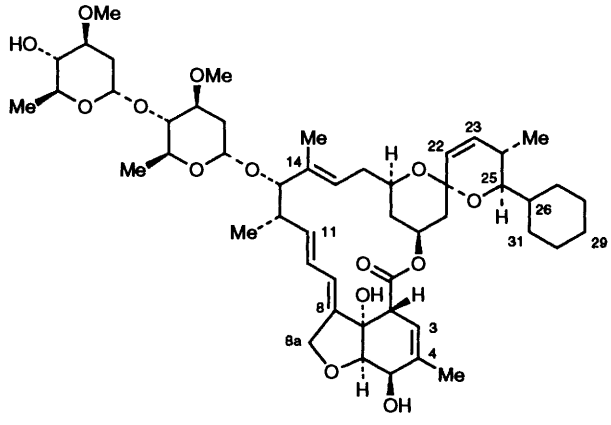
Structure of Doramectin

Christopher J. Dutton,[a] Stephen P. Gibson,[a] Michael Kinns,[a] Alistair G. Swanson[a] and Jon Bordner[b]
[a] *Pfizer Central Research, Ramsgate Road, Sandwich, Kent, UK CT13 9NJ*
[b] *Pfizer Central Research, Eastern Point Road, Groton, Connecticut 06340, USA*

The structures of doramectin **1** and its close analogue, 25-cyclohexyl-avermectin B2 **2**, have been determined by proton and carbon NMR spectroscopy, mass spectrometry and X-ray crystallography. Modern two-dimensional techniques were used to assign the proton and carbon NMR resonances. Several signals in the close-related naturally-occurring avermectins have been re-assigned.

Doramectin **1** [25-cyclohexyl-5-*O*-demethyl-25-de(1-methylpropyl)avermectin A1a] and its close analogue, 25-cyclohexylavermectin B2 **2**, are new avermectins which exhibit improved broad-spectrum antiparasitic activity. Doramectin has been developed as an endectocide for use in cattle[1] and is marketed as Dectomax®. These new avermectins were obtained by the technique of mutational biosynthesis in which the precursor, cyclohexanecarboxylic acid, was fed to a mutant strain of *Streptomyces avermitilis*.[2,3] Due to the commercial importance of these compounds and their unique method of production, it became important to know whether they retained the structural features of the naturally occurring avermectins and whether the cyclohexanecarboxylic acid had been incorporated at the C-25 position as predicted from a consideration of the biosynthesis. Thus, the compounds were examined by proton and carbon NMR spectroscopy, mass spectrometry and X-ray crystallography.



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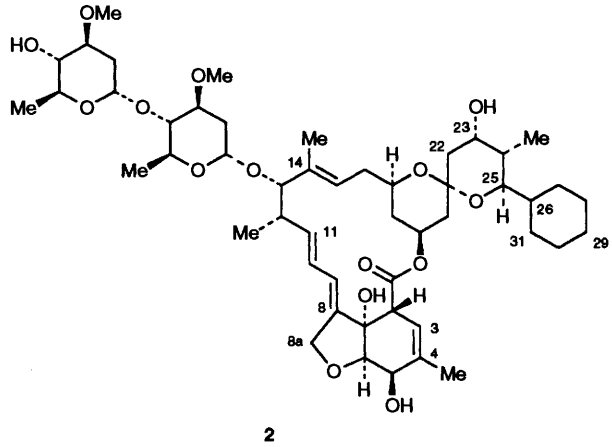

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Experimental

NMR Spectroscopy.—The ^1H and ^{13}C resonance assignments for both compounds in $CDCl_3$ were examined using a range of modern 2D NMR experiments on a Varian Unity 500 spectrometer. HMQC,[4] HMBC,[5] ROESY[6] and TOCSY[7] spectra (Figs. 2–8) made it possible to assign all the ^1H and ^{13}C signals except for certain cyclohexane ring signals. *J*-Values are given in Hz.

Experimental conditions. Solvent: deuteriochloroform ($CDCl_3$) 99.99% D. Concentration: 5 mg/0.75 cm^3 or 20 mg/0.75 cm^3; no detectable dependence of chemical shift on concentration in ^1H or ^{13}C spectra. Spectrometer and probeheads used: Varian UNITYplus 500 (499.843 MHz for ^1H and 125.697 for ^{13}C) 5mm ^1H (^{13}C, ^{15}N) probehead (^1H, TOCSY, HMQC and HMBC); a 5 mm broad band probehead tuned for ^{13}C observation was used to obtain the ^{13}C and DEPT spectra. Probe temperature: 30 °C. Referencing: all spectra were referenced to the ^{13}C and/or residual ^1H resonances of the $CDCl_3$ at 77.0 and 7.26 ppm downfield from Me_4Si, respectively. ^1H Spectrum: sw = 8 ppm, 30 degree pulses, at = 4 s, nt = 32. ^{13}C Spectrum: sw = 230 ppm, ^1H decoupling, 30 degree pulses, dl = 1.7 s, at = 1.3 s, nt = 1024. ^{13}C DEPT: 45, 90, 135 degree ^{13}C pulses in separate acquisitions for spectrum editing.

All 2D experiments (except for ROESY): sw = 8 ppm, np = 2 k, at = 0.26 s, ni = 64 × 2; States-Haberkorn phase cycling; processing, Gaussian apodization in t2, fn = 2 k, extended to 256 complex points in t1 by linear prediction followed by Gaussian apodization in t1, fn1 = 1 k. TOCSY: sw1 = 8 ppm, d1 = 1 s, nt = 4, spin-lock field strength = 10 600 Hz, mixing times = 15 ms, 75 ms. ROESY: sw1 = 8 ppm, np = 2 k, d1 = 1.5 s, nt = 8, spin-lock field strength = 3500 Hz, mixing time = 500 ms, resonance offset compensation, ni = 256 × 2; Processing, Gaussian apodization in both dimensions, fn1 = fn = 2 k. HMQC: sw1 = 200 ppm, d1 = 1.5 s, j = 140 Hz, null = 0.45 s, nt = 4 or 8, WALTZ-16 ^{13}C decoupling during acquisition. HMBC: sw1 = 240 ppm, d1 = 4.68 s, nt = 128, j = 7 Hz; processing, data displayed phase sensitive in ω2 (^1H) and power mode in ω1 (^{13}C).

Abbreviations. sw = spectral width, sw1 = spectral width in the indirectly detected dimension (^1H for TOCSY, ^{13}C for HMQC and HMBC), np = total number of data points acquired, at = acquisition time, d1 = delay between transients to allow for relaxation, nt = number of transients recorded, fn = total number of data points used for Fourier transform, ni = number of increments in the indirectly detected dimension for 2D experiments, fn1 = total number of data points used for Fourier transform in the indirectly detected dimension, j =

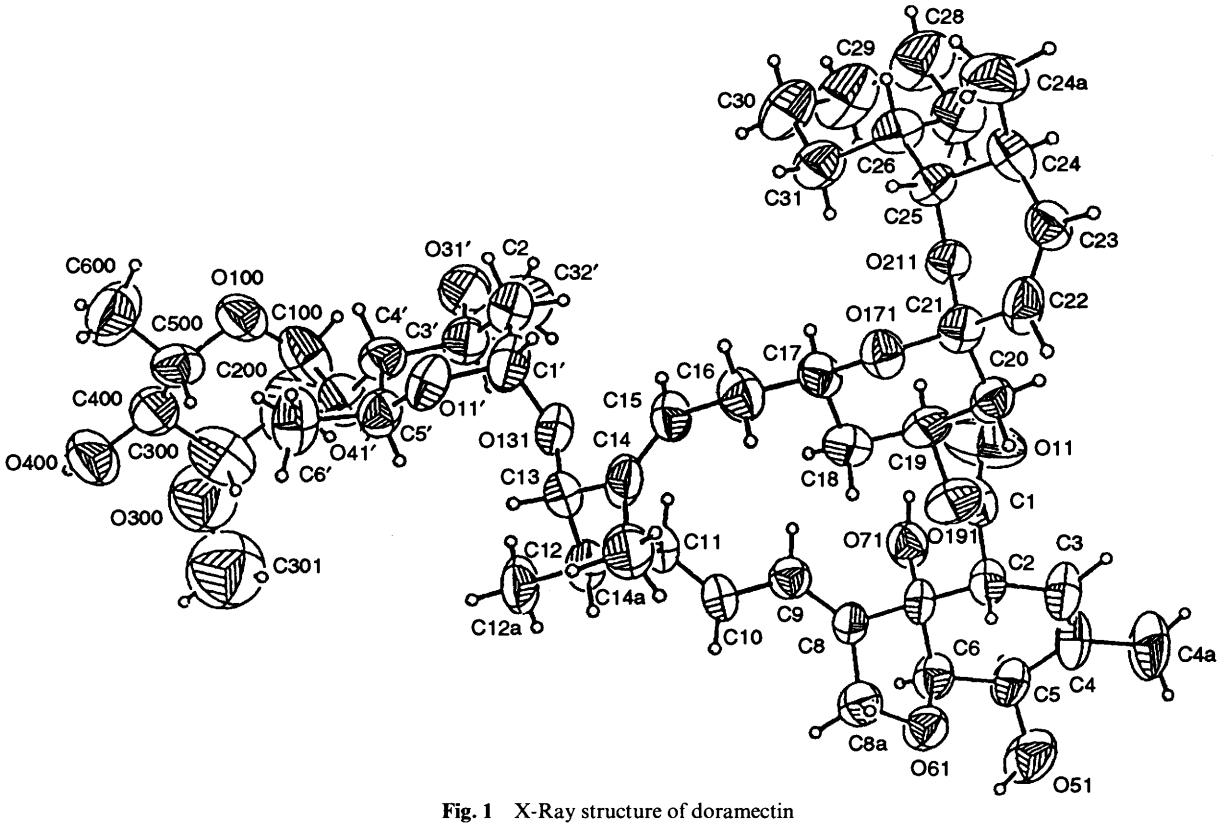

Fig. 1 X-Ray structure of doramectin

parameter used to define the coupling constant for optimum ^{1}H–^{13}C polarization transfer for the HMQC and HMBC experiments, null = delay optimized to minimize signal from hydrogens attached to ^{12}C in the HMQC experiment.

Mass Spectrometry.—FAB mass spectra were recorded on a VG 7070E-HF mass spectrometer using a sample matrix of glycerol, thioglycerol and aqueous sodium chloride. EI mass spectra were recorded on a VG 7070F mass spectrometer.

X-Ray Crystallography.—Crystals of doramectin were obtained from a saturated solution in methanol–water, 90:10. A representative crystal was surveyed and a 1 Å data set (maximum sin $\theta/\lambda = 0.5$) was collected on a Nicolet R3m/μ diffractometer. Atomic scattering factors were from the *International Tables for X-Ray Crystallography.*[8] All crystallographic calculations were facilitated by the SHELXTL[9] system. All diffractometer data were collected at room temperature. A trial structure was obtained by direct methods. This trial structure refined routinely. Hydrogen positions were calculated wherever possible. The methoxy hydrogens were located by difference Fourier techniques. Because of the large thermal parameters associated with the water and methanol molecules, their hydrogens were not located. The hydrogen parameters were addded to the structure factor calculations but were not refined. The shifts calculated in the final cycle of least squares refinement were all less than 10% of their corresponding standard deviations. The final *R*-index was 0.061. A final difference Fourier revealed no missing or misplaced electron density.

The refined structure was plotted using the SHELXTL plotting package (Fig. 1). The absolute configuration was inferred by analogy to the natural avermectins and could not be assigned directly from this analysis.

Crystal data. $C_{50}H_{74}O_{14}\cdot CH_3OH\cdot H_2O$, $M = 949.3$, $a = 39.088(8)$, $b = 10.043(2)$, $c = 14.729(2)$ Å, $\alpha = 90.0°$, $\beta = 108.21(1)°$, $\gamma = 90.0°$, $V = 5492(2)$ Å, space group *C*2, molecules/unit cell = 4, density obs. = 1.14 g cm^{-3}, density calc. = 1.15 g cm^{-3}, linear absorption coefficient = 6.09 cm^{-1}.

Structure analysis and refinement. No. of reflections = 3034, non-zero reflections = 2647, *R*-index = 0.061, GOF = 1.29, scale factor = 1.238(2), secondary extinction coefficient = 18(2) × 10^{-4}.

Results and Discussion

Assignment of Proton and Carbon Spectra.—The ^{13}C and ^{1}H chemical shifts and all the measurable ^{1}H–^{1}H coupling constants in $CDCl_3$ are listed in Tables 1 and 2 for cyclohexyl B2 and doramectin, respectively.

Alkenic carbons (C-4, -8 *and* -14). The HMBC experiment gives correlations between hydrogens and carbons through two and three bonds. This makes it ideal for identifying quaternary carbons with nearby hydrogens. In the spectrum of cyclohexyl B2 in Fig. 2 correlations between the C-4 methyl, 8a-H and C-14 methyl proton signals and the respective adjacent quaternary carbons are labelled. The assignments of the C-4, -8 and -14 signals in Table 1 were confirmed. Traces (*a*), (*b*) and (*c*) in Fig. 3 show slices parallel to the ^{13}C axis from an HMBC spectrum of doramectin at the ^{1}H frequencies of the C-4 methyl, 8a-H and the C-14 methyl, respectively.

22,23-*Double bond in doramectin.* The original assignments[10] for the C-22 and -23 resonances of avermectin B1a and B1b gave the C-22 signal at δ 136.2 and the C-23 signal at δ 127.9. The corresponding hydrogen resonances were later assigned by 2D correlation experiments to be δ 5.75 for 22-H and δ 5.54 for 23-H based on the earlier carbon assignments.[11] These assignments have been questioned following NOE studies on a synthetic avermectic fragment and 2D INADEQUATE spectroscropy of avermectin B1a aglycone.[12] Our results confirm that both the carbon and the hydrogen resonances were misassigned. The two hydrogens on the 22,23 double bond of doramectin appear at δ 5.54 and 5.74. The HMQC spectrum in Fig. 4 shows that these hydrogens are connected to carbons

Table 1 Cyclohexyl B2 NMR data

Atom no.	δ_C	H[a]	δ_H	Multiplicity	J/Hz
1	173.59	0	—		
2	45.75	1	3.29	m	≈2.3, unresolved
3	117.98	1	5.43	m	
4	138.09	0	—		
5	67.71	1	4.30	br t	6.3, ≈7
6	79.16	1	3.97	d	6.3
7	80.43	0	—		
8	139.63	0	—		
8a	68.46	2	4.69	dd	2.4, 14.4
			4.66	dd	
9	120.48	1	5.87	dt	2.1, 10.3
10	124.70	1	5.73	m	
11	138.12	1	5.75	m	
12	39.78	1	2.53	ddq	2.0, unresolved, 7.0
13	81.72	1	3.95	m	unresolved
14	135.62	0	—		
15	117.98	1	4.98	br t	7.4
16	34.21	2	2.30	m	
17	68.21	1	3.87	m	
18	36.57	2	0.850 (ax)	q	12.0
			1.77 (eq)	m	
19	67.30	1	5.36	m	
20	40.70	2	1.42 (ax)	t	11.9
			1.98 (eq)	ddd	11.9, 1.3, 4.5
21	98.51	0	—		
22	41.20	2	1.65	dd	2.6, 14.2
			1.96	dd	3.4, 14.2
23	69.93	1	3.75	m	
24	35.16	1	1.62	m	
25	72.59	1	3.41	m	
4-Me	19.99	3	1.88	s	
12-Me	20.21	3	1.17	d	7.1
14-Me	15.20	3	1.495	s	
24-Me	13.77	3	0.91	d	6.9
26	38.15	1	1.51	m	
27	26.51	2	1.80	m	
			1.30–1.15	m	
31	26.94	2	1.80	m	
			1.30–1.15	m	
28	26.51	2	1.66	m	
			1.3–1.15	m	
30	24.53	2	1.60	m	
			1.23	m	
29	31.21	2	1.5	m	
			1.65–1.55		
1′	94.86	1	4.77	dd	3.2, unresolved
2′	34.63	2	1.58 (ax)	m	
			2.23 (eq)	ddd	1.2, 4.8, 12.0
3′	79.36	1	3.61	ddd	4.8, 8.6, 11.2
4′	80.41	1	3.24	't'	≈9
5′	67.56	1	3.82	dq	6.2, 9.4
5′-Me	18.62	2	1.253	d	6.2
1″	98.51	1	5.395	dd	3.3, unresolved
2″	34.25	2	1.51 (ax)	m	
			2.32 (eq)	ddd	1.1, 4.7, ≈12
3″	78.19	1	3.48	ddd	4.7, 9.1
4″	76.13	1	3.16	br t	1.8, 9.1
5″	68.46	1	3.76	m	
5″-Me	17.89	3	1.273	d	6.4
3′-OMe	56.50	3	3.42	s	
3″-OMe	56.39	3	3.41	s	
5-OH			2.36	d	
7-OH			3.98	s	
23-OH			3.50	d	10.0
4″-OH			2.49	d	1.8

[a] Number of hydrogen atoms.

which resonate at δ 127.93 and 136.4, respectively. Conventional methods using vicinal ^1H–^1H couplings fail to give unambiguous assignments for 22- and 23-H since both resonances are double doublets due to coupling with each other and 24-H. Moreover, the magnitude of the couplings to 24-H are similar for both protons and the 23-H resonance is overlapped by both 10- and 11-H. Thus, it became necessary to use either long range ^1H–^{13}C couplings or through space (NOE or ROE) interactions to assign these resonances. The hydrogens of the C-24 methyl group are three bonds away from C-23 and

Table 2 Doramectin NMR data

Atom no.	δ_C	H[a]	δ_H	Multiplicity	J/Hz
1	173.89	0	—		
2	45.92	1	3.30	m	≈2.3, unresolved
3	118.26	1	5.43	m	
4	138.16	0	—		
5	67.91	1	4.295	br t	≈7
6	79.32	1	3.97	d	6.2
7	80.59	0	—		
8	139.70	0	—		
8a	68.65	2	4.70	dd	2.4, 14.3
			4.67	dd	2.4, 14.3
9	120.70	1	5.885	dt	2.4, 10.4
10	124.88	1	5.73	dd	10.4, 15.5
11	138.38	1	5.78	dd	9.0, 15.5
12	39.93	1	2.53	ddq	2.3, 6.9, 9.3
13	81.92	1	3.94	m	
14	135.30	0	—		
15	118.38	1	5.00	br d	11.0
16	34.56	2	2.29	m	
17	68.49	1	3.87	ddt	2.0, 4.5, 11.0
18	36.92	2	0.855 (ax)	q	12.0
			1.79 (eq)	m	
19	68.30	1	5.43	m	
20	40.55	2	1.48 (ax)	t	11.8
			2.005 (eq)	ddd	1.9, 4.9, 11.8
21	95.90	0	—		
22	127.93	1	5.54	dd	2.6, 9.8
23	136.40	1	5.74	dd	1.8, 9.8
24	30.21	1	2.28	m	
25	77.54	1	3.31	br d	≈9, unresolved
4-Me	20.18	2	1.87	't'	1.7
12-Me	20.43	2	1.17	d	7.0
14-Me	15.38	2	1.495	s	—
24-Me	16.84	2	0.925	d	7.2
26	38.89	1	1.56	m	
27	27.18	2	1.35–1.20	m	
			≈1.80	m	
31	26.82	2	1.35–1.20	m	
			≈1.80	m	
28	26.71	2	1.35–1.20	m	
			1.71–1.63	m	
30	25.77	2	1.35–1.20	m	
			1.71–1.63	m	
29	31.63	2	1.55	m	
1′	95.00	1	4.79	dd	1.4, 3.1
2′	34.79	2	1.56 (ax)	m	
			2.24 (eq)	ddd	1.4, 4.5, 12.8
3′	79.58	1	3.62	ddd	4.7, 8.5, 11.2
4′	80.56	1	3.24	't'	9.0
5′	67.41	1	3.825	dq	6.2, 9.3
5′-Me	18.62	3	1.25	d	6.2
1″	98.68	1	5.395	dd	1.1, 3.4
2″	34.37	2	1.51 (ax)	m	
			2.33 (eq)	ddd	1.1, 4.7, 12.8
3″	78.39	1	3.48	ddd	4.7, 8.8, 11.4
4″	76.27	1	3.16	br t	≈1, 9.3
5″	68.40	1	3.77	dq	6.2, 9.3
5″-Me	17.89	3	1.275	d	6.2
3′-OMe	56.74	3	3.43	s	
3″-OMe	56.60	3	3.42	s	
5-OH			2.42	d	
7-OH			4.06	s	
4″-OH			2.60	d	1.0

[a] Number of H atoms.

four bonds from C-22, therefore only a correlation to C-23 would be expected in an HMBC spectrum. Trace (*d*) in Fig. 3 shows a slice through an HMBC spectrum of doramectin at the ^{1}H frequency of the C-24 methyl group (δ 0.925). The only strong correlation to the alkenic region is to the carbon at δ 136.4, suggesting that this is C-23. Because of the resonance overlap of 24-H with 16-H, it was not possible to obtain unambiguous confirmation of the assignment of C-22 from the HMBC spectrum by taking a slice through the spectrum at the 24-H frequency.

ROESY spectra give cross peaks between hydrogens which are close together in space. This experiment is more useful than NOE difference for avermectins since 'conventional' NOEs are very small for molecules of this size at 500 MHz. The high frequency is needed to resolve overlapped signals. Fig. 5 shows a 500 MHz ROESY spectrum of doramectin and Fig. 6 shows a

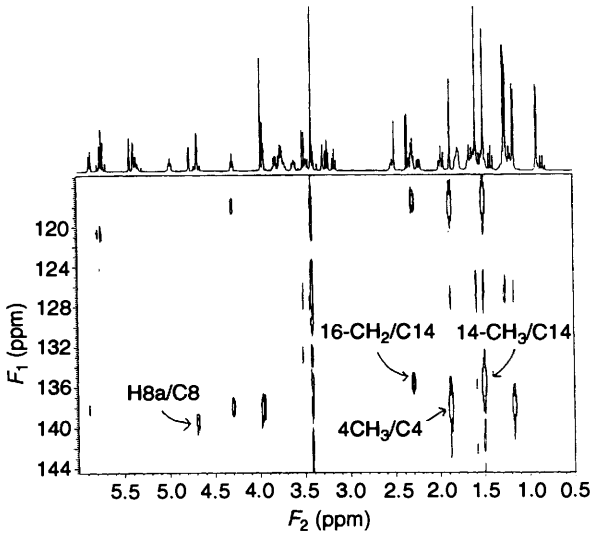

Fig. 2 Partial HMBC spectrum of cyclohexyl B2

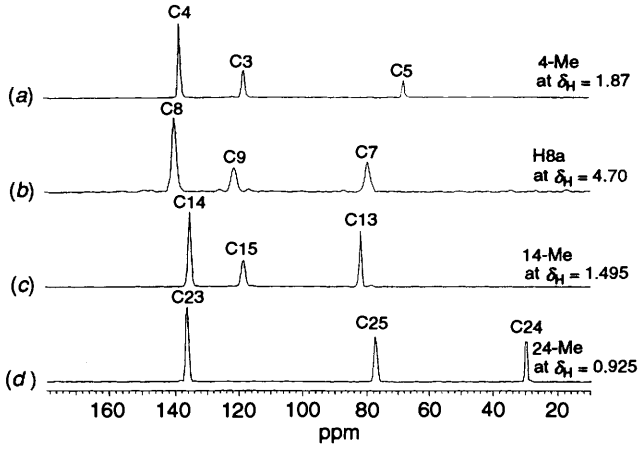

Fig. 3 Traces parallel to the ^{13}C axis of a 500 MHz HMBC ^{1}H–^{13}C spectrum of doramectin

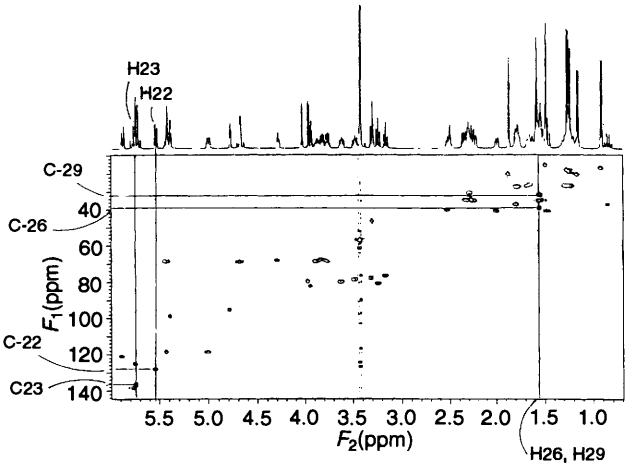

Fig. 4 500 MHz ^{1}H–^{13}C HMQC spectrum of doramectin

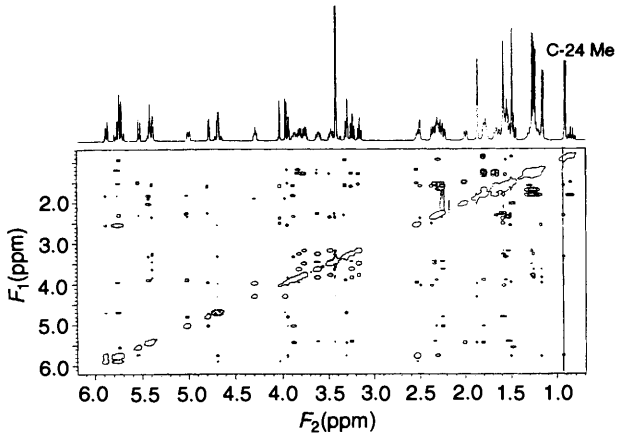

Fig. 5 500 MHz ROESY spectrum of doramectin

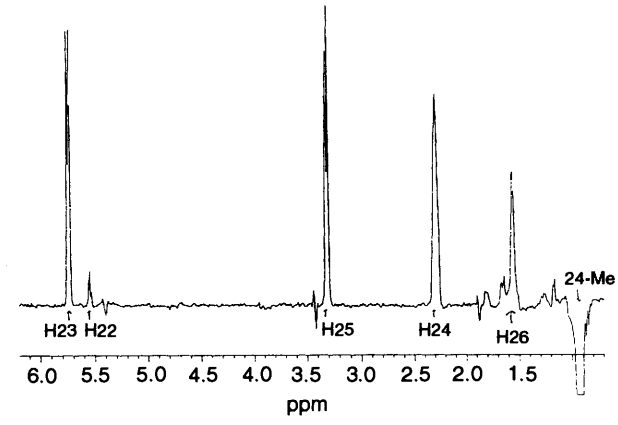

Fig. 6 C-24 Methyl slice of the 500 MHz ROESY spectrum of doramectin; positive peaks correspond to ROEs

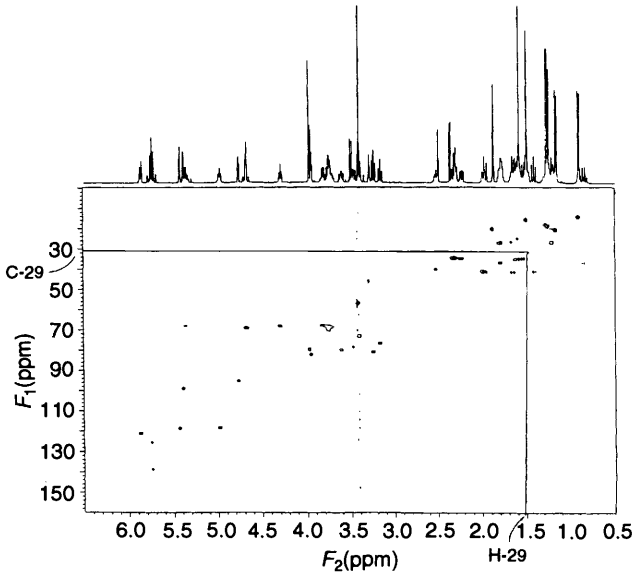

Fig. 7 HMQC spectrum of cyclohexyl B2

trace extracted from the spectrum at the frequency of the C-24 methyl resonance. There is a strong peak at δ 5.74 and only a weak one at δ 5.54. Because of the geometry of the six-membered tetrahydropyran ring, the 24-methyl group must be closer to 23-H than to 22-H. This confirms the assignment of 23-H (and hence also of 22-H, C-23 and -22).

Cyclohexane ring in doramectin and cyclohexyl B2. The chemical shifts of 26- and 29-H in the cyclohexane ring were easily identified from the HMQC and TOCSY spectra shown in Figs. 4, 7 and 8. The other four methylene groups could not be uniquely assigned because of overlap of their ^{1}H resonances.

Mass Spectrometry.—An $(M + Na)^+$ signal was observed at *m/z* 921 for doramectin and 939 for cyclohexyl B2 in the FAB spectrum. The mass spectra of the natural avermectins have been described previously[5] and in the EI spectrum the new avermectins exhibited the same characteristic fragmentation patterns but with the appropriate mass difference for those

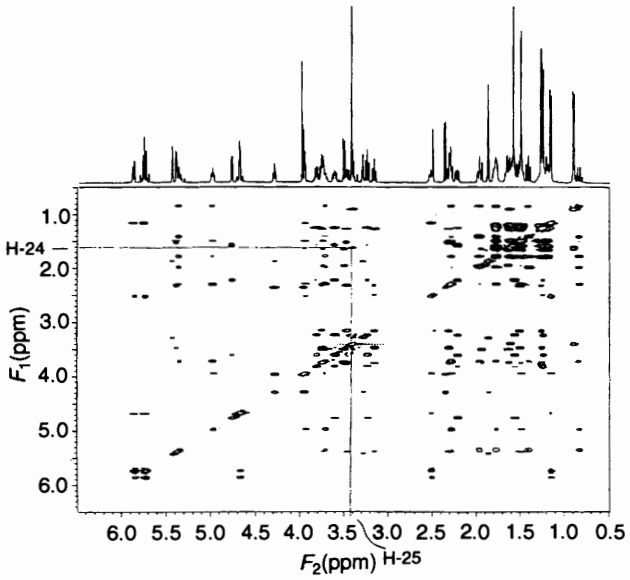

Fig. 8 2D TOCSY spectrum of cyclohexyl B2 (τm = 75 ms)

fragments which contained the cyclohexyl group. Thus, for doramectin the observed fragments were *m/z* 592, 331, 257, 247, 219, 195, 145, 127, 113, 95 and 87. For cyclohexyl B2 the fragments were *m/z* 610, 482, 349, 331, 275, 265, 257, 179, 145, 127, 113, 94 and 87. These results are in agreement with the respective structures **1** and **2**.

Conclusions

Using a range of modern 2D NMR techniques, unambiguous resonance assignments have been obtained for all the hydrogens and carbons in doramectin and 25-cyclohexylavermectin B2 with the exception of certain signals in the cyclohexane ring. The data are in close agreement with those listed for the natural avermectins.[10] The only differences are the presence of signals due to the cyclohexane ring, a feature absent in the natural avermectins and the absence of any signals due to an isobutyl or 2-methylbutyl group which are the C-25 substituents in the natural avermectins. The X-ray study confirms that the C-25 substituent of the natural avermectins has been replaced by a cyclohexane ring in doramectin and 25-cyclohexyl avermectin B2 and that this ring is attached to the C-25 carbon atom in the equatorial configuration. This fully confirms our assumption that new avermectins are derived from the precursor cyclohexanecarboxylic acid which was fed to the fermentation. Our data supports assignments for the C-22 and -23 carbon and hydrogen atoms in doramectin which are reversed compared with those previously proposed for the natural avermectins. This strongly suggests that the original assignments were incorrect.

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