of carbon atoms in the skeleton is possible. Therefore, INFER2D attempts no further substructural analysis and forwards the signal connectivity directly to COCOA. In running this problem, there was no editing of the ACF shortlist and no user-defined substructures were entered. SESAMI produced seven structures (Figure 7), all of which conform to the observed carbon–carbon signal connectivity. Structure **1** was assigned by Olah.[21] (The same seven structures were produced by INTERPRET2D/ASSEMBLE2D using the same input information.[4])

Conclusions

It has been demonstrated that the addition of 2-D NMR data interpreting capability (INFER2D/2-DNMRPRUNE) to SESAMI, a computer-based system of structure elucidation, substantially enhances its power to solve real-world structure problems. A significant advantage of INFER2D is that it allows the structural implications of *all* through-bond signal correlations, including the all-important long-range carbon–hydrogen signal correlations, to be utilized. Inherent in the commonly encountered, large set of long-range correlations is substantial ambiguity, often more than the chemist can contend with if the information is to be used to directly construct compatible structures. SESAMI's ability to do so is made possible by a new structure generating procedure (COCOA) that prospectively utilizes alternative interpretations of spectroscopic data. The same procedure permits SESAMI to readily accomodate unknowns possessing molecular symmetry. The power of SESAMI is further enhanced by its interactive nature. Information known to the user may be conveniently communicated to the program.

Although SESAMI already possesses some problem-solving capability, considerable enhancements to it are envisaged and made possible by an underlying framework for computer-enhanced structure elucidation of considerable promise.

Experimental Section

NMR spectra for the Wasserman compound were recorded on a Bruker Model AM 400 spectrometer (400 MHz for ^1H and 75 MHz for ^{13}C). Multiplicities of ^{13}C NMR signals were determined from APT data. Standard pulse sequences were used for the ^1H–^1H COSY, ^1H–^{13}C COSY, long-range ^1H–^{13}C COSY, and HMBC experiments.

INFER2D is written in FORTRAN. Earlier program development was on a Prime 450 minicomputer dedicated to this project. Currently program development is continuing on a VAXstation 3500 running under VMS 5.1. COCOA is a Pascal program.[16]

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Structure Elucidation of a Novel Antibiotic of the Vancomycin Group. The Influence of Ion–Dipole Interactions on Peptide Backbone Conformation

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Abstract: UK-69542, a novel glycopeptide belonging to the vancomycin group of antibiotics, has been isolated from *Saccharothrix aerocolonigenes* fermentation broth and has had its structure determined by a combination of fast atom bombardment mass spectrometry (FABMS) and two-dimensional NMR. The aglycon of the antibiotic is identical with that of aridicin but contains novel groups attached to the peptide core. Proton NMR studies revealed that this novel antibiotic exists in two conformational forms in DMSO solution. The use of NOESY experiments implicated a cis to trans amide bond isomerization as the cause of the conformational difference. This marked conformational change observed for UK-69542, but not for aridicin, is deduced to arise from a charge–dipole interaction involving an aryl sulfate ester; this functional group is not present in aridicin. The observation of this change highlights the ability of electrostatic interactions to stabilize polypeptide secondary structure.

Introduction

The vancomycin group of antibiotics have received considerable attention in recent years owing to the lack of bacterial strains developing resistance to their antibiotic activity.[1] For this reason, vancomycin—the original member of the group to be isolated and brought into clinical use—has become the main line of defense against methicillin resistant *Staphyloccocus aureus* infections.[2] Antibiotics belonging to this group of antibiotics have been isolated from *Actinomycete* cultures obtained from soil samples from diverse geographical locations. The pharmaceutical industry has been actively involved in isolating these novel antibiotics in the hope of finding compounds either with increased efficacy, with a wider spectrum of activity, or with fewer side effects than those currently in use. The determination of structure for the novel antibiotics of the group[3] and the preparation of semisynthetic derivatives[4] have helped to determine which structural features are needed for the antibiotics to bind to peptide analogues of bacterial cell wall components.[5] More importantly, NMR ex-

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periments have allowed the determination of molecular geometry for the complex between the antibiotics and peptides terminating in D-alanyl-D-alanyl[6] (bacterial cell wall biosynthesis is inhibited by the antibiotic binding to such peptides in nascent peptidoglycan[7]).

The structure of vancomycin was determined by a combination of chemical degradation,[8] NMR,[9] and X-ray crystallography.[10] However, other antibiotics in this family have not given rise to crystals of quality suitable for X-ray analysis; hence, their structures have been determined by NMR and chemical methods. A comparison of the NMR data for various antibiotics of this type (especially the short interproton distances determined from the nuclear Overhauser effect on NOE) suggested that their C-terminal residues were very similar.[11] The realization that residues two, four, five, six, and seven are highly conserved in the nature of their side chains and side chain cross linkages has simplified the analysis of the NMR spectra of novel antibiotics. In many cases, it is possible to determine the structure of such antibiotics with a high degree of certainty by proton NMR alone, even though a completely rigorous determination would involve a large amount of chemical degradation studies or extensive ^{13}C NMR spectroscopy. Such an NMR-based determination of structure has been applied in this paper to the novel glycopeptide antibiotic UK-69542. The antibiotic has been isolated from the supernatant liquid of a *Saccharothrix aerocolonigenes* fermentation and purified by a combination of affinity chromatography,[12] ion exchange chromatography, and reversed-phase HPLC.

The proton NMR assignment procedure relies on the identification of the peptide backbone and aromatic side chain *J*-coupling networks with use of two-dimensional double quantum filtered correlated spectroscopy (DQF-COSY). These spin systems are then connected together with use of the short-range (less than 5 Å) proton–proton contacts obtained from two-dimensional nuclear Overhauser enhanced spectroscopy (NOESY). At this stage, knowledge of the structures of known antibiotics can be of assistance in using the NOE information to formulate the spin systems into a complete molecule. The dependence of NOE size on irradiation time for vancomycin[9b] and molecular modeling studies on aridicin[13] have shown that the observed NOEs are consistent with one particular set of stereochemistries and side-chain cross linkages. The nomenclature used to describe the proton resonances of UK-69542 is similar to that used for teicoplanin[14] and A-40926.[15] The protons are given a two-symbol code indicating their residue (numbered from the N terminus) and a letter corresponding to their position in the residue (w = amide, x = α proton, and z = β proton; ring positions labeled alphabetically from the carbon attached to the backbone—see Figure 5 below). The "front" face of the antibiotic refers to the uppermost face when the N terminus is placed on the right-hand side, with the side chains of residues two, four, and six forming the top edge of the molecule.

Results

Analysis of UK-69542 by positive-ion FABMS did not reveal the intense molecular ions usually observed for antibiotics of this type.[16] Weak ions could be observed at m/z 1410 and 1490 with very acidic matrix conditions (thioglycerol with added trichloroacetic acid). In negative-ion mode, a very abundant molecular ion was observed at m/z 1488. Previous experience in this laboratory suggested that this unusual behavior may be due to the presence of a sulfate ester within the antibiotic.[17] One other glycopeptide antibiotic of the vancomycin group containing such a functional group has been reported.[18] In the negative-ion spectrum, fragment ions were also observed at m/z 1454 (loss of 34 mass units due to replacement of aromatic chlorine by hydrogen from the matrix) and 1374. The NMR data indicated that this latter fragment was due to loss of a 2,3,6-trideoxy sugar. At the conclusion of the NMR analysis, a molecular formula of $C_{65}H_{55}O_{24}N_7S_1Cl_4$ could be proposed, consistent with the observed molecular weight of 1489 mass units.

Proton NMR Studies. The next stage in the structural investigations on UK-69542 involved proton NMR studies. Previous studies on these antibiotics have employed deuterated DMSO as solvent due to its ability to dissolve polar molecules while preserving their amide proton resonances. It was noted that a freshly dissolved sample of the antibiotic in DMSO at room temperature contained resonances of narrow line width (about 2–3 Hz), which were well-dispersed over the range 0–10 ppm. However, over a period of minutes, the intensity of the initial set of resonances decreased and a second set of resonances appeared. After 60 min, the two sets of resonances were of approximately equal intensity (removal of the DMSO or storage of the sample in solid DMSO at −10 °C for 5–6 days produced a temporary reduction of the second set of signals). HPLC analysis of this sample, with use of a variety of solvent conditions, revealed that a single species was present. These two observations can be explained in terms of a conformational change rather than a decomposition. The change is slow on the NMR time scale (hence, the two sets of sharp resonances) but fast compared to the time taken for HPLC analysis. Further evidence against the two sets of signals arising from an epimerization of the backbone is given below.

The slow nature of the exchange on the NMR time scale was emphasized by conducting variable-temperature studies on the antibiotic in DMSO solution. Heating to 350 K produced a sharpening of all resonances, with no sign of a change from slow to intermediate exchange. It was found that maintaining the sample at this elevated temperature for more than 30 min caused a further set of resonances to occur. HPLC carried out on this sample did reveal the presence of a second component of longer retention time than the pure antibiotic. This peak of longer retention time was analyzed by FABMS and was also found to produce negatively charged ions at 1488 mass units, indicating that the heating had produced an isomerization or epimerization of the antibiotic. The decomposition product was not analyzed further, and all subsequent NMR was conducted on the parent antibiotic at room temperature. In the following discussion, UK-69542-A refers to the conformer whose resonances were observed in a freshly dissolved sample of DMSO, while UK-69542-B refers to the conformer whose resonances appeared over the 60 min following dissolution.

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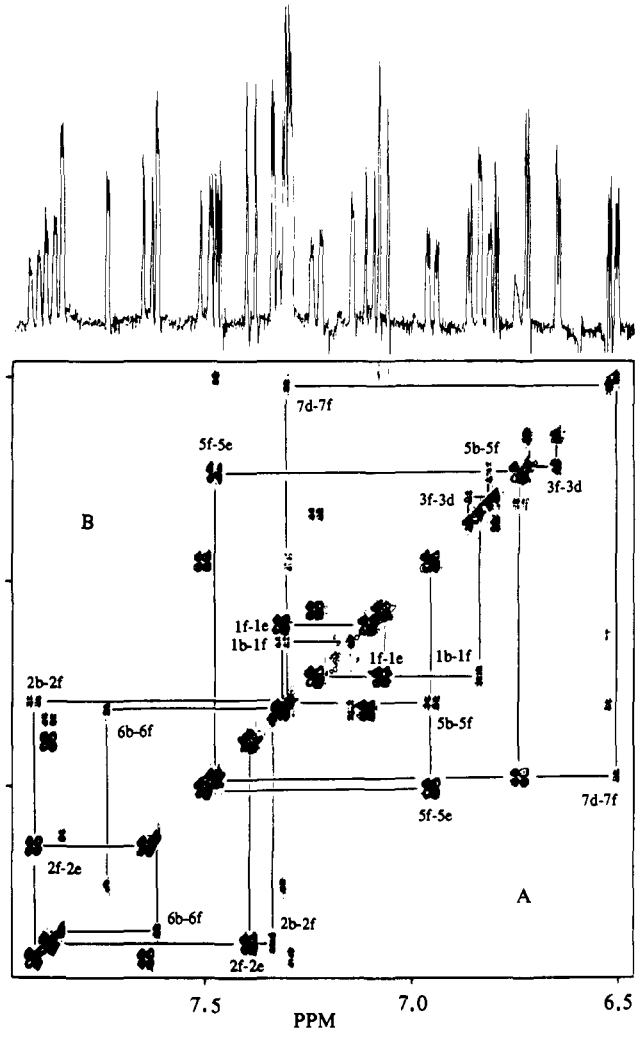

Figure 1. Section of the DQF-COSY of UK-69542 in (d_6)DMSO containing 10% CD_3OD at 400 MHz and 300 K containing the 12 most downfield aromatic spin systems. The couplings within UK-69542-B are marked in the top left-hand portion, while those of UK-69542-A are marked in the lower right section. The peaks of negative phase are shaded. Above the contour plot is the relevant section of the one-dimensional proton spectrum.

The analysis of the proton NMR spectrum was begun by acquiring of DQF-COSY of UK-69542 in (d_6)DMSO, which revealed a wealth or aromatic and amide NH–αCH spin systems. A further COSY was acquired after the addition of 50 μL of CD_3OD to the 500 μL of DMSO. This had the advantage of removing all amide protons by isotopic exchange; hence, only cross-peaks arising from aromatic protons remained in the downfield region of the spectrum. By elimination, any other peaks seen in the first COSY must have arisen from backbone connectivities. The result of this process was to locate 14 aromatic spin systems and 12 NH–αCH couplings. (A section of the COSY revealing the 12 most downfield spin systems is depicted in Figure 1). Thus, the presence of two species was confirmed, with both species consisting of heptapeptides containing seven aromatic side chains. Despite the presence of two antibiotics, the separation of cross-peaks in the DQF-COSY suggested that it was possible to assign the spectra of both species simultaneously. To aid this assignment, a phase-sensitive NOESY was acquired. As discussed below, the NOESY data also allowed a determination of the conformational differences between the two molecules.

Structure of UK-69542-A. The assignment of the resonances of UK-69542-A was initiated from the methyl singlet at 2.51 ppm. The chemical shift of this resonance is characteristic of the N-methylated N-terminal group commonly found in this group of antibiotics.[9b] Proton x1 was located by an NOE from the *N*-methyl group, and the ring protons 1f and 1b were located by their

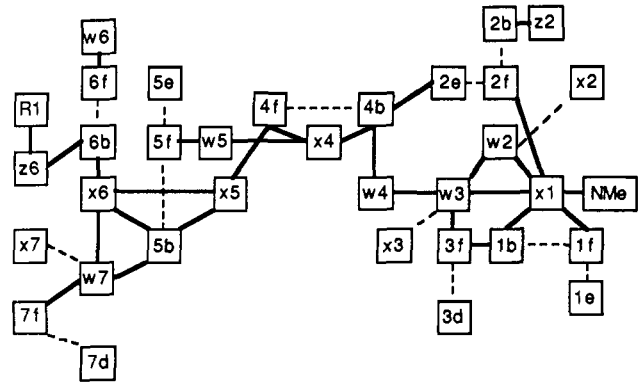

Figure 2. Schematic representation of the *J* coupling and NOE networks used to assign the aglycon resonances of UK-69542-A. Broken lines represent scalar (*J*) coupling, while solid lines represent dipolar (NOE) coupling.

NOE to x1. Ring 1 was found to contain a 1,3,4-trisubstituted aromatic ring. Interresidue NOEs from x1 and w2 and to 2f allow the resonances of residue 2 to be assigned. The couplings from w2 and 2f indicate that residue 2 is a β-hydroxytyrosine, with aromatic chlorination at the rear of the ring (position 2c).

The resonances of ring 3 were located by an NOE from 1b to 3f; this NOE arises because of the biphenyl ether linkage between ring 1 and ring 3 usually found in these antibiotics. Proton 3f was found to have a single coupling partner, indicating that ring 3 contains four substituents. The ring proton meta-coupled to 3f has no NOEs to the backbone protons of residue 3 indicating that it is in position 3d. The lack of a proton in position 3b can be ascribed to aromatic chlorination in this position. Such substituents have been observed previously in aridicin,[13] the kibdelins,[19] and A-40926.[15] The ring-4 spin system was located by a small NOE from 2e to 4b, while the residue-4 amide proton was located by an NOE from w3 to w4. The unusually low chemical shifts of protons 4b and 4f (5.82 and 5.15 ppm, respectively) result from the ring current shifts of ring 2 and ring 6.[20]

The resonances of the C-terminal residues were assigned by a series of couplings and NOEs, starting with the NOEs from 4f to x5 and from x4 to 5f. Proton x5 has an intraresidue NOE to 5b, and this latter resonance then has NOEs to x6 and w7. Couplings and intraresidue NOEs allowed the remaining protons of UK-69542-A to be assigned. Residue 6 was found to contain two aromatic chlorines (in positions 6c and 6e), while proton z6 had an NOE to a doublet at 4.59 ppm. This latter resonance is the anomeric proton of a deoxy sugar; the NOE to z6 indicates that it is attached to the benzylic hydroxyl of residue 6. The coupling connectivities and NOEs used in the assignment of the aglycon resonances are shown in Figure 2. The result of this analysis reveals that the aglycon of UK-69542-A is identical with those of the aridicins[13] and the kibdelins.[19] The chemical shifts and coupling constants of UK-69542-A and aridicin A are compared in Table I. The remaining ambiguities in the structure lie in the nature of the sugar attached to residue 6 and the location of the aromatic sulfate ester.

The sugar first was dealt with first; couplings could be observed from the sugar anomeric proton to two high-field protons, indicating a 2-deoxy sugar. Further couplings from this methylene group were restricted to the upfield region of the spectrum, implying that position 3 was also deoxygenated. Analysis of a DQF-COSY with relatively high digital resolution (1.2 Hz/point in f_2 and 4.7 Hz/point in f_1) allowed a proper assignment of all the geminal and trans diaxial couplings in this region. The sugar was found to be a 2,3,6–trideoxy species; the chemical shifts are listed in Table II. The relative stereochemistries of the sugar

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Table I. Proton Chemical Shifts (ppm) and Coupling Constants (Hz) for Aridicin, UK-69542-A, and UK-69542-B in (d_6)DMSO-Based Solvents[a]

	UK-69542-A (DMSO)		UK-69542-B (DMSO)		Aridicin (DMSO)		UK-69542 (D_2O/CD_3CN)
assgnt	chem shift	coupling	chem shift	coupling	chem shift	coupling	chem shift
NMe	2.51	s	2.55	s	2.51	s	
x1	5.32	s	5.38	s	5.41	s	5.33
1b	6.78	d 2.0	7.06	d 0	6.83	d 2.2	7.20
1e	7.03	d 8.2	7.10	d 8.2	7.01	d 8.5	7.15
1f	7.20	dd 0	7.28	dd 0	7.21	dd 8.5, 2.2	7.37
w2	7.92	d 0	8.28	d 7.3	7.79	d 8.2	
x2	5.09	C	5.05	C	5.20	dd 6.6, 6.5	5.16
z2	5.10	C	5.10	C	5.20	d 6.6	5.32
2b	7.24	d 0	7.19	d 0	7.25	d 2.0	7.39
2e	7.28	d 8.2	7.55	d 8.2	7.21	d 8.5	7.37
2f	7.83	dd 8.2, 2.0	7.87	dd 8.2, 2.0	7.83	dd 8.5, 2.0	7.78
w3	7.78	d 10.3	5.83	d 10.3	7.55	d 10.5	
x3	5.98	d 10.3	5.83	d 10.3	6.08	d 10.5	5.97
3d	6.63	d 2.9	6.69	d 2.9	6.65	d 2.5	6.81
3f	6.57	d 2.9	6.79	d 2.9	6.51	d 2.5	6.25
w4	7.10	d 8.2	6.65	d 7.9	6.95	d 8.0	
x4	5.75	d 8.2	4.96	d 7.7	5.64	d 8.0	5.44
4b	5.82	b s	5.55	d 1.2	5.86	d 1.8	5.90
4f	5.15	d 0	5.76	d 1.8	5.12	d 1.8	5.19
w5	8.78	d 5.8	9.40	d 9.5	8.41	d 5.8	
x5	4.38	d 5.8	5.13	d 0	4.42	d 5.8	4.53
5b	7.13	d 0	6.69	d 0	7.12	d 2.2	7.25
5e	7.40	d 8.8	7.38	d 8.5	6.74	d 8.0	7.55
5f	6.81	dd 8.8, 2.3	6.53	dd 8.5, 2.3	6.78	dd 2.2, 9.0	6.80
w6	6.95	d 11.7	6.16	d 9.6	6.58	d 12.0	
x6	4.13	b d 0	5.18	m	4.42	dd 1.1, 12.0	4.19
z6	5.10	d 0	5.10	C	5.10	d 1.1	5.20
6b	7.64	d 0	7.25	d 0	7.78	d 1.8	7.69
6f	7.74	d 1.8	7.63	d 1.8	7.57	d 1.8	7.61
w7	8.28	d 5.6	8.98	d 5.8	8.36	d 5.8	
x7	4.63	d 5.6	4.58	d 5.6	5.53	d 6.0	4.70
7d	7.38	d 2.3	7.22	d 2.3	6.43	d 2.0	7.28
7f	6.36	d 2.3	6.39	d 2.3	6.33	d 2.0	6.68

[a] Key for coupling constants: s, singlet; d, doublet; m, multiplet; b, broad; C, almost coincident in chemical shift with coupling partner; and 0, coupling observed in COSY, but partial coincidence prevents measurement of coupling constant. Data for Aridicin in DMSO and UK-69542 in aqueous solvent are also included for comparison.

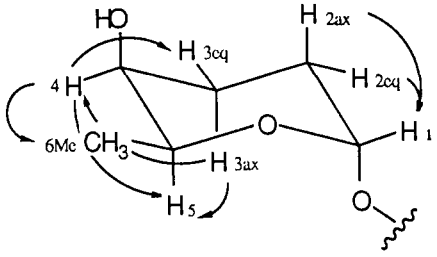

Figure 3. Structure of the trideoxy sugar found in UK-69542. The arrows show the NOEs, which define the relative stereochemistries.

carbons were determined by the NOEs shown by arrows in Figure 3. The FAB fragment ion 129 mass units below the molecular ion is consistent with the loss of a group of empirical formula $C_6H_{11}O_2$, obtained by cleavage of the glycosidic bond of this trideoxy sugar.

Comparison of the aglycon chemical shifts of UK-69542 and aridicin provided a means of positioning the aryl sulfate ester (Table I). It had been previously noted that the presence of an aryl sulfate ester on ring 1 of A-47934 produced a downfield shift of more than 0.6 ppm for proton 1e (adjacent to the site of attachment of the sulfate) relative to the antibiotic without the sulfate ester.[18] The chemical shifts of 7d and 5e had moved downfield by 0.99 and 0.73 ppm, respectively, in the case of UK-69542-A. Location of the sulfate at position 5d or 7c would allow the deshielding effect on protons 7d and 5e to occur. This ambiguity was resolved by using ^{13}C NMR spectroscopy. It was reasoned that the ^{13}C chemical shifts of the aromatic rings would be less influenced by through-space shielding effects and that any changes in chemical shift would arise mainly through changes in substituent on the same ring. The carbon NMR spectrum of

Table II. Proton Chemical Shifts of the Trideoxy Sugar Pesent in UK-69542[a]

proton	UK-69542-A	UK-69542-B
	δ	δ
R1	4.59	4.61
$R2_{eq}$	1.54	1.30
$R2_{ax}$	1.88	1.85
$R3_{eq}$	1.45	1.47
$R3_{ax}$	1.87	2.09
R4	3.28	3.44
R4OH	4.30	4.30
R5	3.72	4.05
R6Me	1.04	1.09

[a] The shifts were measured on a DMSO solution of the antibiotic at 500 MHz and at a temperature of 300 K.

UK--69542 was partially assigned with use of a proton-detected one-bond C–H correlation experiment (HMQC).[21] A comparison of the ^{13}C chemical shifts of UK-69542 (A and B) with the corresponding shifts of the teicoplanin aglycon is made in Table III. The expected changes in ring carbon resonance position on sulfonation of a phenolic hydroxyl have been reported previously and are included in the notes to Table III.[22] The large downfield shift of 5e (4.1 ppm) and only slight changes of 7d (1.5 ppm) and 7f (1.9 ppm) are consistent with attachment of the sulfate to position 5d.

The HMQC spectrum itself was recorded with a 6-mg sample of the antibiotic in 500 μL of DMSO. This corresponds to a concentration of 4 mM for each of the antibiotic species present.

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Table III. Carbon Chemical Shifts of UK-69542,5-A and -B[a]

carbon	UK-69542-A	UK-69542-B	teico planin
x1	62.5	62.5	54.6
x2	61.0[1]	61.9[1]	55.6
z2	70.3[2]	70.3[2]	na
x3	53.8	54.3	58.2
x4	53.8	55.0	54.7
x5	53.3	56.5[1]	53.6
x6	60.6	56.5	71.7
z6	70.3[1]	73.4[1]	71.5
x7	56.5	55.3	56.6
1b	120.0	118.5	120.0
1e	118.8	117.8	118.3
1f	127.5[4]	127.8[3]	126.7
2b	130.3[3]	130.3[4]	131.0
2e	124.7[3]	128.3	124.8
2f	126.7	127.2	130.6
3d	106.0	105.4	105.0
3f	104.8	103.2	102.6
4b	108.7	105.4	107.7
4f	104.2	104.6	104.5
5b	134.6	130.4	135.6
5e	121.0	122.2	116.6
5f	125.6	124.1	125.8
6b	127.5	128.4[3]	127.2
6f	124.7	127.5	127.4
7d	105.2	106.5	103.7
7f	107.7	108.0	105.8

[a] Values for teicoplanin aglycon (reference) are included for comparison. Values are in parts per million relative to (d_6)DMSO at 39.9 ppm. Assignments bearing the same superscript (1, 2, 3, or 4) have protons sufficiently close in chemical shift that the ^{13}C signals cannot be unambiguously defined. In these cases, assignments have been tentatively made on the basis of ^{13}C chemical shift and comparison with teicoplanin. The expected chemical shift changes for the addition of a sulfate ester to a phenolic hydroxyl are C_1, −2.8; ortho, +7.8; meta, +1.6; and para, +7.4 (ppm (from ref 22)).

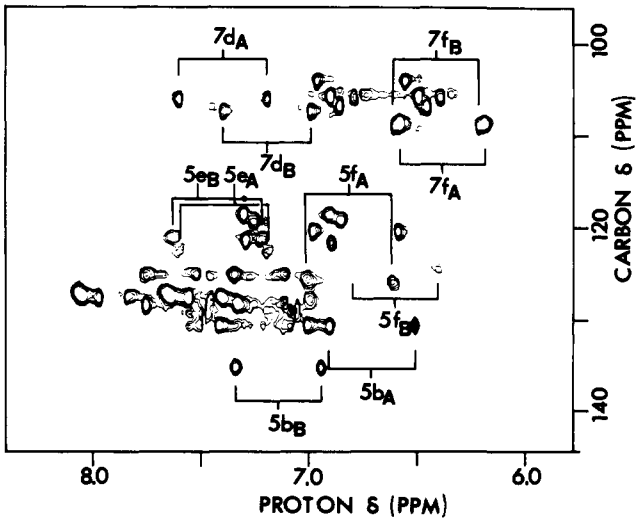

Figure 4. Section of the proton-detected heteronuclear multiple quantum filterd correlation spectrum (HMQC) obtained on an 8 mM sample of UK-69542 in DMSO at 400 MHz (4 mM with respect to the A and B forms of the antibiotic). The region shows the correlations between aromatic proton and carbon resonances. The cross-peaks arising from rings 5 and 7 are labeled.

The section of the two-dimensional spectrum containing correlations between the aromatic proton and carbons is shown in Figure 4. The correlations to the protons of rings 5 and 7 are marked in this diagram. Despite the low sample concentration, the signal:noise ratio obtainable from the proton-detected correlation experiment (at 400 MHz with a total acquisition time of about 14 h) clearly allows the carbons directly bonded to protons to be assigned.

Further evidence of the location of the sulfate on ring 5 was achieved by magnetization transfer from the residual water peak in the DMSO to the phenolic hydroxyl protons. This has the effect of partially saturating the phenolic hydroxyl protons, which in turn produces an NOE to all ring protons adjacent to the hydroxyls.[14a] When the data obtained with irradiation of the water peak were subtracted from a control data set, the resulting spectrum contained large peaks due to the hydroxyl protons attached to sp^3 carbons and less intense NOE to protons 7d, 7f, 1f, and 3d but none to 5e. This situation indicates that position 5d is the only acceptable candidate for location of the sulfate. The complete structure of UK-69542-A is shown in Figure 5.

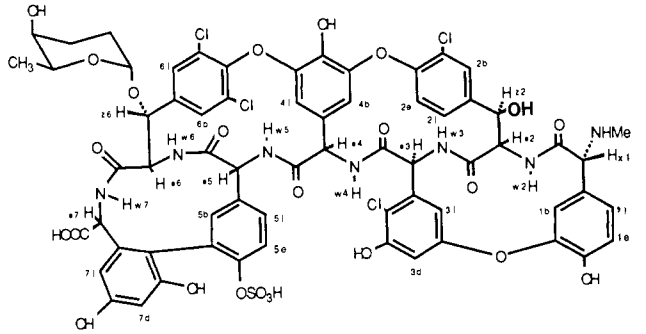

Figure 5. Structure of UK-69542-A.

Table IV. Proton Resonances Differing in Chemical Shift by More Than 0.4 ppm in UK-69542-A and UK-69542-B

resonance	UK69542-A	UK69542-B	Δ
w4	7.10	6.65	−0.45
x4	5.75	4.96	−0.79
4f	5.15	5.76	+0.61
w5	8.78	9.40	+0.62
x5	4.38	5.13	+0.75
5b	7.13	6.69	−0.44
w6	6.95	6.16	−0.79
x6	4.13	5.18	+1.05
6b	7.13	6.69	−0.44
w7	8.28	8.98	+0.70

Structure of UK-69542-B. Inspection of the half-assigned DQF-COSY spectrum revealed that all the spin systems present in UK-69542-A were duplicated in the resonances of the B form. Once again, this indicates that the B from differs in terms of stereochemistry or conformation. An analysis of the NOE data for UK-69542-B was initiated from the N-terminal methyl group. For residues 1, 2, 3, and 4, the interresidue NOEs were cmoparable with those found in the A form; hence, their assignment was relatively straightforward. In fact, the chemical shifts of residues 1, 2, and 3 were also similar in both forms (Table I). This was not found to be the case for the residues in the C-terminal half of the molecule.

By comparison of coupling networks and to a lesser extent chemical shifts, the three remaining aromatic spin systems could be assigned to residues 5, 6, and 7 of the B molecule. Backbone resonances for these residues were assigned by intraresidue NOEs. The major differences between the A and the B forms lie in the interresidue NOEs at the C-terminal end of the molecule and in the chemical shifts of some protons in these residues. The drastic changes in chemical shift listed in Table IV suggested that an alteration in the backbone was taking place in the region of residues 4, 5, and 6. This change caused x6 to move out from underneath ring 6 into a less shielded environment, hence, its increase in chemical shift; the opposite effect took place with w6. the change also caused ring 4 to rotate about the α-carbon-ring bond, changing the shielding effects and relative chemical shifts of 4b and 4f. The change in the backbone is also reflected by the ^{13}C chemical shifts of the α-carbons of residue 4, 5, and 6 (Table III).

The section of the COSY used in the analysis of the sugar attached to UK-69542-A also shows the correlations within the sugar of UK-69542-B. The couplings are duplicated in the second form of the antibiotic. Hence, the trideoxy sugar is not the source of difference between the antibiotics. In the light of the proton

Table V. Selected NOEs for the C-Terminal Region of UK-69542-A and -B

proton	UK-69542-B NOE size	UK-69542-B NOE size	aridicin NOE	aridicin distance
x4–w6	z	s		
x4–w5	l	l	l	2.39
w4–4b	m	l	s	3.15
x5–4f	m	z		
x5–x6	l	c	l	1.83
x5–5b	l	m	l	2.36
x5–6b	m	0	s	3.91
x5–w7	m	s	m	3.25
x6–5b	l	m	s	2.54
x6–w7	m	m	m	2.11
w6–w5	z	l		
w6–4f	z	m		
w6–5f	z	s		
w6–6f	l	m	s	2.73
w7–5b	l	l	m	1.88
w7–6b	m	s	s	3.90
w5–4f	l	m	s	3.10
5b–6b	m	z		
5b–4f	s	z		

[a] NOE sizes: zero; s, small; m, medium; l, large; 0 = cross-peak coincidence with other cross-peak; c protons too close in chemical shift to determine their NOE size. Also included are the NOEs and molecular modeling derived distances observed for aricidin (ref 13).

assignment, it was possible to assign partially the carbon spectrum of UK-69542-B from the HMQC spectrum. The chemical shifts of 5e, 7d, and 7f are very similar in the A and B forms, suggesting that, in both cases, the sulfate is attached to ring 5 (Table III). Once again, no NOE was observed to 5e on saturation of the phenolic hydroxyl protons by irradiation of the residual water peak in the DMSO.

Jeffs et. al. have performed a series of molecular mechanics calculations on a large number of the possible epimers of aridicin.[13] They concluded that the crude assessment of interproton distances available from NOESY spectra permitted only one set of relative stereochemistries for the α-carbons of residues 2, 3, 4, 5, and 6. For these calculations, all amide bonds were assumed to be trans, except that between residues 5 and 6. The cis nature of this bond had been noted in the crystal structure of vancomycin (CDP-1)[10] and was supported by the NMR data on aridicin.[13] The sizes of NOEs observed in aridicin and UK-69542-A are in close agreement, and pinpoint the stereochemistries as *R*, *R*, and *S* for the α-carbons residues 4, 5, and 6, respectively. However, none of the trial structures of Jeffs have distances consistent with the NOEs observed for UK-69542-B (Table V). All of the structures contain at least one distance less than 2.5 Å where a weak or zero NOE occurs or one distance greater than 3.0 Å where a large NOE is observed. This information confirms that the difference between UK-69542-A and -B does not arise from a backbone epimerization.

The NOEs listed in Table V provide the greatest information about the changes taking place in UK-69542-B. Figure 6 and 7 show sections parallel to the f_2 axis of a NOESY spectrum of UK-69542 at f_1 frequencies corresponding to various resonances. Figure 6a–d shows the NOEs in UK-69542-A and -B from x4 and w6; both of these protons are at the rear of the molecule. It can clearly be seen that $w6_B$ has large NOEs to 6f, w5, 4f, and x4, while $w6_A$ only has an NOE to 6f. Figure 7a–d shows the NOEs for 5b and w7, both of which are at the front of the molecule. On going from the A to the B form, the NOEs 5b ↔ 6b, 5b ↔ 4f, and w7 ↔ x5 are substantially decreased in intensity. Figure 7d also shows that the x6/z6 coupling constant has increased sufficiently in UK-69542-B to produce a noticeable doublet structure for z6 and a triplet appearance for x6 (actually a double doublet).

In UK-69542-A, the carbonyl of residue 5 intervenes between w6 and the other protons at the rear left-hand side of the molecule (Figure 8a). There is an obvious need to reposition this carbonyl in UK-69542-B, so as to allow w6 to approach closer to w5. The

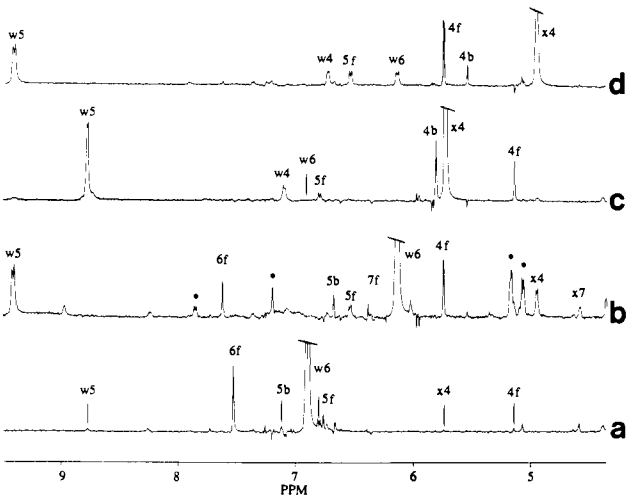

Figure 6. Sections through a Noesy spectrum of UK-69542 taken parallel to the f_2 axis. The spectrum was recorded in DMSO at 500 MHz with a mixing time of 400 ms. The sections are at the f_1 frequencies of (a) $w6_A$, (b) $w6_B$, (c) $x4_a$, and (d) $x4_B$. Resonances marked by stars arise from protons at similar f_1 frequencies (these can be recognized as such in a contour plot of the data).

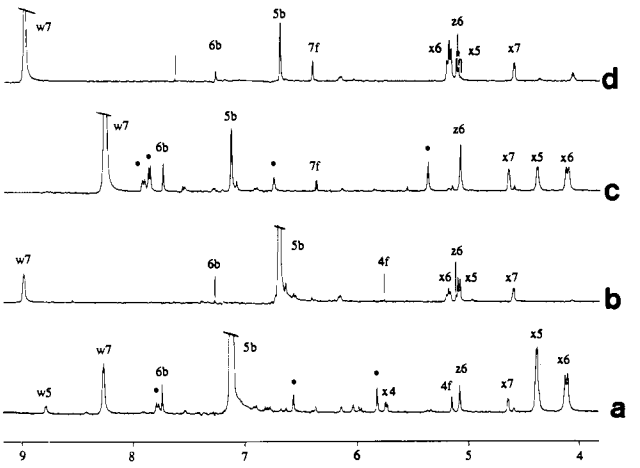

Figure 7. Sections through a NOESY spectrum of UK-69542 taken parallel to the f_2 axis. The spectrum was recorded in DMSO at 500 MHz with a mixing time of 400 ms. The sections are at the f_1 frequencies of (a) $5b_A$, (b) $5b_B$, (c) $w7_A$, and (d) $w7_B$. Resonances marked by stars arise from protons at similar f_1 frequencies (these can be recognized as such in a contour plot of the data).

simplest means by which this can occur is the reorganization of the 5–6 amide bond to a trans form, with the amide proton (w6) still at the rear and with the carbonyl oxygen moving through to the front face of the molecule (Figure 8b). Inspection of space-filling models reveals that this isomerization can take place without reorganization of the covalent structure. Rotation of the carbonyl group through the 16-membered macrocyclic ring formed by the side chain of residues and 6 is possible, although the fit is tight. This process is shown schematically in Figure 9. Previous studies on vancomycin have shown that rotations of amide bonds in macrycyclic ring systems generally occur more readily than might be expected from the mobility of models.[23]

The rate constant for the interchange between the cis and trans forms of UK-69542 could be estimated with proton NMR by following the equilibrium process of a sample freshly dissolved in DMSO. The changes of peak areas for resolved resonances over a period of 1 h are shown in Figure 10a. These data permitted the calculation of the first-order rate constant for the interchange (Figure 10b). The rate of 6×10^{-4} s^{-1} at 300 K

(23) Waltho, J. P.; Williams, D. H.; Stone, D. J. M.; Skelton, N. J. *J. Am. Chem. Soc.* **1988**, *110*, 5638–5643.

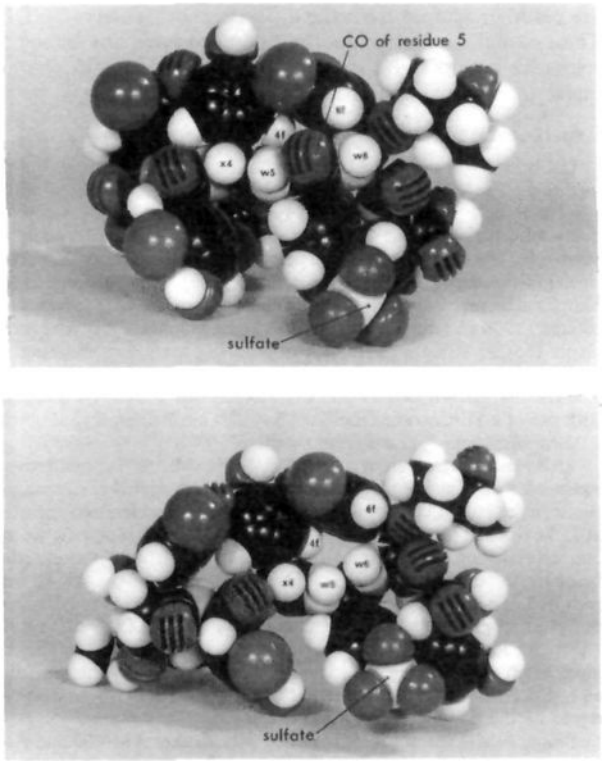

Figure 8. (a, top) Space-filling model showing the rear of the C-terminal end of UK-69542-A. Note that no NOE is observed between w5 and w6 because the cis nature of the 5–6 amide bond places the carbonyl oxygen of residue 5 between them. (b, bottom) Same as (a), but for UK-69542-B. Due to the amide bond isomerization, w5 and w6 are much closer together; hence, an NOE is seen between them.

indicates a barrier to rotation of 17–20 kcal mol^{-1}. This experimental value compares well with the barriers to rotation of 20 kcal mol^{-1} measured for simple acyclic amide bonds.[24] The value of 17–20 kcal mol^{-1} observed for the exchange in UK-69542 is also consistent with there being little or no steric penalty involved in moving the carbonyl oxygen of residue 6 in the manner shown in Figure 9.

The observation of seven trans amide bonds has not been notoed for any other member of the group of antibiotics. This is curious because the covalent structure of the aglycons of UK-69542-B and aridicin-des-sulfate are identical, and yet the unusual conformer is not observed for aridicin. This implies that the groups attached to the aglycon of UK-69542-B may be inducing the change. This matter is discussed in more detail below. A second point of consideration concerns the isolation of UK-69542 by affinity chromatography. This part of the purification process depends on the ability of the antibiotic to bind to D-alanyl-D-alanine immobilized on an agarose gel.[12] From inspection of space-filling models of UK-69542-B, it is not certain that this form of the antibiotic is capable of binding to such peptides.

This problem of isolation of UK69542-B by affinity column was tackled by the introduction of Ac-D-Ala-D-Ala into a DMSO solution of the antibiotic. The hope was that the binding process might induce a trans–cis isomerization. Unfortunately, conditions of pH suitable for binding to take place in DMSO could not be found. A slight broadening of the A resonances was observed, but the B resonances underwent no appreciable change.

In order to circumvent this problem, experiments were initiated to study the antibiotic in aqueous media, with a view to introduction of dipeptide once assignments had been made. Surprisingly, a single set of resonances was observed in a solvent mixture of D_2O and CD_3CN (4:1 v/v). Assignment of these resonances was possibly by a combination of DQF-COSY and NOE difference spectroscopy (see right-hand column of Table I). The NOE between w6 and w5 cannot be observed, and the large NOEs between x5 and x6 and between x6 and 5b establish the presence of a conformer with a cis conformation for the amide bond. Additionally, the chemical shifts of backbone protons more closely resemble those of UK-69542-A in DMSO.

Even with 20% acetonitrile in the solvent, the majority of the signals still had line widths in excess of their DMSO equivalent. The possibility remained that this was due to relatively fast exchange between the cis and trans froms and not simply due to greater aggregation in the aqueous medium (relatively broad lines for vancomycin and very broad lines for ristocetin A are observed in aqueous solvents, this being attributed to aggregation). Lowering of the sample temperature as far as 270 K did not clarify the situation—no transfer to slow exchange was observed. The existence of a fast equilibrium was finally discounted by addition of increasing amounts of D_2O to a 500-μL DMSO solution of the antibiotic. As the D_2O was added, the intensity of the trans resonances decreased. However, the decrease was not accompanied by a broadening of resonances, indicating that the rate of cis-trans isomerization was not changed by the D_2O. After the addition of 100 μL of D_2O and a delay of 30 min for equilibration to occur, only resonances due to the A form remained.

Discussion

The analysis outlined in the results section has shown that, in DMSO solution, the antibiotic UK-69542 exists in two equally populated conformations. As mentioned above, the groups attached to the aglycon are reasoned to induce this change, as the peptide component of aridicin is identical with that of UK-69542. The major difference in covalent structure between UK-69542 and aridicin lies in the sulfate ester of ring 5 (the sugars are highly variable in this family of antibiotics and have not previously been shown to have any influence of the aglycon structure). Somehow, the presence of this charged group must be influencing the relative stabilities of the cis and trans amide bond geometry.

The space-filling models show that the sulfur atom is approximately 7 Å from the carbonyl oxygen of residue 5 in UK-69542-A. Thus, the electrostatic interaction between these two negatively charged or polarized components raises the energy of this backbone conformation. In UK-69542-B, the sulfate is not close enough to form a hydrogen bond directly to the amide protons w5 or w6 in UK-69542-B. However, the charge–dipole interaction between the sulfate and the positively polarized amide protons will lower the energy of this backbone conformation. The interactions between the sulfate anion and the amide bond components will seek to induce a cis–trans isomerization. The energies of these interactions are not great—they are only sufficient to equalize the energies of the two conformations. For small linear peptides, the trans amide bond is generally about 2 kcal mol^{-1} more stable than the corresponding cis form.[24] This indicates that the interaction involved in changing the relative proportions of the cis and trans conformers need only be of the order of a few kcal mol^{-1}.

The observation of only one set of resonances (and one backbone conformation) in aqueous solution highlights the fragile balance of energies involved in determining the backbone conformation. Changing from DMSO to a solvent of higher dielectric constant such as water or even water/DMSO mixtures is sufficient to decrease the electrostatic interactions that favor UK-69542-B (the dielectric constant for DMSO is about 40, and that for water about 80). In solvents of lower dielectric constant, the UK-69542-B conformation would be expected to be even more favorable than in DMSO. Unfortunately, the antibiotic is only sparingly soluble in such solvents, and this hypothesis cannot be tested.

UK-69542 existing in two conformations in DMSO raises questions about the role of these conformations in the activity of the antibiotic against bacteria. Given that the unusual trans form is not observed in aqueous solvent (or in water/DMSO mixtures), it seems very unlikely that it offers any great change in antibacterial action. Indeed, inspection of the space-filling models suggests that UK-69542-B would bind very poorly to peptides

(24) Shulz, G. E.; Schirman, R. H. *Principles of Protein Structure*; Springer-Verlag: New York, 1985; p 25.

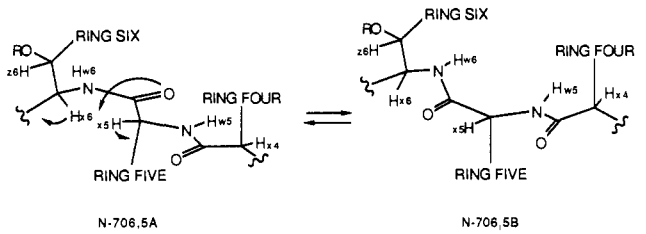

Figure 9. Proposed mechanism by which the cis–trans isomerization takes place.

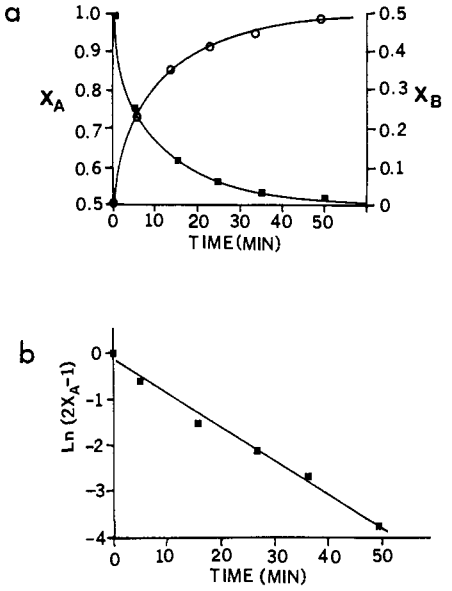

Figure 10. (a) Variation of the mole fractions of N-706,6-A and -B with time after initially dissolving the sample in DMSO at 300 K. The amounts of each species were judged by comparing the intensities of resolved peaks. (b) First-order kinetics of the isomerization process. From these data, the rate constant was determined to be 6×10^{-4} s^{-1}.

terminating in D-alanyl-D-alanine. Thus, the observation seems to be merely a product of the characterization procedure. However, the fact that the unusual trans transform is observed does have wider implications in the area of polypeptide or protein structure.

First, the case of UK-69542 highlights how charge-dipole interactions can affect the conformations adopted by the amide units of a peptide's backbones. This interaction may be important even if the charge and dipole cannot approach close enough to form a hydrogen bond. In the general case of polypeptide conformation, this type of electrostatic interaction would be expected to influence the orientations of trans amide units, rather than inducing cis–trans isomerization, as in UK-69542. Second, the observation that changing solvent markedly changes the effects of charge–dipole interactions highlights the importance of the medium surrounding the charge and the dipole. Factors such as the pH and ionic strength of the surrounding solvent or the exclusion of solvent by other portions of peptide will dictate the amount by which these weak electrostatic interactions are able to change the polypeptide conformation.

Conclusion

This work has shown how the structure of novel glycopeptide antibiotics can be determined by a combination of FABMS and NMR spectroscopy. The similarity in the observed set of NOEs among antibiotics of this class greatly simplifies the analysis of the proton NMR data. This was found to be the case for UK-69542-A. For this particular glycopeptide antibiotic, the presence of an aryl sulfate ester was found to dramatically change the conformation of a relatively rigid macrocyclic system. However, the successful determination of the structure of UK-69542-B indicates that differences in covalent structure or conformation are readily apparent from the different set of observed NOEs. The unusual conformer does not seem to have any biological relevance but does give insight into the ways in which charge–dipole interactions can influence peptide structure.

Experimental Section

UK-69542 was produced by fermentation of a *Steptomycete* culture medium. The compound was isolated from the broth supernatant by adsorption onto Amberlite XAD-2 resin. The glycopeptide was selectively adsorbed from the resin elutes onto agarose-bound D-alanyl-D-alanine.[12] The antibiotic was then desorbed from the affinity support by eluting with aqueous ammonia/acetonitrile (7:3). The purification was completed by reversed phase HPLC with a Waters 10μ C-18 μ-Bondapak column (19 × 150 mm) eluting with 0.1 M ammonium formate/acetonitrile (85:15) at a flow rate of 8 mL/min. Fractions containing the antibiotic were pooled, desalted, and lyophilized to give a white powder. The thermal decomposition product was isolated from pure antibiotic with use of a Hi-Chrome ODS-2 (2.5 × 250 mm) column eluting with 0.02 M ammonium acetate/acetonitrile (86:14).

FAB mass spectra were recorded on a Kratos MS-50-TC spectrometer equipped with a standard FAB source and an extended range magnet (mass range 10000 Daltons at 8 keV) and a PAD accelerator (operating at 15 keV). The instrument was fitted with an Iontech saddle-field atom gun accelerating xenon atoms to 6–9 keV, which were then used to bombard the sample and matrix. The FAB spectra of UK-69542 were recorded in negative-ion mode with 10–20 nmol of compound dissolved in 2–3 μL of glycerol (Aldrich Chemical Co.) with 15-Cr-5 crown ether (Aldrich) added to 5% v/v. The secondary ions produced by the fast atom beam were accelerated to 8 keV before analysis by the spectrometer. Spectra were calibrated with the oligomeric matrix ions also produced by the impinging fast atom beam.

Proton NMR spectra were recorded with Bruker AM-400 and AM-500 spectrometers equipped with aspect 2000 computers. Samples for analysis in DMSO (commonly 5–10 mg) were prepared by dissolving in 100 μL of (d_6) DMSO and removal of this solvent in vacuo over phosphorous pentoxide. The samples were then dissolved in 500 μL of fresh 99.96 atom % (d_6) DMSO (Aldrich). This procedure was not employed for the time-course experiments to observe the UK-69542-A ↔ UK-69542-B rate of interchange. In this case, the samples were simply dried in vacuo over phosphorous pentoxide. Spectra were recorded over spectral widths of 4000 or 5000 Hz (at 400 and 500 MHz, respectively) with quadrature detection employed throughout. Chemical shifts were measured relative to the internal DMSO peak at 2.50 ppm. Samples for analysis in D_2O were lyophilized two or three times from D_2O to reduce the intensity of the HOD peak.

Two-dimensional proton NMR spectra were acquired in the phase-sensitive mode with use of quadrature detection in f_2 and with the time-proportional phase incrementation method of Marion and Wüthrich in f_1.[25] Initially, data sets resulting from 400–512 increments of t_1 were acquired (and zero filled to 1024 points), with each FID composed of 2048 data points. However, many of the cross-peaks to the more crowded regions of the one-dimensional spectrum could only be resolved by increasing the digital resolution. Thus, 4096 data points were acquired in t_2, and 800–950 increments in t_1 (zero filled to 2048 points) were performed. The sum of acquisition time and recycle delay between transients was typically 1.5–2.0 s, this being greater than twice the T_1 values of the protons concerned. NOESY spectra were recorded with mixing times of 400 ms, varied by 20–30 ms between t_1 values to minimize zero quantum artifacts. Typically, 32 or 64 transients were recorded for each increment of t_1, giving a total acquisition time of 12–17 h. The final data sets were multiplied by Lorentzian–Gaussian functions before Fourier transformation.

One-dimensional saturation transfer experiments were performed with a recycle delay of 2 s and an irradiation time of 1.5 s with use of a decoupler power of about 0.2 W. Sets of 32 transients were acquired with the decoupler alternately off resonance and on resonance with the residual water in the DMSO. The resulting FIDs were subtracted and then subjected to Fourier transformation. NOE difference spectra on the antibiotic in D_2O/CD_3CN were recorded in a similar fashion, with irradiation times of 0.8 s employed.

The proton-detected heteronuclear correlation spectrum were acquired at 400 MHz by the method of Bax et. al[21] with use of the phase cycling of Cavanagh and Keeler.[26] A 6-mg sample of the antibiotic in DMSO was used (corresponding to a 4 mM solution with respect to UK-69542-A or UK-69542-B). Values of t_1 (256) were acquired, with each FID

(25) Marion, M.; Wüthrick, K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967.
(26) Cavanagh, J.; Keeler, J. H. *J. Magn. Reson.* **1988**, *77*, 356–362.

consisting of 160 transients recorded over 2048 data points. Four dummy scans were included before each experiment, and a nonspinning sample was used to minimize artifacts in the spectrum. The data was multiplied by Lorentzian line-broadening functions and zero filled to 1024 data points in f_1 prior to transformation.

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Siroheme Model Compounds: Vibrational Spectra and Normal Modes of Copper(II) Octaethylisobacteriochlorin

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Abstract: Resonance Raman (RR) and infrared spectra are reported for the Cu(II) complexes of octaethylisobacteriochlorin (OEiBC) and the *meso*-deuteriated compounds OEiBC-γ-d_1, OEiBC-β,γ,δ-d_3, and OEiBC-$\alpha,\beta,\gamma,\delta$-$d_4$. These compounds represent a series in which each of the three symmetry inequivalent methine bridges of the macrocycle is systematically perturbed. The RR spectra of the complexes are obtained at a variety of excitation wavelengths which span the B_x, B_y, Q_x, and Q_y absorption bands of the complexes. The observed RR intensity enhancement patterns provide insight into the scattering mechanisms that are active for the tetrahydroporphyrin macrocycle. All of the vibrational data are used in conjunction with semiempirical normal coordinate calculations to obtain a set of assignments for the observed high-frequency (above 1000 cm^{-1}), in-plane skeletal modes of CuOEiBC. The normal coordinate calculations and, more importantly, the isotope-shift patterns observed for the complexes deuteriated at specific methine bridges indicate that the forms of the vibrational eigenvectors of many of the modes of the OEiBC macrocycle differ substantially from those of porphyrins, chlorins, or bacteriochlorins. The differences in the general appearance of the vibrational eigenvectors of these various classes of macrocycles can be rationalized in terms of structural changes that occur upon reduction of the pyrrole rings.

I. Introduction

Metallohydroporphyrins (porphyrins with one or more reduced pyrrole rings) serve as the prosthetic groups in a number of proteins which perform a variety of biological functions. Metallochlorins (one reduced pyrrole ring) are found in the photosynthetic proteins of green plants (chlorophylls),[1] leukocyte myeloperoxidase,[2-8] bacterial heme *d*,[9,10] and sulfmyoglobin and sulfhemoglobin.[6,11-17] Metallobacteriochlorins (two opposite reduced pyrrole rings) constitute the active sites in bacterial photosynthetic systems (bacteriochlorophylls),[18,19] whereas metalloisobacteriochlorins (two adjacent reduced pyrrole rings) are found in assimilatory nitrite reductase of green plants[20-22] as well as in the nitrite and sulfite reductases of bacteria.[23-26] A metallocorphinoid (four reduced pyrrole rings) constitutes the methylreductase cofactor, F_{430}, of methanogenic bacteria.[27-29] Presumably, the unique ring structures found in these various metallohydroporphyrin prosthetic groups impart more efficacious biological activity to the specific systems.

Resonance Raman (RR) spectroscopy has proven to be a useful probe of the structural properties of metallohydroporphyrins both in model compounds and in proteins (for a review, see ref 30). The metallochlorins are the most extensively investigated class of metallohydroporphyrins. Vibrational assignments have been proposed for these complexes[31-38] and their vibrational frequencies

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