the peptide motilin that causes duodenal contractions.[5,6]

Medicinal chemists have invested considerable effort to characterize the binding of motilides to the motilin receptor and have developed structure−activity relationships for an impressive number of derivatives.[5−7] Biological data on these derivatives have provided the basis for the development of quantitative structure activity relationships (QSAR) and pharmacophore maps.[8−10] The literature contains a small number of structural studies relevant to motilin and the erythromycin enol ether family of motilides. Jarvet et al. determined the three-dimensional structure of motilin in SDS micelles from NMR data.[11] However, the structure of the motilin receptor remains poorly characterized. Faghih et al. applied unconstrained molecular mechanics to the determination of the conformation of erythromycin enol ether; ABT-229, a close analogue; and products of the reduction of 8,9-double bond of ABT-229.[12] Their analysis of the biological properties of these compounds was also based on crystal structures.[13] In contrast to the situation with erythromycin, no solution-phase structures of the enol ether have been published. We initiated our application of NMR spectroscopy to the problem in order to fill this gap in the literature with the expectation that the results would complement the insights gained from the existing structures and the QSAR studies. As anticipated, a comparison of its NMR structure with motilin was instructive. The functional portion of motilin has a β turn,[11] and we concluded from an analysis of the structures of erythromycin enol ether and erythromycin A that they are β-turn mimics.

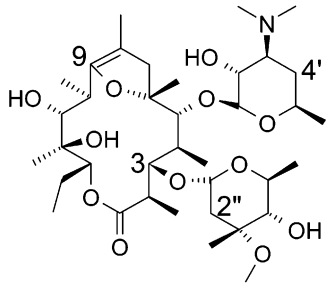

Figure 1. Covalent structure of erythromycin enol ether.

Results and Discussion

Determination of the Structure of Erythromycin Enol Ether. Structurally, erythromycin enol ether consists of a central 14-membered bicyclic ring, the macrolide ring, to which are attached two cyclic sugars, α-L-cladinose and β-D-desosamine. In the use of constrained molecular mechanics to determine the three-dimensional structure, the bond angles and bond lengths are for the most part determined by the force field. The major challenge is the determination of the torsional angles that are less well defined by the force field. To this end, we employ NMR data following the method pioneered by the Wüthrich group.[14] Full details on our adaptation of the Wüthrich methodology to small molecules are provided in our paper on biaxin.[3]

The NMR phase of the determination of the structure had two steps: assignment of the spectrum and deter-

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mination of constraints. Dichloromethane was selected as a solvent because it has a comparatively low dielectric constant, 8.9, and was assumed to provide a better match to the presumed hydrophobic environment of the motilide binding site than highly polar water. However, in the absence of a structure for the binding site, the selection of the optimal NMR solvent is conjectural. An assignment of the proton spectrum was generated by standard methods from the integrated one-dimensional spectrum, a COSY spectrum, and ROESY spectra. Alam et al. previously produced an assignment of the proton and carbon-13 spectra of the enol ether in D_2O, CD_3-OD, and $CDCl_3$ and our results agree with theirs.[15] 44 distance and 8 torsional constraints were obtained from the NMR spectra and are available as Supporting Information. Torsional constraints were obtained by applying the Karplus equation to vicinal proton−proton scalar coupling constants as previously described.[3] To generate distance constraints, a series of ROESY spectra with mixing times of 125, 250, and 500 ms were measured. A plot of the volume of the cross-peak versus mixing time showed linearity up to 250 ms but not at 500 ms. Therefore, the distance constraints in the form of upper bounds on interproton distances were based on the integrated cross-peaks from the 250 ms spectrum and a total of 44 distance constraints were obtained, 29 sequential, 5 medium-range, and 10 long-range.

The calculation of the structure from the NMR constraints consisted of three stages: searching conformational space, energy minimization, and reminimization with the enol ether embedded in a matrix of 36 solvent molecules. Two strategies were employed to ensure a thorough search of conformational space. Eight thousand cycles of the SYBYL Random Search algorithm in which randomly chosen torsional angles are tweaked were combined with several cycles of molecular dynamics conducted at 1000 and 2000 K. This was followed by an energy minimization of each structure whereby the NMR distance constraints were incorporated by adding a penalty function to the force field for each constraint. This constrained energy minimization of the structures that were generated in the search of conformational space yielded a set of 10 low-energy structures with energies in the range 826.0−843.1 kJ/mol that maintained chirality and completely satisfied the NMR constraints. These 10 structures constitute the in vacuo solution to the determination. In this case, the contribution of the solvent to the conformation of the enol ether is made indirectly through the use of the distance constraints that are based on the NMR measurements in solution. The potential role of the solvent was examined more explicitly in a final refinement step in which SYBYL simulated solvation by placing an enol ether molecule in a matrix of solvent molecules. The "solvation" was followed by minimization of the energy of the entire ensemble of solute and solvent molecules. This approach applied on the molecules in the in vacuo solution set produced a second in CD_2Cl_2 solution set that is virtually identical with the in vacuo solution set. Similar results were obtained in our determination of the aqueous-phase structure of biaxin where the molecule was embedded in a matrix of water molecules.[3] The comparison of the two solution sets shows that the polarity of the solvent has a minor effect on the molecular energetics. The results indicate that we can dispense with the final "solvation" step in future studies.

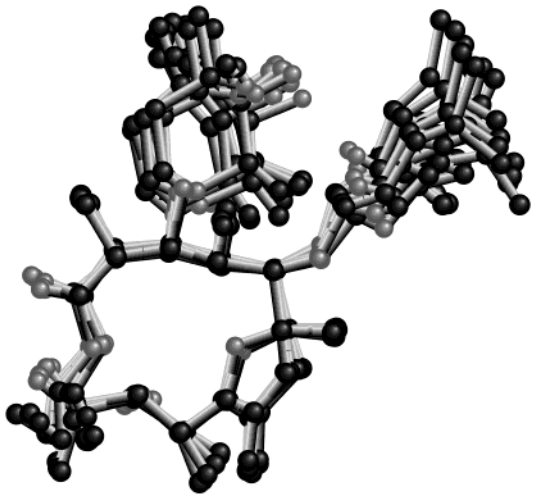

Figure 2. Superposition of the 10 structures of the in CD_2Cl_2 solution set for erythromycin enol ether. Only the heavy atoms are shown. The superposition is based on the heavy atoms in the macrolide ring.

The structural results are displayed graphically as a superposition of the 10 structures in Figure 2. Values of the torsional angles defining the macrolide ring and the orientation of the two sugar rings are given in the Supplemental Information. Visually, the conformation of the macrolide ring is well defined and the carbonyl group in each structure has the same orientation. Some variation in the orientation of the sugar rings is evident. This spread might reflect conformational diversity in solution or insufficiency of constraints. The measurement of proton−carbon vicinal coupling constants would resolve this question. The qualitative impression is supported by the values of the torsional angles and the following statistics. When the heavy atoms in the macrolide backbone are used to superimpose the structures in a pairwise manner, rms values in the range 0.018−0.51 Å and 0.035−0.37 Å were obtained for the in vacuo and in CD_2Cl_2 solution sets, respectively. The average rms values are 0.19 ± 0.14 Å and 0.16 ± 0.11 Å, respectively. In contrast, the range of rms values obtained in the in vacuo biaxin structure was 0.18−0.83 Å with an average of 0.55 ± 0.29 Å.

The values of the torsional angles provide a quantitative measure of the structure of erythromycin enol ether but some qualitative comments can also be made. As in the case of erythromycin, the two sugar residues adopt a chair conformation. When the backbone atoms in the three rings are fit to planes, the angles between these planes indicate their relative orientation. The desosamine ring is nearly perpendicular to the macrolide ring and the cladinose ring is oriented close to 45°. Because of the conformational impact of the dihydrofuran ring, we prefer not to use the terms folded in and folded out to describe the conformation of the macrolide ring but note the following interatomic distances which are diagnostic: 3H-11H, 2.28 ± 0.12 Å; 4H-11H, 3.90 ± 0.14 Å; 4H-6MeC, 4.14 ± 0.02 Å. Accordingly, the ROE at 250 ms between the 3H and 11H protons is approximately 6 times stronger than the weak ROE between the 4H and 11H protons. No ROE was observed between 4H and the protons on the

6-methyl group. Hydrogen bonding is indicated by a short distance (1.72 ± 0.05 Å) between the lactone carbonyl oxygen and the 12-hydroxyl hydrogen in 8 of the 10 structures. The orientation of the lactone carbonyl group is well defined. In constrast with biaxin where it was found to be approximately perpendicular to the plane of the macrolide ring, it is roughly in the plane of the ring and pointing out.

Biological Relevance of the Structure. A β or tight turn is a prominent feature in the structure of proteins.[16] Many bioactive peptides and proteins contain a β turn on the surface, which is usually stabilized by hydrogen bonding. The well-defined architecture of the turn contains a scaffold of backbone heavy atoms to which the side chains are attached. The β turn is a well-designed tool to present oriented functional groups to a binding site. Small organic molecules that mimic these essential features have proven to be effective drugs and the design of β-turn mimics has been a productive approach to the design of new drugs.[17,18] Earlier an exhaustive search of the Cambridge Structural Database for β-turn mimics turned up erythromycin A. In analyzing our NMR structure, we found that erythromycin enol ether is no exception. Its backbone atoms 13C through 5C constitute a β turn that closely matches a type I turn. An rms value of 0.36 Å is obtained when these atoms are superimposed on the backbone atoms of a peptide whose φ and ψ angles have been set for a classic type I turn.[16]

Jarvet et al. determined the structure of motilin in a SDS micelle.[11] The same group also determined its structure in an aqueous solution of hexafluoro-2-propanol.[19] In our analysis we have used the structure in a micelle because the aqueous-phase structure is highly disordered and we assumed that the well-defined micelle structure is more likely to be in the bioactive conformation. In the micelle residues 10−16 form an α helix but more importantly residues 3 through 6 (Pro-Ile-Phe-Thr) of the 22-residue peptide define a β or tight turn. The structure at the N terminus is important as an exhaustive study of analogues of motilin showed that substitution of residues 1−7 would eliminate biological activity and demonstrated that they constitute the minimal unit of binding.[20] The enol ether and motilin structures were superimposed using the seven backbone atoms 13C through 5C in the enol ether and Thr-6 C_α through Ile-4 C_α atoms in the peptide. An average rms value of 0.17 ± 0.035 Å and visual examination (Figure 3) show that the backbones of the two molecules closely match. The lactone carbonyl group is parallel with the carbonyl on Phe-5, suggesting that this group might form a hydrogen bond to a residue in the receptor. The superposition of the biaxin solution-phase structures yielded a significantly poorer rms, 0.57 ± 0.13 Å. Furthermore, when the crystal structure[21] of biaxin is used, the rms is even larger, 0.90 Å. In the aqueous-phase and crystal biaxin structure, the lactone carbonyl is oriented 90° with respect to the Phe-5 carbonyl and would not be able to participate in hydrogen bonding in the same way as the peptide without significant reorganization of the macrolide ring. Erythromycin enol ether is a more potent motilide than erythromycin by a factor of 10.[5,6] These results suggest that the better fit of erythromycin enol ether to a β turn and the availability of the lactone for hydrogen bonding might be the structural features responsible for the difference in activity. The primary role then of the dihydrofuran ring would be to define the conformation of the macrolide ring and render it more rigid.

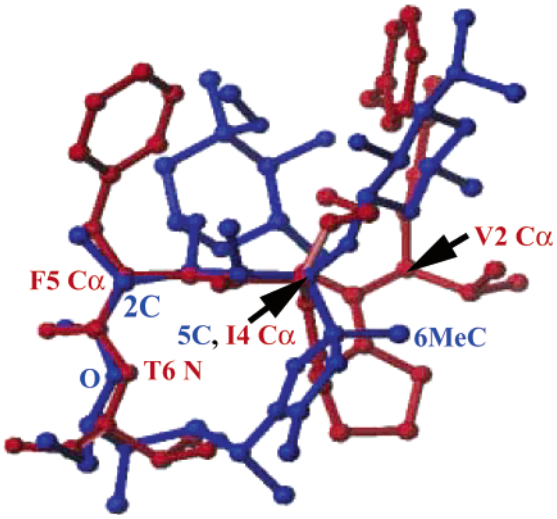

Figure 3. Superposition of the lowest-energy NMR structure of erythromycin enol ether in CD_2Cl_2 with the NMR structure of motilin in a SDS micelle.[12] Only the heavy atoms and the residues Phe-1 through Thr-6 of motilin are shown. The amino terminus of the peptide chain is at the right. Blue trace, erythromycin enol ether; red trace, motilin.

An examination of the adjacency of functional groups in the superposition of the enol ether and motilin structures lends further support to our model. The following pairs of groups are adjacent: the aromatic ring in Phe-5 and the cladinose ring, the aromatic ring of Phe-1 and the desosamine ring, and the aromatic ring in Tyr-6 and the ethyl group attached to 13C. The amino nitrogen on the desosamine ring is within 2 Å of carbon 4 on the phenyl ring of Phe-1. The cladinose/Phe-5 overlap can be improved by making small adjustments in the 3H−3C−O−1″C and 3C−O−1″C−1″H torsional angles. These adjacencies are more than coincidental. Extensive data on derivatives of erythromycin enol ether show that its activity as a motilide can be fine-tuned by modifying the sugar residues.[5,6,7] Furthermore, macrolides that lack the cladinose ring or the amine group on the desosamine do not possess activity as motilides.[22]

The adjacency of the desosamine ring and the phenyl ring of Phe-1 in a superposition of the enol ether and motilin has also been indicated in QSAR studies. Khiat and Boulanger applied the APEX 3D QSAR method to a set of 18 motilides in the erythromycin enol ether family.[8] They derived two models. One, model 1, gave better statistics (R^2 of 0.98 versus 0.94) and fewer components (4 versus 6). Model 1 showed a correlation of the substituted amino group on the desosamine ring with biological activity. Furthermore, they associated the amino site with the side chain of Phe-1 from the superposition of the motilide structures with the motilin NMR structure. In a parallel study Gouda et al. employed a combination of conformational analysis and a novel approach to superposition to identify common structural features responsible for biological activity.[9] They stressed the importance of the substituents on the amino group in the desosamine substituent and their model showed an alignment between the substituent

and the Phe-1 side chain. They also suggested an adjacency of Tyr-7 with the ethyl group on the macrolide ring.

The determination of structures of motilides in the erythromycin enol ether family has provided insights into the relationship between the conformation of the macrolide ring and biological activity. Faghih et al. correlated the activity of diastereomers obtained from the reduction of the 8,9-double bond of ABT-229 with differences in the positions of heavy atoms 11C through 13C.[12] In this study we report for the first time a second important structural feature, a β turn defined by heavy atoms 13C through 5C that places the cladinose and desosamine rings in an orientation essential for activity. Work is in progress to test this hypothesis with the structure of other motilides.

Experimental Section

The erythromycin enol ether used in the initial stages of the study was provided by Abbott Laboratories. Additional material was synthesized following the procedure described by Alam et al. and recrystallized from a hexanes−ethanol mixed solvent.[15] A solution of the macrolide in 99.95 atom-% CD_2Cl_2 (Aldrich) was transferred to an NMR tube. Dissolved oxygen was removed by a series of freeze−thaw cycles on a vacuum line and the tube was sealed.

NMR measurements in CD_2Cl_2 were performed at 400.13 MHz on a Bruker DPX spectrometer with a broad-band, inverse-detection probe with a single-axis gradient coil. Version 2.6 of XWINNMR was used to acquire and process the data. The one-dimensional proton spectrum was acquired with 16k data points; the free induction decay (fid) was zero-filled once. An absolute-value COSY spectrum was acquired with a relaxation delay of 1.7 s, 2048 points in the t_2 dimension, and 256 values of t_1. The fid was zero-filled twice in the t_1 dimension. Phase-sensitive ROESY spectra were measured at 25 °C and 30 °C with mixing times of 125, 250, and 500 ms using a TPPI pulse sequence designed to suppress TOCSY cross-peaks.[23] The relaxation delay was 2.0 s and 4096 points in the t_2 domain and 512 values of t_1 were employed. The fid was zero-filled once in the t_1 domain. A squared-cosine bell apodization was applied in both dimensions.

The modeling calculations were run on a SGI Indy workstation running under Irix 6.5 and using Version 6.8 of SYBYL (Tripos, Inc., 1669 S. Hanley Road, St. Louis, MO) and the 1994 release of the Merck Molecular Force Field (MMFF94). Random Search parameters were set to default values with the following exceptions: Bump Factors, 0.02; Ring Bond Closure, 10 Å; energy cutoff, 100 kcal/mol; interations for Energy Minimization, 2000. Solvent molecules were added using the Silverware droplet algorithm with two solvent shells.

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Supporting Information Available: NMR spectra, assignments of the proton NMR spectrum, NMR constraints, parameters characterizing the structures, a SYBYL mol2 file of the global minimum structure. This material is available free of charge via the Internet at http://pubs.acs.org.

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