Structure Elucidation of UK-72,051, a Novel Member of the Vancomycin Group of Antibiotics

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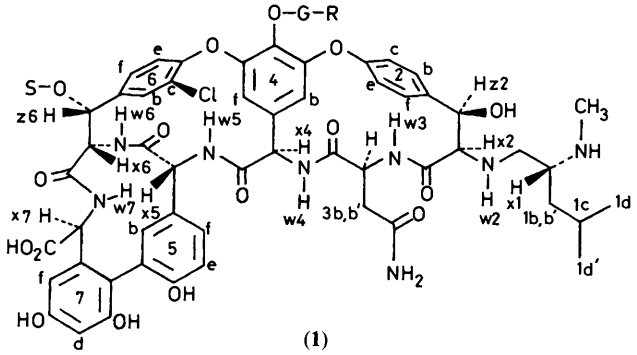
A novel antibiotic structurally related to vancomycin has been isolated from a streptomycete fermentation broth. The structure has been elucidated by a combination of FAB-MS and ^{1}H proton n.m.r. studies, and is found to differ from vancomycin in the type of sugars present, and in the positions of aromatic chlorination.

UK-72,051 is a member of the vancomycin glycopeptide family of antibiotics produced by the fermentation of a new strain of *Amycolatopsis orientalis*. The antibiotic could be absorbed from broth filtrates by the affinity chromatography procedure using D-alanyl-D-alanine immobilised on agarose.[1] The crude eluates were finally purified by reverse-phase h.p.l.c. The structure of UK-72,051, as reported in this paper, was determined by fast-atom bombardment mass spectrometry (FAB-MS), chemical derivatisation, and ^{1}H n.m.r. spectroscopy.

During the preparation of this manuscript, it came to our attention that compounds of a similar, and in one case identical, nature had been isolated and characterised by other workers. The glycopeptide complex A82846[2] has been shown to contain 4-*epi*-vancosamine, by degradation of the complex and isolation of the amino sugar residues. The complete structure of the antibiotic A 82846A has recently been presented,[3] and is an isomer of UK-72,051 from which it differs by containing chlorine at position c of ring 2, rather than at position c of ring 6 [see structure (**1**)]. A compound identical to UK-72,051 has been isolated[4] and characterised.[5] However, full details of the structural assignment of the compound (named orienticin A) were not presented. Most recently,[6] orienticin A (and hence UK-72,051) has been produced by catalytic dechlorination of A 82846B, which contains chlorine at both positions 2c and 6c [see (**1**)]. Thus, the catalytic reduction selectively reduces out the chlorine atom at the 2c position.

The strategy involved in our structure determination was similar to that used for the structure lucidation of the antibiotics teichoplanin[7] and A 40926[8] in this laboratory. The ^{1}H n.m.r. studies involved one- and two-dimensional techniques, including 2D correlated spectroscopy (COSY) to determine coupling connectivities, and 2D nuclear Overhauser enhanced spectroscopy (NOESY), to explore the spatial relationships between protons. The vancomycin group of antibiotics have a high degree of structural similarity,[9] particularly in their peptide portions, and this fact is used to aid the generation of a structure from the spectral data. In particular, the structure of UK-72,051 was found to be similar to that of vancomycin,[10,11] and reference will be made to differences and similarities in the structures.

The nomenclature used in this paper is the same as that used in references 7 and 8. With this nomenclature, aglycone positions are described by a letter (small case) and a number. The number refers to the residue number, counting from the N-terminus, and the letters correspond to the position in the residue (w = amide NH; x = α-CH; z = β-CH; a, b, c, d, e, and f = position on aromatic ring counting 'a' as the site of attachment to the backbone). Upper-case letters correspond to sugar resonances, with the number being the position around the ring, counting from the anomeric carbon. Aliphatic side-chains are labelled alphabetically from 'b', the β position.



(**1**)

Structure of UK-72,051 (**1**). -G-R = 4-*epi*-vancosaminyl (1 ⟶ 2)-glucosyl, -S = 4-*epi*-vancosaminyl

Table 1. Peaks observed in the FAB mass spectrum of UK-72,051

m/z for $[M + H]^+$	Mass loss from *m/z* 1 557	Ion
1 557	0	Molecular ion
1 523	34	Aromatic Cl replaced by H from matrix
1 380	143	Loss of amino sugar (isomeric with vancosamine)
1 218	143 + 162	Loss of hexose and amino sugar
1 075	143 + 162 + 143	Loss of hexose and 2 amino sugars

Results and Discussion

Fast-atom Bombardment Mass Spectrometry.—FAB-MS carried out on the h.p.l.c.-purified material showed it to have a single molecular ion with $[M + H]^+$ at *m/z* 1 557, and with an isotope distribution in this region consistent with the presence of one chlorine atom. Less abundant fragments were also observed, and they are listed in Table 1. These fragment ions give information about the number, and type, of sugars attached to the aglycone.

The antibiotic was next treated with 1M-HCl in methanol at room temperature. Aliquots of the reaction mixture analysed by FAB-MS showed both methanolysis of the sugars and esterification to be occurring. After four hours, the most

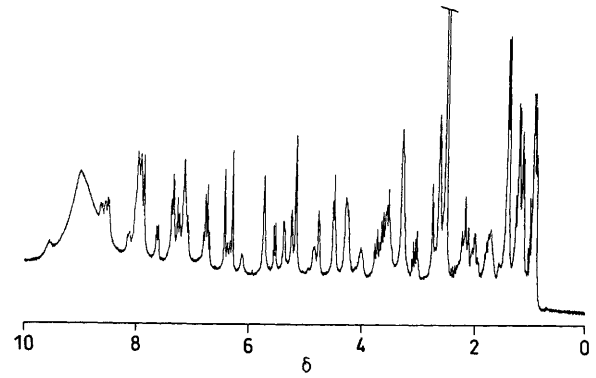


Figure 1. ^{1}H n.m.r. spectrum of UK-72,051, *ca.* 5 mM in $(CD_3)_2SO$. Recorded at 250 MHz and 335 K with 64 transients

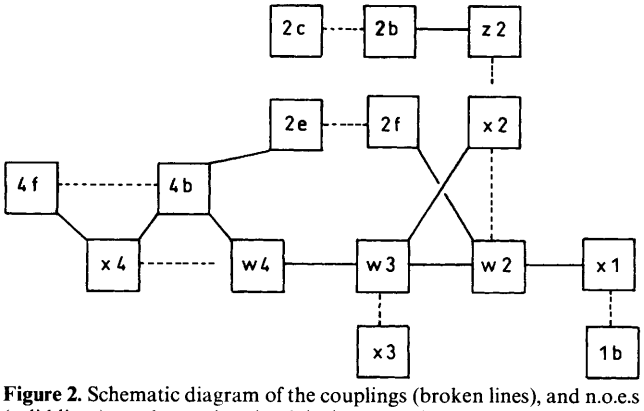


Figure 2. Schematic diagram of the couplings (broken lines), and n.o.e.s (solid lines), used to assign the right-hand portion of UK-72,051

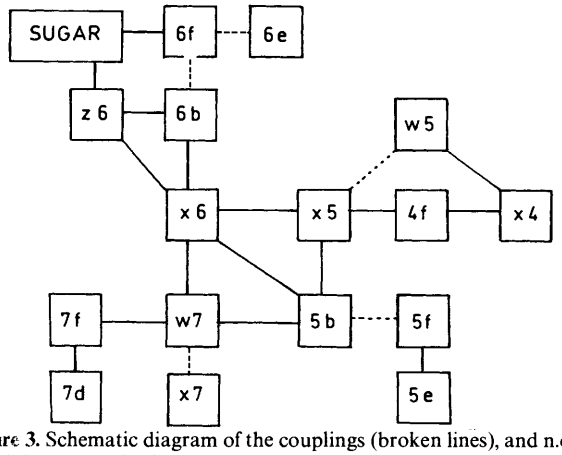


Figure 3. Schematic diagram of the couplings (broken lines), and n.o.e.s (solid lines), used in the assignment of the left-hand side of UK-72,051

abundant ion corresponded to loss of 305 mass-units, with a peak a further 143 mass units below this, at *m/z* 1 109, also being visible. Both peaks were accompanied by less abundant ions 14 mass-units heavier due to methyl ester formation. After eight hours, all four peaks in the spectrum were of equal intensity, with no apparent formation of a dimethyl ester. These results show that UK-72,051 contains a single carboxylic acid and 3 sugars: two isomeric with vancosamine (*m/z* loss 143), and one hexose (*m/z* loss 162).

^{1}H *Nuclear Magnetic Resonance Spectroscopy.*—Spectra were acquired from solutions of the intact antibiotic in $(CD_3)_2SO$. Variable-temperature studies revealed a sharpening of most non-exchangeable resonances as the sample was warmed to 330 K. Analysis of a phase-sensitive double-quantum-filtered COSY (DQF-COSY) spectrum at this temperature revealed only four α-CH–NH couplings, rather than the expected six. This was due to an exchange phenomenon causing broadening of some resonances (see later), and thus prevented the observation of a COSY crosspeak to their coupled partners. Acid was added to the dimethyl sulphoxide (DMSO) solution to alleviate this problem. Trifluoroacetic acid (TFA) (*ca.* three equivalents) was added and the following changes noted:

(1) The residual water peak in the solvent broadened and moved downfield from δ 3.3 to δ 9.0.

(2) Protonation of the amine groups in the molecule produced a sharpening of the amine signals to give two broad singlets at δ 7.94 and 8.06 (each of intensity approximately equal to three protons).

(3) The downfield shift of the methyl singlet at δ 2.32 to δ 2.62 was attributed to protonation of an *N*-methyl group. Two other methyl singlets moved downfield by 0.15 p.p.m. to δ 1.41 and δ 1.39, these being attributed to methyl groups adjacent to the amines undergoing protonation.

(4) Certain other resonances also sharpened, in particular the amide protons at δ 6.37 and 8.64.

These changes allowed the ^{1}H n.m.r. spectrum to be assigned, as described below, and lead to the structure (**1**) for the antibiotic. The ^{1}H n.m.r. spectrum after the addition of TFA is shown in Figure (1).

The assignment of the peptide aglycone of this antibiotic follows the general strategy described for teicoplanin and A 40926 described recently in the literature.[7,8] The method employs COSY[12] spectra to observe the backbone and aromatic spin systems. The coupling networks do not extend across either the amide linkages, or link aromatic and benzylic protons. In order to complete the assignment and elucidate a structure unambiguously, short-range through-space connectivity data, obtained from a NOESY[13] spectrum, are used to link the spin systems together. As already mentioned, the antibiotics of this group have structural similarities, particularly in residues 2, 4, 5, 6, and 7, and this fact simplifies analysis of the data obtained from the NOESY spectrum.

The DQF COSY spectrum of the acidified sample at 330 K and 250 MHz revealed six backbone NH–α-CH connectivities; hence the combination of heating and addition of TFA removed the broadening phenomena observed in the initial sample. Two of the backbone spin systems could be extended: one to include a β-CH, and the other to include a β-CH_2 unit. A further aliphatic spin system, consisting of α-CH–β-CH_2–γ-CH–δ-$(CH_3)_2$, could also be identified. This was reasoned to be due to the N-terminal residue by virtue of the lack of coupling to an amide proton, and also because the α-CH moved downfield by 0.90 p.p.m. on addition of TFA; a weak n.O.e. was also found linking this α-CH to the *N*-methyl group. Thus, the N-terminal residue is an *N*-methyl-leucine. Finally, the COSY spectrum defined five aromatic spin systems: one 1,4-disubstituted ring, two 1,3,4-trisubstituted rings, and two 1,3,4,5-tetrasubstituted rings, one of which was at anomalously high field (this is characteristic of the ring-4 protons, which experience an upfield ring current effect due to their position near the centre of rings 2 and 6[14]).

The crosspeaks obtained from a phase-sensitive NOESY spectrum, obtained at 400 MHz and 330 K, permitted an assignment of the residues, starting from x1, the α-CH of the N-terminal residue. The coupling and n.O.e. pathways by which this was achieved are shown in Figures 2 and 3, whilst the chemical shift and n.O.e. data are listed in Table 2, with the corresponding data for vancomycin as a comparison. This analysis led to the conclusion that residue two was a β-hydroxytyrosine, and that residue three contained the β-CH_2. Thus, residue three was deduced to be an asparagine by comparison of chemical shifts with vancomycin, and also by the molecular weight determined for the entire molecule.

Table 2. Chemical shifts, couplings, and n.o.e.s observed in the aglycone of UK-72,051

Resonance	Coupling[a] (Hz)	Chemical shifts[b]	Observed n.O.e.s[c]	Vancomycin[d] chemical shift
NMe	bs	2.62	x1(w)	2.41
x1	bs	4.00	w2(m), NMe(w)	3.99
1b/b′	m	1.73		1.75
1c	m	1.73		1.75
1d/d′	d 5.5	0.94/0.90		0.90/0.93
w2	d 8.1	8.64	x1(m), 2f(w), w3(w), x3(w)	8.00
x2	bs	4.84	2b(m), w3(m), z2(s), x3(w)	4.86
z2	d 2.8	5.25	2b(s), x2(2)	5.15
2b	0	7.37	z2(s), x2(m), 2c(s)	7.42
2c	0	7.16	2b(s), 4b(m)	
2e	dd 8.2/2.2+	7.11	2f(s), 4b(w)	7.20
2f	bd 8.1	7.64	w2(s), 2e(s)	7.57
w3	bs	6.13	x2(m), w2(w), w4(w)	6.59
x3	0	4.25	3b/b′(w), x2(w)	4.38
3b/b′	0/d 15.1+	2.57/2.19	x3(w)	2.50/2.18
w4	d 7.6	8.16	w3(w), 4b(m)	8.43
x4	0	5.74	4f(m), w5(s)	5.71
4b	0	5.74	2e(w), 2c(m)	5.63
4f	0	5.17	x4(s), 6e(s), w5(w), x5(m)	5.21
w5	d 5.0	8.57	x4(l), 4f(w), 5f(m)	8.14
x5	0	4.50	4f(m), x6(s), 5b(s), 6b(m) w7(w)	4.50
5b	0	7.16	x5(s), x6(s), z6(w), w7(s)	7.19
5e	d 8.6	6.73	5f(s)	6.73
5f	dd 8.6/2.1+	6.80	5e(s), w5(w)	6.78
w6	d 11.6	6.37	6f(w)	6.50
x6	0	4.25	x5(s), 5b(s), z6(s), 6b(m) w7(s)	4.22
z6	0	5.17	x6(s), 6b(s), w7(m), S1(s)	5.13
6b	bs	7.88	x5(w) x6(s), z6(s)	7.87
6e	d 8.4	7.26	6f(s), 4f(w)	7.28
6f	0	7.37	w6(w), 6e(s), S1(s)	7.48
w7	d 6.2	8.51	5b(s), x6(s), z6(m), 6b(m)	8.39
x7	d 6.2	4.47		4.50
7d	d 2.0	6.44		6.44
7f	d 2.0	6.30	w7(w)	6.30

[a] s = singlet, d = doublet, dd = doublet of doublets, m = multiplet, b = broad ($J < 2$ Hz), 0 = overlapping resonance precludes direct measurement, + = data obtained at 250 MHz and 335 K before addition of TFA. [b] Data acquired at 250 MHz and 335 K. [c] Data acquired at 400 MHz and 330 K. s = strong, m = medium, w = weak n.O.e. observed. [d] Values for comparison, obtained from refs. 7 and 8 [(0.1M-vancomycin in $(CD_3)_2SO$ at 340 K].

For the antibiotic to function, the amide protons of residues two, three, and four all need to be at the front of the molecule to provide a binding pocket for the carboxylate anion of peptides terminating in D-alanyl-D-alanine[15,16] (this binding is the means by which these antibiotics exert their influence over gram-positive bacteria). It was noted that, even after the addition of TFA, the resonance assigned to w3 was broad, and had n.O.e.s to w4, w2, and x2. The n.O.e.s to w2 and x2 cannot be satisfied by a single conformation, and this suggested that w3 was present at both the front (n.O.e.s to w2 and w4) and the rear (n.O.e. to x2) of the antibiotic. The exchange between these two conformations probably involves the rotation of the whole *trans* amide bond, as shown in Figure 4. A conformational change of this kind has been reported for vancomycin,[17] and would also explain the broad nature of the w3 resonance. Heating to 330 K increases the rate of this exchange and causes w3 to move into fast exchange and sharpen sufficiently to allow its couplings to be observed in the COSY spectrum.

As mentioned earlier, the addition of acid produces a sharpening of the amide proton at δ 8.64, now assigned as w2. It has been noted that a similar effect occurs in vancomycin.[17] This was shown to be due to w2 undergoing exchange with the residual water in the DMSO. This exchange was approximately 500 times faster than the exchange of any other amide proton (sufficient to put w2 into the intermediate exchange regime, giving rise to a very broad peak), and is mediated by the free base at the N-terminus. Hence, upon protonation of the N-terminal residue, the exchange rate of w2 decreased and its signal sharpened. It seems likely that a similar mechanism operates for w2 and the *N*-methyl-leucine of UK-72,051. It is interesting to note that w6 also sharpens dramatically on addition of acid. A similar exchange process may be occurring, this time involving the free amine on sugar S as the basic catalyst. This sugar is not present in vancomycin, hence, w6 is not unduly broad.

Assignment of the protons contained in the left-hand portion of the antibiotic (Figure 3) reveals it to be identical with vancomycin. Thus, residue six was found to be a β-hydroxytyrosine, in spite of the lack of coupling between x6 and z6. This must be a consequence of their torsion angle being held at ~90°; the small size of this coupling has been noted elsewhere.[10] The benzylic hydroxy group of residue six provides

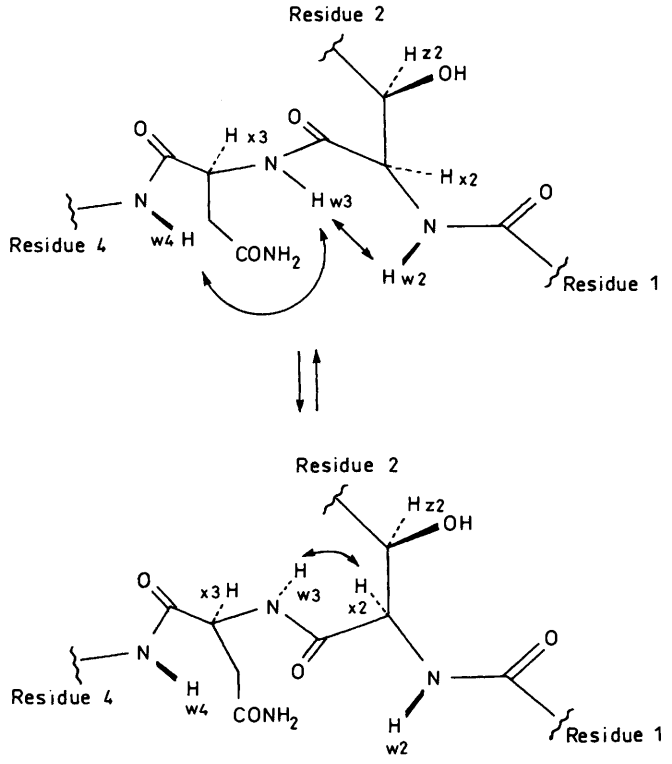


Figure 4. N-terminal residues of UK-72,051 showing the interconversion between the two orientations of the w3 amide bond. N.O.e.s are shown with arrows

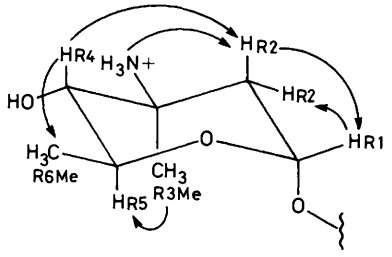


Figure 5. Structure of the amino sugars 'R' and 'S'. Some of the stereochemistry-defining n.O.e.s are shown with arrows

the site of attachment for one of the amino sugars, as will be discussed below. This conclusion is evident from the n.O.e.s from the anomeric proton to z6 and 6f.

Turning now to the carbohydrate portion of the molecule, inspection of the DQF-COSY spectrum reveals three possible connectivities to anomeric protons in the δ 4.5—6.0 range. It has already been noted that one of these, S1, is attached benzylically to residue six. The coupling and n.O.e. networks associated with this anomeric proton indicate that this corresponds to one of the sugars isomeric with vancosamine, determined by the FAB-MS fragment ions. The n.O.e.s leading to this conclusion are shown in Figure 5. The position of the S3-methyl group adjacent to the amine is supported by the slight downfield shift in its chemical shift upon the addition of TFA to the sample. The assignment of these resonances to a 4-*epi*-vancosamine is confirmed by the coupling constants: the S1–S2 coupling is small (J 3.2 Hz), implying that the α-anomer is present, the S2–S2′ coupling is large (15 Hz) due to its geminal nature, and finally the S4–S5 coupling constant is greater than 5 Hz, suggesting a *trans*-diaxial relationship. This last coupling could not be measured directly from the 1D spectrum as both S4 and S5 were partially coincident with other resonances. Although exact measurement of the coupling constant by inspection of the DQF-COSY crosspeak is precluded by the low digital resolution in f_2, a lower limit of 5 Hz could be set by this means. The analogous coupling in vancosamine is only 0.7 Hz, due to two *trans*-coplanar electronegativity effects.[18]

By a similar process of coupling and n.O.e. connections, the sugar containing R1 as the anomeric proton is also found to be a 4-*epi*-vancosamine. The chemical shift and coupling data for both amino sugars are given in Table 3. The location of sugar R within the antibiotic may be determined by the n.O.e. from its anomeric proton, R1, to the proton G2 of the hexose, as discussed below. Both *epi*-vancosamine residues are assumed to have the same absolute configuration as vancosamine in vancomycin.[9]

By elimination, the final anomeric proton G1 must belong to the hexose discovered by the FAB-MS fragments. The 8 Hz coupling to the proton G1 indicates that G1 and G2 have a *trans*-diaxial configuration, and that this sugar is present as the β-anomer. Once again the problem of coincident resonances prevented the direct observation of the ring coupling constants. Nevertheless, the chemical shifts of all the resonances can be found by the COSY crosspeaks (Table 3), and the relative stereochemistries can be deduced by the n.O.e.s observed. In particular, G1 has an n.O.e. to G3 and G5, whilst G2 has an n.O.e. to G4. These linkages suggest that all the ring protons are in axial positions and that the hexose is glucose, assumed to be of D absolute configuration, as in vancomycin.[9]

It has already been noted that G2 has an n.O.e. to R1, and thus the disaccharide 4-*epi*-vancosaminyl-(1 ⟶ 2)-glucosyl is present in the antibiotic. No n.O.e.s could be found from G1 to link the disaccharide to the aglycone. This could, however, be a consequence of the correlation time between G1 and any proximate aglycone protons being such that their n.O.e. is zero. This situation may be remedied by acquiring a CAMELSPIN spectrum.[19] Here, the nuclear Overhauser effects build up in the rotating frame, and give positive enhancements between all spatially close protons regardless of their correlation times.

Table 3. Chemical shifts, couplings, and n.O.e.s observed for sugars of UK-72,051. Notes as for Table 2

Resonance	Coupling[a] (Hz)	Chemical shift[b]	Observed n.O.e.[c]
G1	d 7.8	5.54	G3(l) G5(l)
G2		3.64	G4(l) R1(l)
G3		3.48	G1(l) G5(l)
G4		3.29	G2(l)
G5		3.29	G1(l) G3(l) G6(l)
G6		3.56	G6′(l) G5(l)
G6′	d 11.4	3.75	g6(l)
R1	d 3.3	5.38	R2(l) R2′(l) G2(l)
R2		1.95	R1(l) R2′(l) RNH_3^+(w) R4(m)
R2′		2.12	R1(l) R2(l) RNH_3^+(w) R3Me(w)
R3Me	s	1.41	R2′(w) $R3NH_3^+$(m) R5(m)
$R3NH_3^+$	bs	7.94	R4(m) R2/R2′(w) R3(m)
R4		3.27	R2(s) $R3NH_3^+$(w) R6(m)
R5		4.25	R6(m)
R6Me	d6.3	1.13	R5(m) R4(W)
S1	d 3.2	4.76	S2(l) S2′(l) Z6(l) 6f(m)
S2		2.01	S1(l) S4(m) $S3NH_3^+$(w)
S2′		2.20	S1(l) S3Me(m) $S3NH_3^+$(w) 6f(w)
S3Me	s	1.39	$S3NH_3^+$(w) S5(l)
$S3NH_3^+$	bs	8.06	S3(m) S2/S2′(w)
S4		3.25	S2(w) S6Me(l)
S5		3.54	S3Me(l) S6Me(l) z6(w)
S6	d 6.1	1.21	S4(m) S5(l) Z6(w)

Indeed, in the 2D-CAMELSPIN spectrum, correlations can be seen between G1 and the aromatic protons, 2c, 2e, and 6c, and hence the disaccharide is linked to ring 4.

Experimental

The antibiotic was isolated by passing the broth filtrate through a column containing agarose-bound D-alanyl-D-alanine. Desorption of the antibiotic was achieved using an eluant of 0.1% aqueous ammonia–acetonitrile (7:3). Further purification was achieved by preparative h.p.l.c. using a waters 10μ-Bondapak column (19 × 150 mm) with a mobile phase of 0.1M-ammonium formate–acetonitrile pH 7.3 (9:1) at a flow rate of 10 ml min^{-1}. Antibiotic-rich fractions were pooled and UK-72,051 obtained as a white solid by lyophilisation.

FAB-MS spectra were recorded on a Kratos MS-50 instrument fitted with an Iontech fast-atom gun, a standard FAB source, high-field magnet, and PAD accelerator. The sample (~10 nmol) was dispersed in matrix (typically 1:1 α-thioglycerol–glycerol) (2 μl) and 1M-trichloroacetic acid (1 μl) was added to increase formation of molecular ions, and improve fragmentation of the molecular ion. The sample was then inserted into the spectrometer and bombarded with 6—9 keV xenon atoms. Several spectra were acquired over a period of minutes to insure that all possible fragment ions were observed. Spectra were calibrated by reference to glycerol oligomer ions arising from the matrix.

^{1}H N.m.r. spectra were recorded on Bruker WH 250 and AM 400 spectrometers equipped with Aspect 2000 computers. Prior to analysis, samples were dissolved in $(CD_3)_2SO$, which was then removed under reduced pressure. This procedure ensures that all volatile salts and residual water are removed from the sample. Samples of the antibiotic were then made up to *ca.* 10mM concentration in $(CD_3)_2SO$. Quadrature detection was used throughout with spectral widths of the order of 2 500 or 4 000 Hz (depending upon the spectrometer used).

DQF-COSY and NOESY spectra were acquired in the phase-sensitive mode using the time-proportional-phase-incrementation method of Marion and Wüthrich[20] in f_1. All 2D spectra were acquired with 400—512 t_1 increments. Each t_1 increment consisted of 2K data points from 32 or 64 transients. The recycle delay was typically 1.0—1.5 s (~1.5 times the longest T1 value in molecules of this size), leading to a total acquisition time in the region of 12—14 h. NOESY spectra were recorded with a mixing time of 0.4 s, and CAMELSPIN spectra were recorded with a 0.2 s spin lock field of 4 KHz frequency. Data sets were multiplied by Lorentzian-gaussian functions, and zero-filled to 1K data points in f_1 prior to Fourier transformation.

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