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APLASMOMYCIN C: STRUCTURAL STUDIES OF A MARINE ANTIBIOTIC

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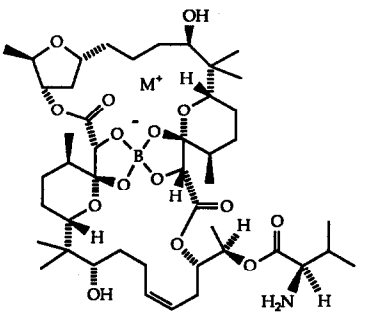
Abstract - The solid and solution state structures of aplasmomycin C, a boron-containing antibiotic, were studied by single crystal X-ray diffraction and NMR techniques. This unusual antibiotic was shown to have a complex and as yet imperfectly understood series of conformations.

Marine natural products have presented numerous structural challenges to chemists. Often a structural analysis is complicated by unusual or unprecedented chemotypes. The usual strategy of generating possible molecules from fragments deduced by spectral techniques and narrowing this list through additional spectral measurements - perhaps using other analytical techniques or interpretation strategies (*e.g.*, ^{1}H-NMR, ^{13}C-NMR, IR, and MS) - may not always succeed. A unique structure may never emerge, or the correct possibility may not have been considered in the original list. In these cases, X-ray diffraction is often the technique of choice. A classic example of this difficulty was the elucidation of the structures of boromycin (**1**)[1,2] and the aplasmomycins (**2**, **3**, **4**)[3-6]. X-ray diffraction not only revealed the final structures of these compounds, but also gave the correct molecular composition.

Boromycin, **1**, was first isolated from an African soil sample containing *Streptomyces antibioticus*, and is an ionophore composed of a Boeseken complex of boric acid surrounded by the unsymmetrical macrolide "hemispheres" seen in Figure 1. Aplasmomycin, **2**, which was originally isolated from *Streptomyces griseus*, found in a shallow sea sediment[3], differs from boromycin by having two chemically identical subunits surrounding the borate complex. *S. griseus* produces several variations of aplasmomycin and the series has been designated as aplasmomycins A, B, and C (**2**, **3**, **4**) where aplasmomycin A is the diol; aplasmomycin B, the monoalcohol monoacetate; and aplasmomycin C, the diacetate. These antibiotics are unique as the only known metabolic products containing the element boron, and the seminal efforts of Floss and co-workers[7-15] have contributed much to the study of the biosynthetic origins of these molecules.

As part of a program to examine marine micro-organisms for biologically active metabolites, we recently re-examined aplasmomycin C by a variety of techniques and found an unexpected family of conformational isomers. The story began with a surface dwelling nocardiaform actinomycete collected from a sandy sediment sample (isolate CNB 222) in La Jolla, CA. Initial chromatographic traces of the isolate indicated a pair of closely related, yet separable compounds, as well as a single distinctly different compound. Analysis of the purified samples by NMR techniques indicated the distinct compound to be deboroaplasmomycin C (5)[16], which to our knowledge is the first time this form of aplasmomycin C has been isolated as a natural product. The pair of compounds was closely related to aplasmomycin C, but how?



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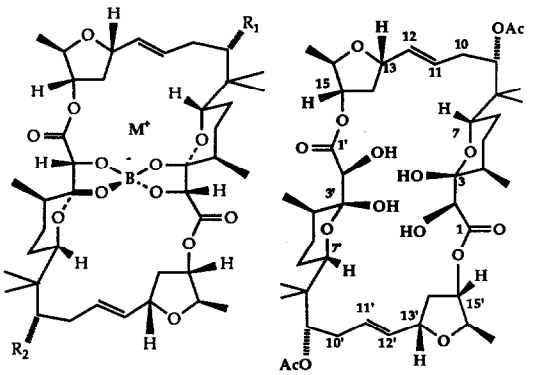


2: R_1 = OH, R_2 = OH
3: R_1 = OH, R_2 = OAc
4: R_1 = OAc, R_2 = OAc

5

Figure 1: Boromycin (1) and the Aplasmomycin Series (2, 3, 4, 5)

Since good quality single crystals (*ca.* 0.8 x 0.6 x 0.2 mm) of the minor chromatographic component of this pair were available, we turned to single crystal X-ray crystallography in the hopes of obtaining precise three-dimensional geometric information about these compounds. Initially grown from the NMR solvent, $CDCl_3$, the crystals were shown by photographic methods to belong to the monoclinic crystal class. Centering on 30 diffraction maxima with $30° \le 2\theta \le 45°$ gave the accurately determined unit cell: a = 11.822(2), b = 19.188(2), and c = 21.179(3)Å, β = 92.75(1)°. All unique diffraction maxima with +h,+k, ±l, and $2\theta \le 115°$ were collected. None of the three moderately strong reflections (454, $36\bar{3}$, and 210) monitored every 97 data points showed any decomposition greater than *ca.* 10%. Examination of the intensity distribution of the complete data set and knowledge of the chiral nature of the molecule showed the space group to be uniquely defined as $P2_1$ (Z=4). Of the 6330 unique data collected, 5279 (83.4%) were judged observed ($|F_o| \ge 4.0\sigma|F_o|$).

Extensive attempts to obtain a phasing model by direct methods were unsuccessful. Thousands of phase sets were calculated with various starting conditions, and while many had good figures of merit, none produced E-maps containing recognizable fragments. This example, with 126 non-hydrogen atoms in the asymmetric unit, shows that not all crystal structures are solvable or at least that some remain unsolved despite considerable effort.

Faced with a setback on the minor compound, we were delighted to discover that a small number of crystals of the major component had appeared in an NMR sample ($CDCl_3$). A small crystal (*ca.* 0.4 x 0.2 x 0.2 mm) was used for analysis by X-ray diffraction at -30°C in a cold N_2 stream, since the sample was observed to lose crystallinity quickly when exposed to air at room temperature. A least squares fit of 30 centered reflections with $25° \le 2\theta \le 40°$ gave a refined, orthorhombic unit cell with dimensions: a = 12.44(2) Å, b = 18.41(1) Å, and c = 23.47(3) Å. While the crystal was far from optimum, it was clearly not isomorphous with that of the other component previously studied (Table 1) and data were collected while a recrystallization was attempted. Examination of the intensity distribution of the complete data set, and knowledge of the chiral nature

Table 1: Relative Unit Cell Parameters

	Aplasmomycin C_1	Aplasmomycin C_2	β-Aplasmomycin C_1	DBAC	A/A - Ag [6]
a	12.43(2)Å	11.822(2)Å	11.341(2)Å	19.310(5)Å	18.572(8) Å
b	18.41(2)Å	19.188(2)Å	21.119(3)Å	9.893(1)Å	21.246(9) Å
c	23.47(3)Å	21.179(3)Å	20.575(4)Å	12.135(3)Å	11.595(5) Å
α	90.00°	90.00°	90.00°	90.00°	90.00°
β	90.00°	92.75(1)°	99.28(1)°	104.91(1)°	90.00°
γ	90.00°	90.00°	90.00°	90.00°	90.00°
Space Group	$P2_12_12_1$	$P2_1$	$P2_1$	C2	$P2_12_12_1$
R-factor	0.136	0.078	0.082	0.035	0.084

DBAC: Deboroaplasmomycin C **A/A-Ag**: Aplasmomycin A - Silver salt[6]

of the molecule showed the space group to be uniquely defined as $P2_12_12_1$ (Z=4). Of the 3971 unique data collected, 2644 (67%) were judged observed ($|F_o| \geq 4.0\sigma|F_o|$). With some effort, a suitable phasing model was obtained by direct methods, which, combined with several cycles of tangent formula recycling, led to the full elucidation of the structure of aplasmomycin C. A computer generated perspective drawing of this molecule is shown in Figure 2.

In addition to a molecule of 4, the asymmetric unit also contained two molecules of chloroform, one of which was highly disordered (accounting for the unstable nature of the crystals), for a total of 70 non-hydrogen atoms. It is believed that the highly disordered nature of the second chloroform molecule, the low data-to-parameter ratio (4.2:1), and the less than optimal quality of the crystal sample all contributed to the relatively high convergence of refinement at a standard crystallo-graphic residual of 0.136. This residual is the most common means of referring to the quality of a model obtained for a molecular structure by single crystal X-ray diffraction methods, and thus a word about its significance is in order. The residual, or *R-factor*, is defined as

$$R = \sum_{hkl} |(|F_o|-|F_c|)| \Big/ \sum_{hkl} |F_o|$$

where F_o refers to the experimentally observed magnitude for a reflection, and F_c refers to the value calculated from the model. Thus, this value is a measure of the percent discrepancy between a measurement and a calculation. One of the advantages of a crystallographic experiment over other analytical means of structure determination is the degree to which the solution is overdetermined. In many cases, the data–to–parameter ratio is on the order of 10:1 or even greater. Also, the utilization of this large amount of data is straightforward. We can feel confident then, that while today's technology can often produce refined crystal structure determinations whose residuals fall below 6% discrepancy, this structure – with an R-factor of 13% and only a 4:1 overdetermination – is certainly correct, and, except in regions of disorder (in this case the solvent), quite reliable.

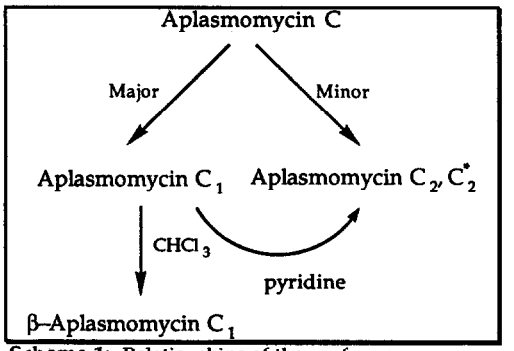
Knowing now that this major component of the closely related pair of isolates was the sodium salt of aplasmomycin C, re-evaluation of the spectral data for the minor component suggested that it was very closely related to the structure we had just solved. In fact, given the molecular formula, we also knew that the crystal structure which we had been unable to solve – the minor component – must contain two independent copies of this molecule, though we knew nothing as yet about their relationship to each other or to the major component. Having observed the similarities of the unit cell dimensions of the crystal

forms studied thus far (Table 1), it was hoped that the molecular conformations of the unknown structure might be at least approximately similar to that of the structure which had already been solved, aplasmomycin C.

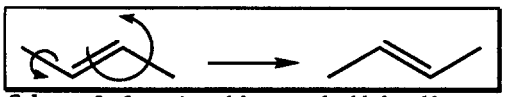
Given this assumption, the method of *molecular replacement* was undertaken. This procedure is one of the most valuable improvements in the solution of crystallographic problems in recent years. Both an advantage and a disadvantage of X-ray crystallography has been the independence of the technique from any prior chemical knowledge. Through *direct methods*, structures may be solved without any chemical information on the part of the crystallographer. On the other hand, it has seemed almost unfair that considerable knowledge about a molecule cannot be put to use in aiding a structure determination by crystallography. For example, a large molecule may have been well characterized at all chiral positions through today's sophisticated NMR techniques except for a single isolated asymmetric center. With direct methods, the crystallographer approaches this problem as though nothing were known.

Molecular replacement attempts to use a molecular structure or fragment to successfully solve a crystal structure. This method depends upon the acquisition of a geometrically accurate model from an X-ray crystal structure or a well-converged molecular mechanics energy minimization (a computational method of molecular representation that has made enormous advances in recent years). The relative positions of the atoms must, within some limit of error, be sufficiently close to their true values that an approximately correct solution may be found.

In the present case, we had available the moderately good crystal structure of the major component, which we hoped shared some geometrical similarity with the minor component based on similar unit cell dimensions and NMR spectra. Using the program PATSEE[18], a preliminary rotational search of the model against the Patterson map of the unsolved data set was performed to determine in what general orientation the molecule might sit in the unit cell. Some 60,000 random orientations of the search model in each case were tested at 7° angular steps. The most promising resultant orientation was then subjected to fine scale searching (3° steps) to find the optimum choices of Euler angles (ϕ_1, ϕ_2, ϕ_3 in three dimensions) to describe the orientation of the search model in the unit cell. The properly oriented model was then used in a translational search to find



Scheme 1: Relationships of the conformers



Scheme 2: Inversion of the trans double bond by concerted free rotation about the C10-C11 and C12-C13 single bonds.

the best position (x, y, z) for the model in the unit cell.

Indeed, this technique led to coordinates for the search model which sufficiently corresponded to those of one of the two molecules in the asymmetric unit that a phasing solution could be obtained by tangent formula recycling. Tangent formula recycling[19] takes phases based on a molecular fragment and improves them to reveal a larger fragment. This is an iterative process whose aim is to take an approximate solution and improve it to the point where the entire structure is revealed. Several cycles of this procedure enabled the calculation of an electron density map which revealed all of the non-hydrogen atoms present in the crystal structure, including those of solvent.

Refinement of this model was pursued by block-diagonal least-squares methods[20] due to the very large number of parameters (≥1,100). All non-hydrogen atoms were refined anisotropically, with the exception of disordered solvent atoms. All hydrogen atoms were generated at geometrically optimum positions and refined isotropically using a riding model. These refinements led to a final crystallographic discrepancy ratio of 0.078. A computer generated perspective drawing of one of the two independent molecules in the asymmetric unit is shown in Figure 3. This molecule is also a sodium salt of aplasmomycin C, but is sufficiently different that

it may be separated from the previously determined structure by chromatography. Clearly a more explicit nomenclature is needed and we have therefore designated the major component as aplasmomycin C_1 (or AC_1), and the minor component as aplasmomycin C_2 (or AC_2).

During this analysis, a successful recrystallization of the major compound, aplasmomycin C_1, from $CHCl_3$/iso-octane by liquid-liquid diffusion gave good quality crystals. Surprisingly, under analysis these crystals now belonged an entirely different crystal class than prior to recrystallization! Least-squares analysis of 40 centered reflections ($10° \leq 2\theta \leq 35°$) gave a refined, *monoclinic* unit cell with $a = 11.341(2)$, $b = 21.119(4)$, $c = 20.576(4)$Å, and $\beta = 99.28(2)°$. We have been unable to regrow crystals of the original orthorhombic form. A total of 6854 unique diffraction maxima were measured (+h,+k,±l), of which 4309 (62.9%) were judged to be observed ($|F_o| \geq 4.0\sigma|F_o|$). This again presented a situation with greater than 100 non-hydrogen atoms (128, in the final analysis) in the asymmetric unit and, with only a moderate percent observed in the data set, a considerable challenge to direct methods strategies. Predictably, as was the case with the minor chromatographic compound, the data proved unyielding to the traditional direct methods approaches to structure solution.

Again, molecular replacement using one of the available crystal structure models for aplasmomycin C was undertaken using PATSEE and Karle recycling and resulted in successful structure determination. The resulting model was also refined by block-diagonal least-squares methods, employing anisotropic heavy atoms and isotropic riding model hydrogens which were generated at geometrically optimum positions. This refinement converged at a standard discrepancy ratio of 0.082. A computer generated perspective drawing of one of these two molecules in the asymmetric unit is shown in Figure 4. Comparison of each of these two molecules with aplasmomycin C_1 shows this crystal form to be a polymorph of aplasmomycin C_1, and so it has been designated β-aplasmomycin C_1, or β-AC_1. By this nomenclature we mean that the molecular conformation is that described as AC_1 (major form), but the organization of these molecules in the solid state is different.

Comparison of each of the crystallographically unique molecules of aplasmomycin C showed a number

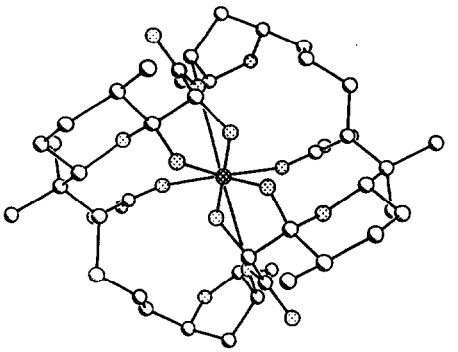


Figure 2: Computer generated perspective plot of aplasmomycin C_1 (4) viewed down the Na–B axis. The enantiomer shown is arbitrary. Hydrogens have been omitted for clarity.

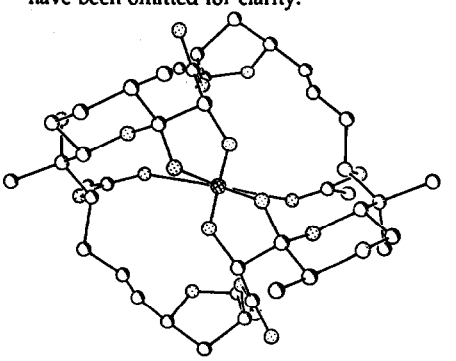


Figure 3: Computer generated perspective plot of aplasmomycin C_2 (4) viewed down the Na–B axis. The enantiomer shown is arbitrary.

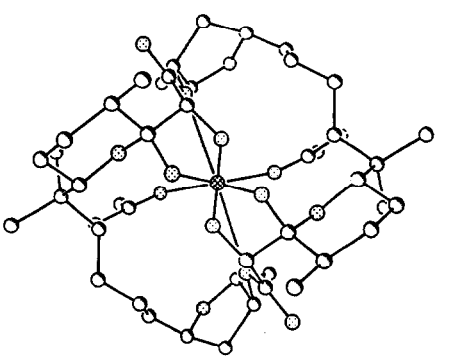


Figure 4: Computer generated perspective plot of ß-aplasmomycin C_2 (4) viewed down the Na–B axis. The enantiomer shown is arbitrary.

Table 2: Selected Torsion Angles

	AC_2	AC_2^*	β-AC_1	β-AC_1	AC_1	DBAC	Silver salt[6]
9-10-11-12	-140.9°	91.3°	-83.6°	-81.9°	-83.6°	109.5°	59°
9'-10'-11'-12'	-167.3°	84.4°	-94.0°	-75.3°	-83.3°	109.5°	12°
10-11-12-13	-166.9°	-173.9°	170.3°	174.3°	168.2°	178.7°	168°
10'-11'-12'-13'	173.2°	176.0°	176.0°	172.5°	176.2°	178.7°	179°
11-12-13-14	67.0°	-143.6°	15.6°	5.4°	15.2°	-5.7°	179°
11'-12'-13'-14'	111.2°	-125.7°	5.6°	-1.3°	5.3°	-5.7°	-153°

of geometric conformers represented in the solid state. One very interesting aspect is the co-crystallization of a pair of atropisomers in the asymmetric unit of the minor component of aplasmomycin C (AC_2). These molecules have been differentiated as *aplasmomycins* C_2 *and* C_2^*, as shown in Scheme 1. The conformers are geometrically similar at all positions except the C11-C12 double bond. While the olefin remains *trans* in all instances, the entire unit has been rotated 180° from one molecule to the next (Scheme 2). A least squares overlay clearly showing the inverse orientations of the C9'-C10'-C11'-C12' regions of the two isomers is shown in Figure 5. The torsion angles throughout this region in each of the conformers are listed in Table 2. Table 2 lists each torsion angle of interest in the left hand column immediately followed by the same angle in the opposing subunit of the same molecule (primed numbers). Individual molecules are listed in successive columns. While the torsional angle defining the olefinic plane is ~180° for each conformer – clearly indicating the trans configuration – in aplasmomycin C_2 the neighboring torsion angles differ in sign for the two conformers reflecting the inverted sense of the carbon chain. While the crystals of this sample grew with a consistent 1:1 pairing of rotomers in each asymmetric unit, *i.e.*, there was no evidence of positional disorder observed in the final difference maps where any residual electron density would be found, there was no indication of a barrier to interconversion between the two form in the NMR studies. The solid state had two distinct molecules, but the solution structure, as judged by NMR, showed only a rapidly averaged structure. The puzzle as to why the crystals grew in this manner is the subject of an ongoing study by computational methods in our laboratories.

An additional point is the degree of similarity of the molecules designated as AC_1 and β-AC_1. A least-squares "best fit" overlay of the single molecule in the asymmetric unit of aplasmomycin C_1 with each of the two molecules in the asymmetric unit of the recrystallized sample, β-aplasmomycin C_1, showed little difference. While the two molecules of β-aplasmomycin C_1 were similar in conformation and differed mostly by non-crystallographic symmetry, each differed from aplasmomycin C_1 in small shifts along the outer loop atoms; the inner core of the sodium borate complex was unchanged. The rms deviations for the least-squares best fit of each of the independent molecules of β-aplasmomycin C_1 to aplasmomycin C_1 were 0.15 Å and 0.27 Å, with the deviations greatest along the outer loop segments. The relative positions of the furan moieties are shifted by an average of ~0.25Å. In a globular structure such as this, these changes may be caused by differences in packing considerations. That these crystals were "built" by a different mechanism of crystal-growth is supported by the different molar composition and location of the solvent molecules included in the crystal lattice. Aplasmomycin C_1 contains 2 chloroform molecules per asymmetric unit, while β-aplasmomycin C_1 contains just one chloroform per asymmetric unit located in the cleft between the two independent molecules and hydrogen bonded to one of the molecules (C1S–H1Sa–O1* 3.27Å; 156.5°). The more severely disordered of the chloroforms in aplasmomycin C_1 forms the same hydrogen bond as in β-aplasmomycin C_1 (C1Sa–H1Sa–O1 2.17Å; 140.7°); while, located in a bridging position between asymmetric units, the well ordered chloroform forms 2 hydrogen bonds (C1Sb–H1Sb–O3a 3.27Å; 173.9° and C1b–H12a–C12 3.73Å; 159.8°). This implies that the crystal lattice of aplasmomycin C_1 is continuously linked with chloroform units "bridging" the asymmetric units, while the crystal lattice of β-aplasmomycin C_1 is comprised of discrete units.

The final and probably most interesting question is how the two conformers, aplasmomycin C_2 and C_2^* (the "minor" component), relate to aplasmomycin C_1 (the "major" component). While aplasmomycin C_1 corresponds in conformation along the outer loop

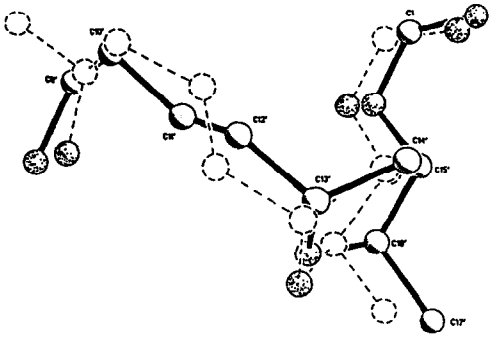


Figure 5: A least-squares best-fit overlap of the C10-C11-C12-C13 regions of AC_2 and AC_2^*.

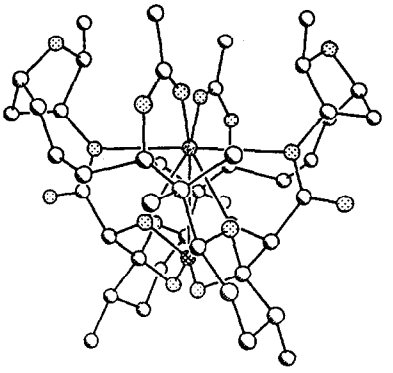


Figure 6: Perspective drawing of AC_1 showing the changes with sodium coordination.

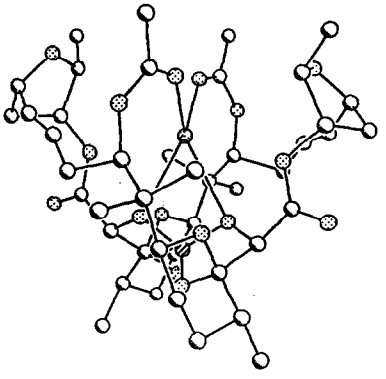


Figure 7: Perspective drawing of AC_2 showing the changes with sodium coordination.

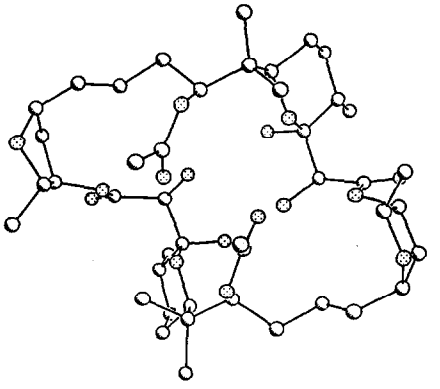


Figure 8: Computer generated perspective plot of deboroaplasmomycin (5). The enantiomer shown is arbitrary. Hydrogens have been omitted for clarity.

to just one of the atropisomers of aplasmomycins C_2, the greatest difference between these conformers lies in the very core of the molecules. As can be seen in the computer generated perspective drawings of the crystallographic models in Figures 6 and 7, the sodium borate salt of aplasmomycin C forms a basket-shaped structure which varies both in the "spread" of the basket opening (the separation between the outer loops) and, concurrently, in the coordination of the sodium ion. For the purposes of our discussion, we have taken the coordination sphere of sodium to include all oxygen atoms within 3.0 Å. Aplasmomycin C_1 and β-aplasmomycin C_1 are the most tightly bound, with six-fold oxygen coordination about the sodium, while aplasmomycins C_2 and C_2^* display a lesser four-fold metal coordination. The variant coordination site involves the drawing in – or releasing – of the ester-linkage oxygen adjacent to C-1 which dictates the separation of the outer loops at the top of this basket-like structure. For example, the average separation of the furan rings (Table 3) is ~6Å in aplasmomycin C_1 and β-aplasmomycin C_1, while in aplasmomycin C_2 and C_2' this distance is nearly 7Å.

This understanding of the various crystal forms observed for the sodium salts of aplasmomycin C sheds some light upon the solvent dependent behavior previously observed by NMR[6,10,13,16]. As can be seen in Figures 9 and 10, the ^{1}H-NMR spectra of aplasmomycin C_1 and aplasmomycin C_2 are clearly distinct. When aplasmomycin C_1 is removed from $CDCl_3$ and placed in pyridine, a quite different spectrum results (Figure 11). On complete removal of the pyridine and return of aplasmomycin C_1 to $CDCl_3$ (Figure 12) the resulting spectrum matches that of aplasmomycin C_2 within experimental error. It may therefore be hypothesized that pyridine promotes the interconversion of aplasmomycin C_1 to aplasmomycin C_2, perhaps by occupying a coordination site of the sodium ion. This process is also currently under study in our laboratories by molecular modeling studies.

To complete the series, a study of the crystal structure of deboroaplasmomycin C (5) was also undertaken by X-ray diffraction. Deboroaplasmomycin C (DBAC) crystallized as exquisitely well formed diamond-shaped flat plates. Observation of the crystals under a polarizing light microscope showed the majority of the highest quality crystals to be a composite of two plates grown together in different alignments. A preliminary rotation photograph confirmed this analysis with two discrete spots observed for each diffraction maximum.

	Na^+ Coord.	"Basket Spread"	Na^+–B (Å)
AC_1	6	5.96Å	2.94Å
β-AC_1	6	6.18Å	2.90Å
β-AC_1'	6	6.38Å	2.88Å
AC_2	4	6.80Å	3.17Å
AC_2^*	4	6.70Å	3.40Å

Table 3: Conformation Changes with Sodium Coordination

Single crystal X-ray diffraction relies on the sample being composed of a single discrete orientation of the crystal lattice. Fortunately, in this case, the different crystalline domains were not interpenetrating and with very careful cutting along the interface, a single half of the composite was obtained (*ca.* 0.5 x 0.4 x 0.1 mm) and successfully used for data collection. Centering on 20 reflections with $10° \leq 2\theta \leq 25°$ gave a refined, C-centered, monoclinic unit cell with parameters: a = 19.310(6), b = 9.893(2), c = 12.135(4)Å, and β = 104.91(2)°. All unique diffraction maxima with +h, +k, ±l, h+k even, and $2\theta \leq 115°$ were collected using θ-2θ scans. Of the 1590 unique reflections measured in this manner, 1547 (97.3%) were judged observed ($|F_o| \geq 4.0\sigma|F_o|$). The space group was uniquely determined to be C2 (Z=1), therefore the asymmetric unit consisted of one-half the molecule. The structure was solved uneventfully with SHELX-86, and refined with full-matrix least-squares minimization of the quantity $\Sigma\omega(F_o-F_c)^2$ to a standard crystallographic discrepancy ratio of 0.0452. A computer generated perspective drawing of the entire molecule is shown in Figure 8. The molecule contains a 2-fold symmetry axis at the approximate center of mass.

In summary, we have been able to use the techniques of direct methods and molecular replacement to study several moderately large crystal structures (>125 atoms/asymmetric unit) which in combination with NMR spectral analysis has allowed us some insight into the conformational complexity of this system of ionophore antibiotics. What began as an almost casual investigation of the structure of an old molecule by new techniques has opened up a new and wholly unexpected vista of aplasmomycin chemistry. We have seen – using X-ray crystallography – some of the stable positions of aplasmomycin C and can now begin to ask how the molecule travels among these conformational minima.

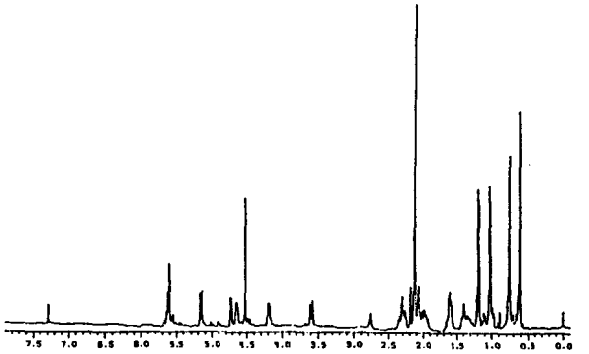


Figure 9: ^{1}H-NMR spectrum of aplasmomycin C_1 in $CDCl_3$

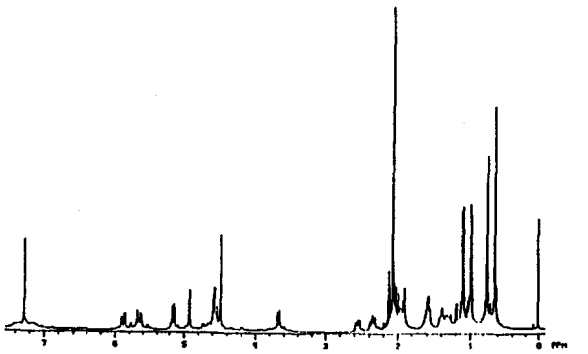


Figure 10: ^{1}H-NMR spectrum of aplasmomycin C_2 in $CDCl_3$

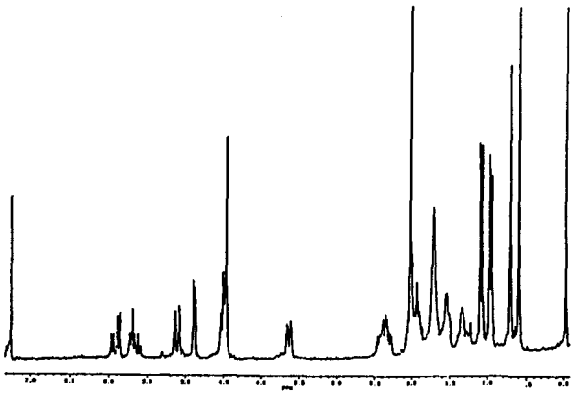


Figure 11: ^{1}H-NMR spectrum of aplasmomycin C_1 in pyridine

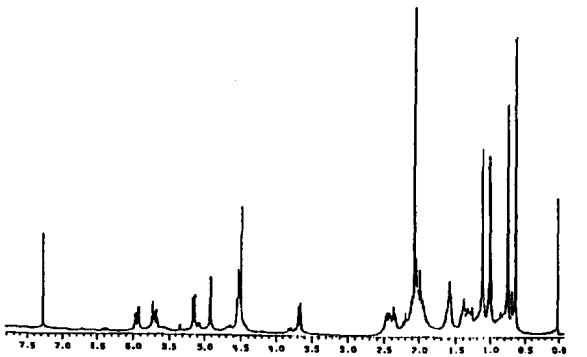


Figure 12: ^{1}H-NMR spectrum of the product obtained by treating AC_1 with pyridine, and then returning to $CDCl_3$ solution.

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