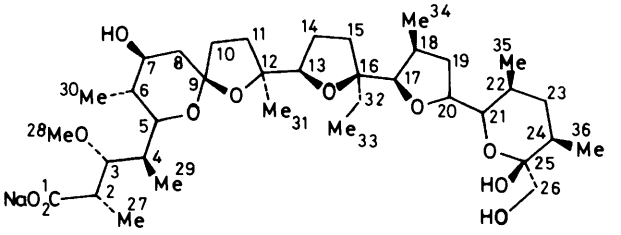
Biosynthesis of the Polyether Ionophore Antibiotic Monensin A: Assignment of the Carbon-13 and Proton N.M.R. Spectra of Monensin A by Two-dimensional Spectroscopy. Incorporation of Oxygen-18 labelled Molecular Oxygen

Abid A. Ajaz and John A. Robinson *
Department of Chemistry, The University, Southampton SO9 5NH
David L. Turner
Department of Chemistry, The University, Leicester LE1 7RH

The total assignment of the carbon-13 and proton n.m.r. spectra of the polyether ionophore antibiotic Na monensin A are reported. These assignments were derived unambiguously by consideration of two-dimensional correlation and *J*-resolved spectra of Na monensin A. These data are essential for the correct analysis by n.m.r. spectroscopy of biosynthetic experiments using stable isotope labelling techniques. The incorporation of molecular oxygen, labelled with oxygen-18, into monensin A, has been used to show that four of the oxygen atoms in the antibiotic are derived from this source.

Important progress has been made recently in unravelling the complex secondary metabolic pathways leading to the polyether ionophore antibiotics. This progress has come in particular through the application of new stable isotope labelling techniques based on the use of carbon-13 with oxygen-18 or deuterium.[1-11] The value of these labelling methods is illustrated most effectively in the case of monensin A (**1**) biosynthesis, where, from a knowledge of the biosynthetic origins of the numerous oxygen atoms[1,2,6] attached to the fatty acid-like carbon backbone, an attractive biogenetic scheme was proposed to account for the formation of the various heterocyclic rings and spiro ketal.[1,2] This concept, shown in Scheme 1, was



Monensin A (**1**)

subsequently generalized into a mechanistic and stereochemical model which can be used to account for the formation of the entire family of polyether ionophore antibiotics.[12]

A crucial requirement, however, for the unambiguous interpretation of such biosynthetic labelling experiments is the total assignment of both the natural abundance ^{13}C and ^{1}H n.m.r. spectra of Na monensin A, since it is through the isotope-induced perturbations on such spectra that the location of sites of enrichment are revealed. Until recently, n.m.r. spectral assignments for molecules as complex as the polyether antibiotics would have been achieved only with great difficulty.[13] Thus, the natural abundance ^{13}C and ^{1}H n.m.r. spectra of Na monesin A contain a large number of nuclei in closely similar chemical and magnetic environments, which result in severe spectral crowding in large parts of each spectrum. Fortunately, the emergence recently of a new family of multi-pulse two-dimensional n.m.r. experiments[14] has now provided an experimental basis for achieving such assignments unambiguously, on a routine basis. This is illustrated below, where we describe assignments of the ^{13}C and ^{1}H n.m.r. spectra of Na monensin A, derived from an analysis of two-dimensional correlation and *J*-resolved spectra. In addition, the incorporation of oxygen-18 label from molecular oxygen into monensin A, using a culture of the producing organism, *S. cinnamonensis*, has been investigated. ^{13}C N.m.r. spectroscopy is used to reveal the sites of isotopic enrichment.

Some preliminary results have been described in two communications.[5,6]

Results and Discussions

Assignment of the ^{13}C N.m.r. Spectrum.—The assignment of an n.m.r. spectrum has traditionally relied upon the evaluation of information from a number of sources; in particular chemical shifts, longitudinal relaxation times, proton-coupled multiplicities, comparisons with model compounds, chemical modification, and isotopic substitution or enrichment. In relatively complex natural products, such as the polyether ionophores, such information can be ambiguous and may result in a tentative or probable assignment. Moreover, the observation of isotopic enrichments, obtained during biosynthetic experiments involving the incorporation of labelled precursors, cannot logically be used to generate unambiguous assignments unless the biosynthetic pathways have been well characterized. These problems apply equally to proton and ^{13}C n.m.r. spectra so that although selective decoupling and two-dimensional shift correlations allow the spectrum of one nucleus to be assigned from the spectrum of a coupled nucleus, for which the assignment is known (*vide infra*), any ambiguities are perpetuated. For the ^{13}C n.m.r. spectra of most organic molecules, however, an unambiguous assignment will usually follow from a knowledge of the number of protons attached to each resonant carbon nucleus, and the pattern of carbon–carbon couplings indicating directly bonded nuclei.

Although it is usually possible to obtain the proton-coupled multiplicities by off-resonance decoupling if the spectrum is reasonably uncrowded, the overlapping lines in more complex spectra, such as those of typical polyether antibiotics, require more subtle techniques. One important approach uses characteristic modulations in spin-echo experiments to distinguish resonances with singlet or triplet structure, from those of doublets or quartets.[15] The most general form of this experiment, a complete two-dimensional analysis of the spin-echo generated by a 180° refocussing pulse, allows the separation of the effects of chemical shift and of proton coupling into two frequency dimensions so that the detailed multiplet structure of all resolvable lines in the proton-decoupled spectrum may be obtained in a single experiment.[16,17] The heteronuclear ^{1}H–^{13}C

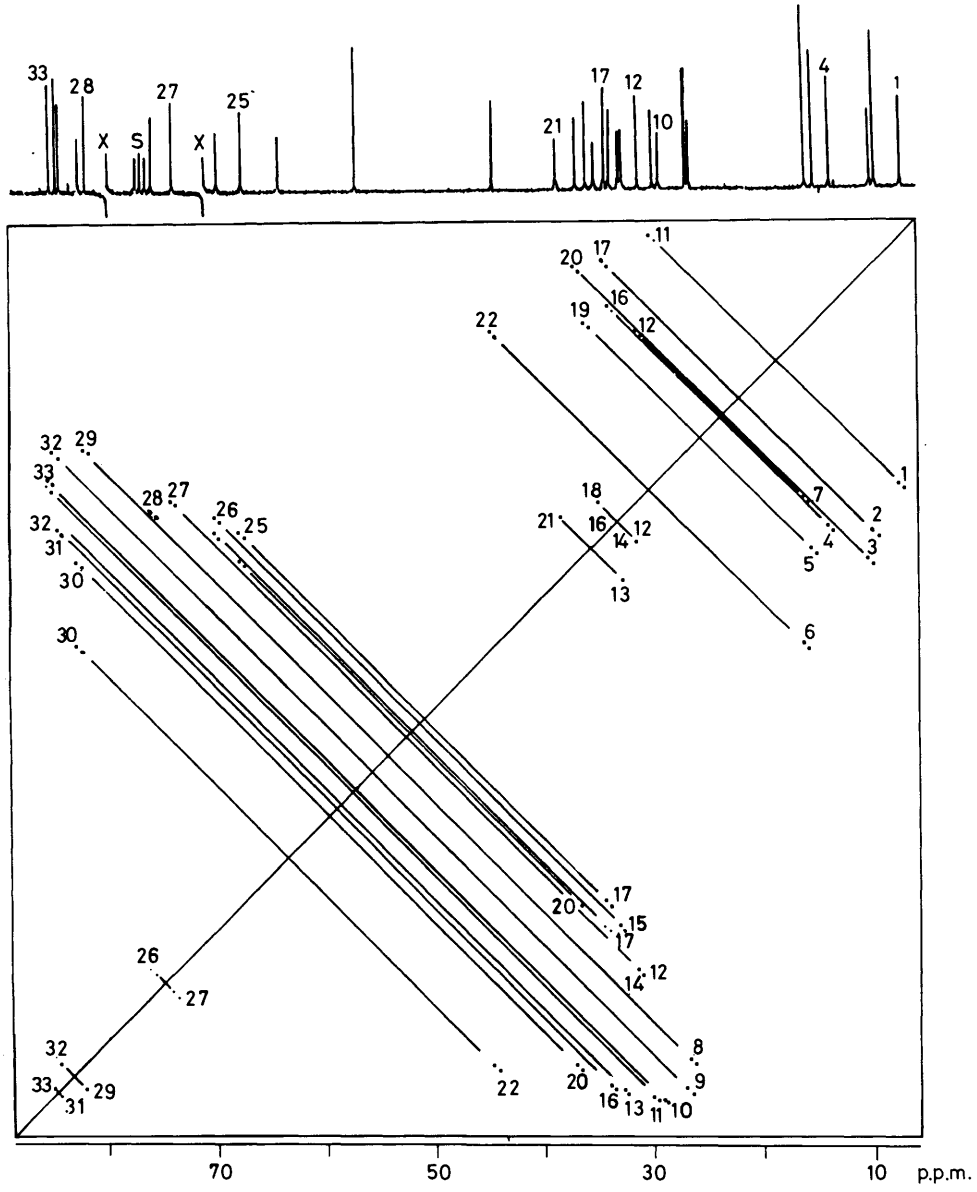


Figure 1. A two-dimensional autocorrelation spectrum of sodium monensin A at 75 MHz. ^{13}C chemical shifts are shown horizontally. Peaks in the ^{13}C spectrum are numbered with increasing chemical shift from 1—36 (see text). Folded lines due to peaks 34 and 35 are marked with an X. Directly bonded nuclei are indicated by cross peaks equidistant from the diagonal. Parameters: $F_2 = 4K$ data points 6 250 Hz; $F_1 = 256$ points over 6 250 Hz with respect to $t_1/2$; 288 scans per increment. An absolute value matrix $1K \times 1K$ symmetrized to remove folded lines is shown. The sample consisted of 1.0 g Na monensin A dissolved in deuteriochloroform (2 ml) in 10 mm tube

J-resolved spectrum of monensin A was published earlier[5] and identifies the number of protons attached to each carbon atom.

The pattern of carbon–carbon bonds may also be obtained by conventional methods; the steadily increasing sensitivity of commercial spectrometers now allows the routine observation of carbon-13 satellities in natural abundance ^{13}C n.m.r. spectra. Resonances may then be identified as those of directly bonded carbon atoms if they have identical splittings in the satellite spectrum.[18] This analysis breaks down, however, for more complex spectra where the narrow range of one-bond carbon–carbon coupling constants leads to ambiguities. In these situations the direct correlation of carbon resonances becomes necessary.

Two-dimensional n.m.r. spectroscopy again offers a method for identifying all coupled resonances in a single experiment.[19–23] This begins with a fixed sequence of three pulses which creates double quantum magnetization arising specifically from doublets having a splitting of *ca.* 35 Hz, the typical one-bond carbon–carbon coupling constant. The phases of the pulses are cycled to ensure that all of the magnetization which is eventually detected has passed through the double-quantum state[24] inaccessible to the simple two-level systems corresponding to isolated carbon-13 nuclei. It is important to suppress the relatively intense singlets from such nuclei, since they might otherwise obscure the weak satellites which are to be measured, and the method used offers a suppression ratio approaching 1 000:1. The selected components of transverse magnetization are then left to precess at their double quantum frequencies (given by the sum of the chemical shifts with respect to the transmitter) for a time $t_1/2$. A 120° pulse is then applied to redistribute the magnetization among the four transitions of the two-spin AX system, selecting the component that will refocus.[23] The effect generated by this pulse is to cause both the A- and X-spins, to acquire a modulation at the sum of the

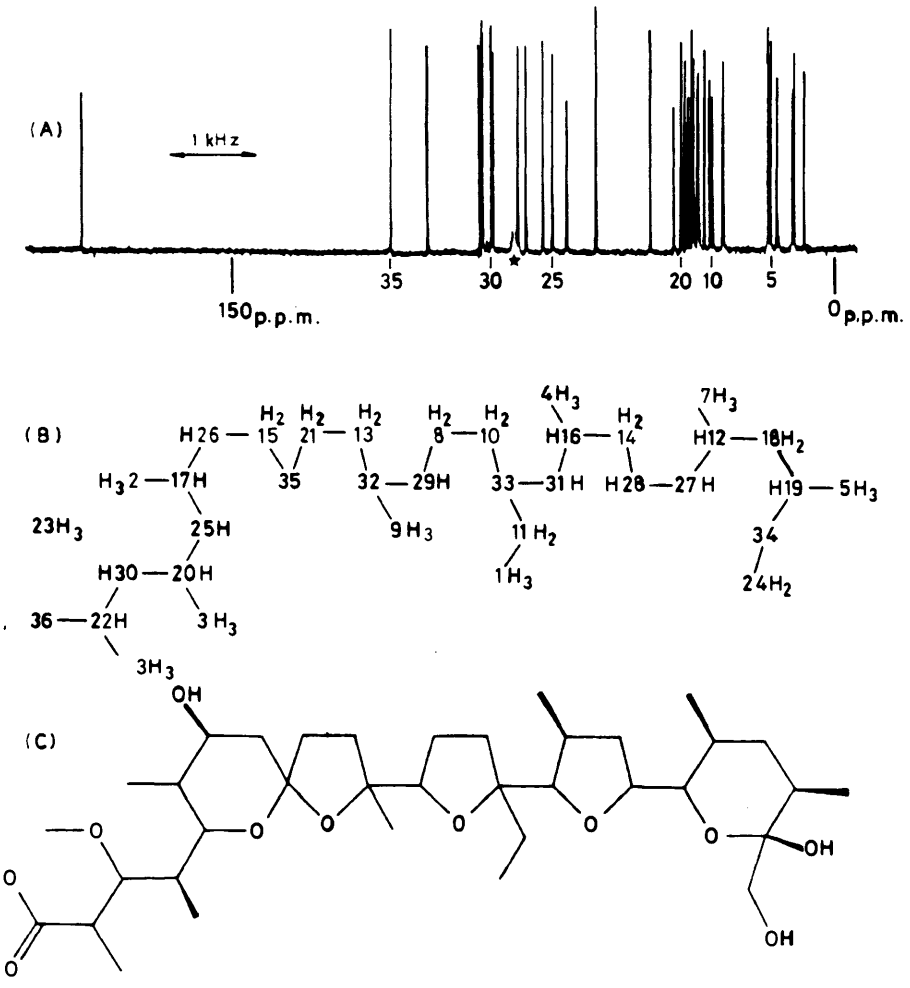


Figure 2. (*a*) The ^{13}C-{^{1}H} decoupled n.m.r. spectrum of Na monensin A at 50.3 MHz: * = deuteriochloroform; (*b*) carbon–proton and carbon–carbon bonds identified by 2D n.m.r. spectroscopy (see text); (*c*) the known structure of monensin A; comparison with the information of (*b*) gives the assignment of the spectrum (*a*)

frequencies of the two spins, as a function of $t_1/2$. A formal treatment of a non-selective 120° pulse shows that the magnetization associated with each double quantum transition before the pulse is distributed equally among the four single quantum transitions of the two-spin system, and the amplitude of the signal from the A-spins detected after a period of refocussing equal to $t_1/2$ is then modulated only by the frequencies of the coupled X-spin, and *vice versa*. The chemical shift of the coupling partner of a particular resonance may then be determined by measuring the frequency of modulation of that resonance as a function of $t_1/2$. This is achieved by repeating the experiment several times with regular increments in the delay between the initial excitation and the 120° mixing pulse; the second Fourier transformation with respect to $t_1/2$ reveals the associated frequencies.

Part of the two-dimensional autocorrelation spectrum of Na monensin A obtained in this way is shown in Figure 1. The peaks in the normal one-dimensional carbon-13 n.m.r. spectrum have been numbered from 1—36 as they occur at increasing chemical shift from tetramethylsilane. Only the chemical shift region between δ 90—4 is included within the spectral width of the 2D-autocorrelation spectrum. The spectrum shown is symmetrized and folded signals therefore do not appear; the connectivity to resonances 34, 35, and 36 are not evident. All other carbon–carbon connectivities can be identified except those between peaks 8 and 10, and peaks 18 and 19. The bonding information derived from this spectrum is summarized in Figure 2. In an earlier communication[5] a similar 2D-spectrum was described, which had been recorded at 50 MHz using a Bruker CXP200 spectrometer. In this case the three triplets 13—15 and the two quartets 6 and 7 were not resolved. However, in the spectrum shown in Figure 1 recorded at 75 MHz, these peaks are resolved allowing each to be assigned with a high degree of confidence. A comparison of the bonding information obtained from these 2D-spectra, with the known structure of monensin A, leaves no doubt over the complete assignment of the ^{13}C n.m.r. spectrum, and these data are given in the Table. It is gratifying that largely the same* assignments have been reached independently by Cane and co-workers,[2] using the more traditional methods.

Finally, it should be noted that although small concentration dependent changes in chemical shifts were observed, the order of

* Our assignments of peaks 13 and 14, are to C(11) and C(19) respectively. However, in the work of Cane and co-workers[2] these two assignments are reversed. We have found that the incorporation of sodium [1-^{13}C]propionate, as well as sodium [1-^{13}C]butyrate, into monensin A, in shake flask cultures of *S. cinnamonensis*, leads to significant enhancements of peak 13 (as well as of other peaks) but not peak 14, whereas the incorporation of sodium [1-^{13}C]acetate enhances peak 14 (again, in addition to other peaks) but not peak 13. These enrichments are consistent with our assignment, based on the accepted origins of C(11) from C(1) of propionate, and of C(19) from C(1) of acetate. Apart from this, our assignments are in complete agreement with those of Cane.[2]

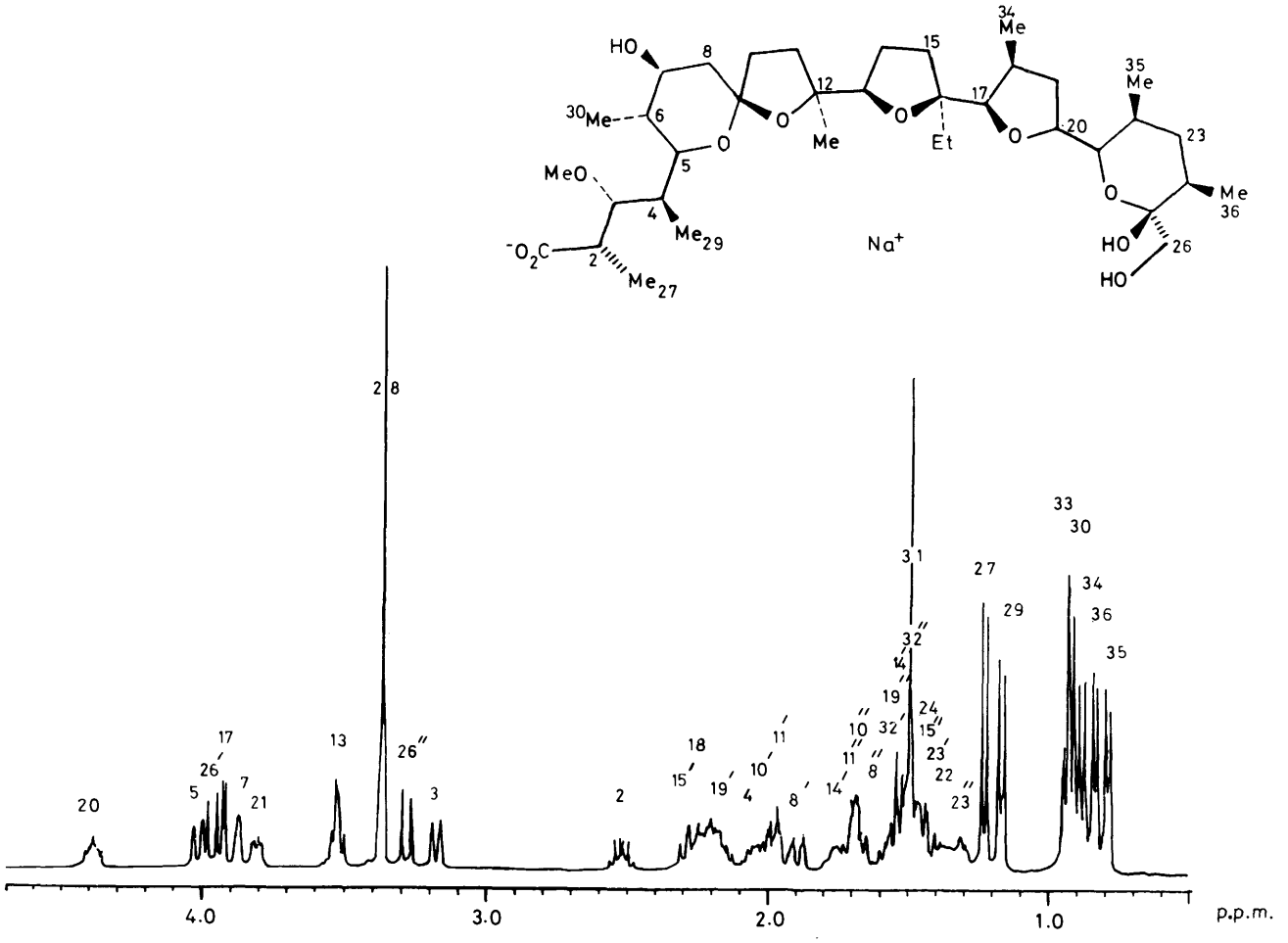


Figure 3. Proton n.m.r. spectrum of Na monensin A in deuteriochloroform, at 360 MHz. Proton resonances are assigned according to their directly attached carbon atoms (shown by numbering)

Table. Assignments of the ^{13}C and ^{1}H n.m.r. spectra of Na monensin A in deuteriochloroform, relative to tetramethylsilane (δ 0.00) internal standard

Carbon-13				Proton		Carbon-13				Proton	
Signal	Multiplicity	Chemical shift (δ)	Carbon	Chemical shift (δ)	Position	Signal	Multiplicity	Chemical shift (δ)	Carbon	Chemical shift (δ)	Position
1	q	8.14	C(33)	0.810	H(35)	21	t	39.28	C(10)	1.779	H(14′)
2	q	10.48	C(30)	0.849	H(36)	22	d	45.07	C(2)	1.911	H(8′)
3	q	10.97	C(29)	0.903	H(34)	23	q	57.84	C(28)	1.981	H(11′)
4	q	14.56	C(34)	0.939	H(30)	24	t	64.92	C(26)	2.011	H(10′)
5	q	16.03	C(36)	0.944	H(33)	25	d	68.33	C(5)	2.066	H(4)
6	q	16.72	C(27)	1.178	H(29)	26	d	70.51	C(7)	2.185	H(19′)
7	q	16.80	C(35)	1.238	H(27)	27	d	74.51	C(21)	2.216	H(6)
8	t	27.26	C(14)	1.32	H(23″)	28	d	76.45	C(20)	2.261	H(18)
9	q	27.47	C(31)	1.36	H(22)	29	d	82.52	C(13)	2.304	H(15′)
10	t	29.91	C(15)	1.41	H(23′)	30	d	83.11	C(3)	2.530	H(2)
11	t	30.60	C(32)	1.46	H(24)	31	d	84.94	C(17)	3.190	H(3)
12	d	31.87	C(22)	1.468	H(15″)	32	s	85.26	C(12)	3.298	H(26″)
13	t	33.25	C(11)	1.507	H(32″)	33	s	85.90	C(16)	3.381	H(28)
14	t	33.33	C(19)	1.507	H(31)	34	s	98.31	C(25)	3.540	H(13)
15	t	33.56	C(8)	1.536	H(14″)	35	s	107.03	C(9)	3.828	H(21)
16	d	34.39	C(18)	1.551	H(19″)	36	s	181.20	C(1)	3.895	H(7)
17	d	34.87	C(6)	1.598	H(32′)					3.938	H(17)
18	t	35.75	C(23)	1.687	H(8″)					3.977	H(26′)
19	d	36.56	C(24)	1.704	H(10″)					4.029	H(5)
20	d	37.50	C(4)	1.718	H(11″)					4.403	H(20)

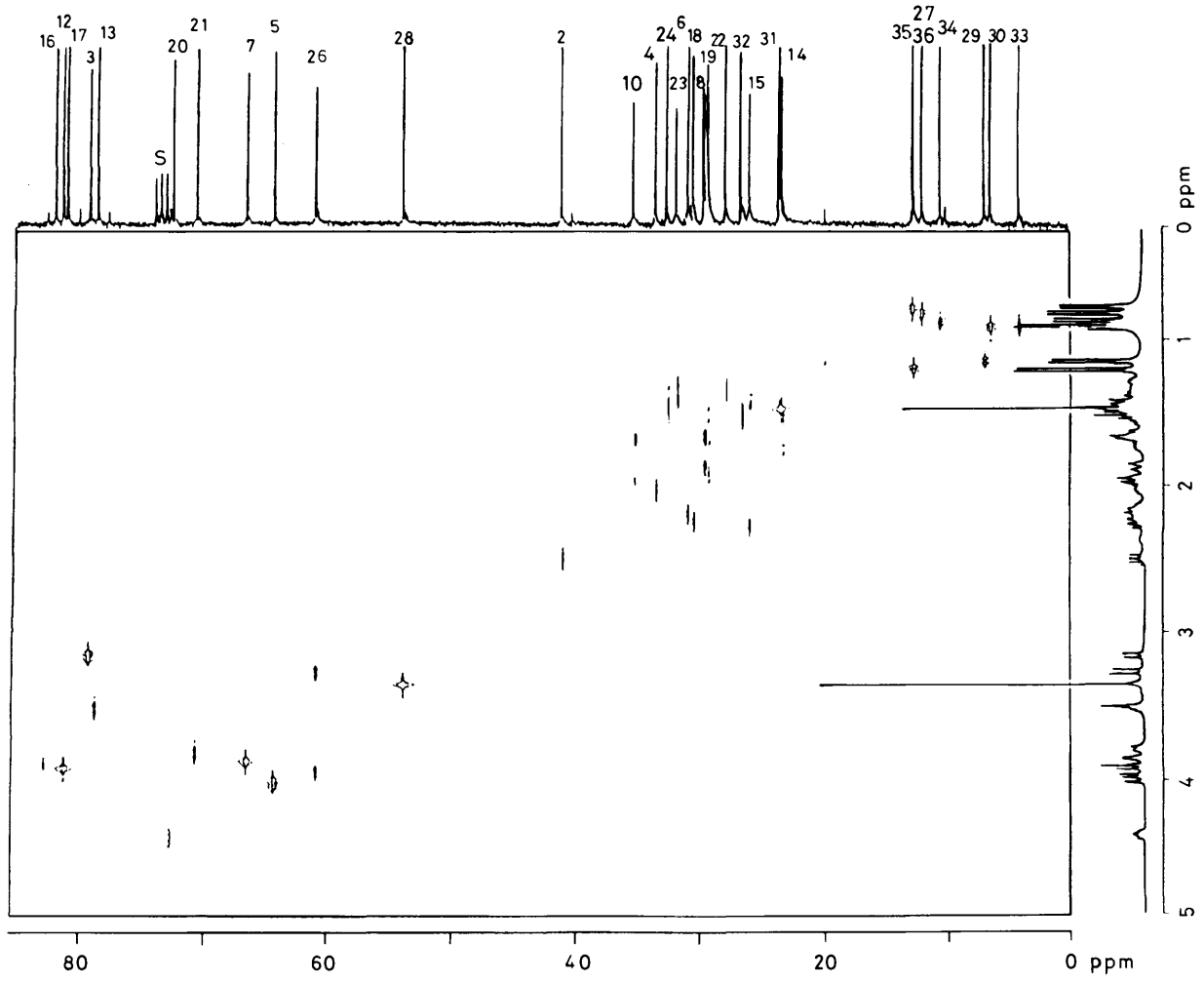


Figure 4. A two-dimensional ^{1}H–^{13}C shift correlation spectrum of Na monensin A in deuteriochloroform, at 300 MHz. Peaks in the contour plot identify those carbon and hydrogen atoms that are directly bonded. Parameters: $F_2 = 2K$ data points; $F_1 = 256$ W zero-filled to 512 W; $SW2 = 6\,450$ Hz; $SW1 = 1\,500$ Hz; delays $\Delta_1 = 4$ ms, $\Delta_2 = 2$ ms and 3 s relaxation delay

the lines remains unchanged in $CDCl_3$ over the range 1 g ml^{-1} to, effectively, infinite dilution.

Assignment of the ^{1}H *N.m.r. Spectrum.*—An assignment of the proton n.m.r. spectrum of Na monensin A can be obtained most straightforwardly by a correlation with the known ^{13}C n.m.r. assignments. Previously such a correlation might have been achieved through a series of single frequency decoupling experiments. However, this method is tedious and can run into serious problems in complex spectra containing overlapping resonances. Exactly this situation is encountered in the normal ^{1}H n.m.r. spectrum of monensin A. The resonances occur in a small spectral window between δ 4.5—0.7 (see Figure 3) except for a broad and D_2O exchangeable signal, occuring typically at δ 9.6 (in deuteriochloroform).

An alternative approach involves an analysis of scalar couplings occurring between related nuclei along the carbon backbone. In fact, a substantial part of the ^{1}H n.m.r. spectrum of Na monensin A had been assigned by Anteunis[25] using double resonance techniques, before the impact of recent 2D n.m.r. methodologies had emerged, and despite severe problems due to spectral overcrowding.

Many of these assignment problems, however, can now be eliminated elegantly, in relatively few experiments, by using 2D n.m.r. techniques. Thus, a typical 2D ^{13}C–^{1}H chemical shift correlation spectrum[26,27] is shown in Figure 4, with the normal ^{13}C and ^{1}H n.m.r. spectra plotted also on the F_2 and F_1 axes. The ^{1}H n.m.r. signals in the region between δ 4.5—3.1 are easily assignable *via* the clear correlation each makes with its directly attached carbon nucleus. Similarly, the multiplet at δ 2.5 [assigned to H(2)], and the signals corresponding to each of the methyl groups can be assigned unambiguously, and all of these assignments are in agreement with those made by Anteunis.[25]

The most crowded region of the ^{1}H-spectrum occurs between δ 2.3 and 1.3, and contains twenty-one overlapping multiplets. Although a detailed analysis of the ^{13}C–^{1}H correlation map (shown in Figure 4) reveals many of the remaining assignments, the process is aided considerably by examining also 2D ^{1}H-homonuclear J-resolved[28] and ^{1}H-homonuclear correlation (COSY) spectra.[29] Thus, J-resolved spectroscopy can again be used to facilitate an analysis of these multiplets; the second frequency dimension is used to spread-out information by separating the effects of chemical shift differences from the effects of coupling. Figure 5 shows a part of the contour plot from the ^{1}H-homonuclear J-resolved spectrum of Na monensin A. In addition, Figure 6 shows the contour plot of a ^{1}H-homonuclear correlation (COSY-90) experiment, which reveals all of the observable scalar couplings in a single spectrum. By considering the spectra shown in Figures 4, 5, and 6, the remaining assignments can be made.

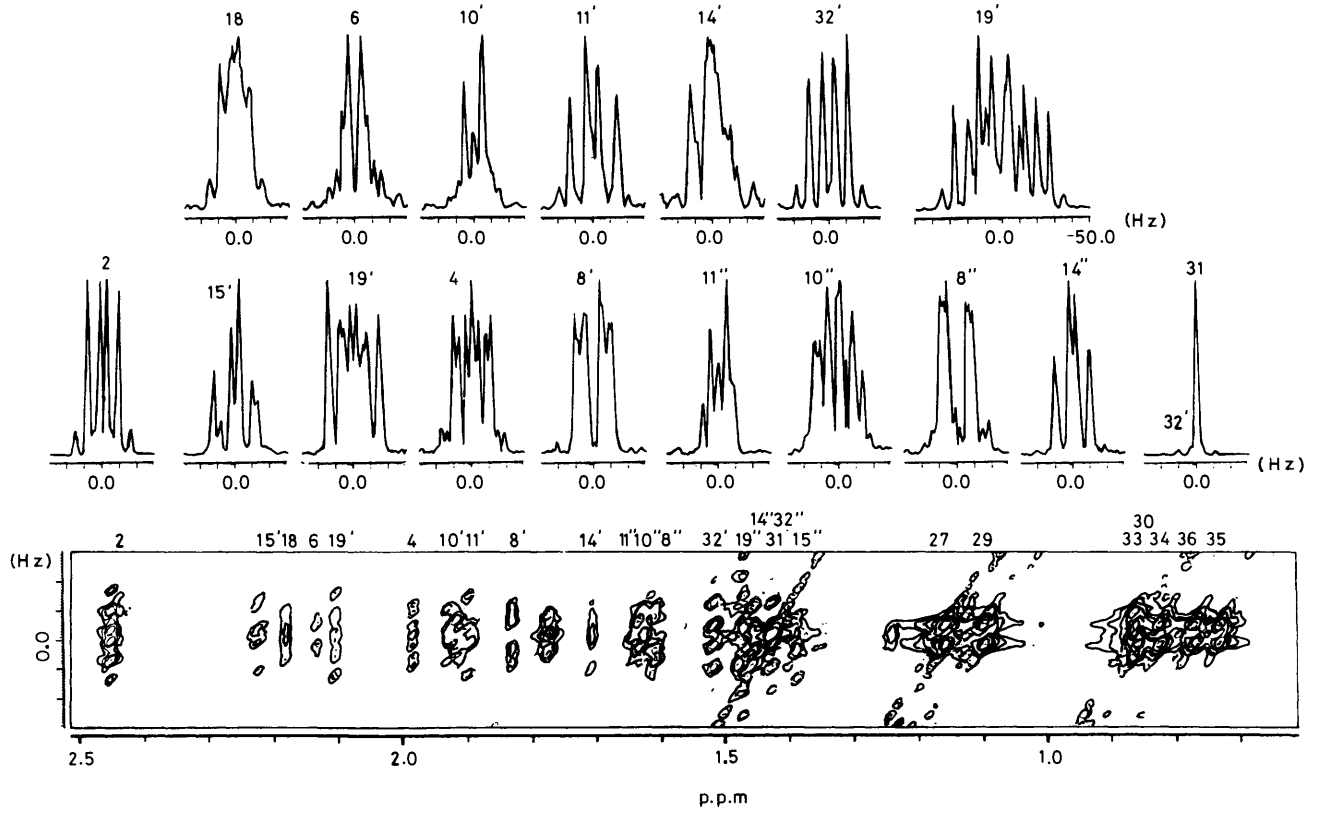


Figure 5. A two-dimensional ^1H homonuclear *J*-resolved spectrum recorded at 360 MHz. Submatrix columns taken from the 2D-plot are illustrated for proton resonaces in the range H(2)—H(31). Parameters: $F_2 = 4K$ data points; $F_1 = 64\ W$ zero-filled to 128 *W*. Transformed with unshifted sine-bell function and shown with a 45° TILT applied. Scale divisions are 10 Hz

Thus, the ^1H-homonuclear *J*-resolved spectrum (Figure 5) clearly reveals four resonances in the region between δ 2.30—2.10, three of which can be assigned from the ^1H–^{13}C correlation spectrum (Figure 4) to H(15′), H(18), and H(6). The fourth signal is H(19′), whose assignment follows most clearly from correlations to H(20) and H(19″) in the COSY spectrum (Figure 6). Another set of four resonances occur between δ 2.1 and 1.8. Again, three of these can be assigned to H(8′), H(10′), and H(4) from the ^1H–^{13}C correlation spectrum, whereas the fourth correlates less clearly to H(11′), due to poor resolution of the carbon signals for C(19) and C(11). The assignments of H(8) and H(4) are reinforced in the COSY spectrum, where correlations of H(8) to H(7), and of H(4) to H(5) are evident. The effects of strong coupling give rise to an additional level of complexity in the ^1H *J*-resolved spectrum at δ 2.0, due to the virtual coincidence of resonances assigned to H(10′) and H(11′). A similar situation occurs at δ 1.71, where the resonances of H(10″) and H(11″) occur, and nearby are seen signals due to H(14′) [at δ 1.78; couples to H(13) and H(14″)] and H(8″) [at δ 1.69; couples to H(7) and H(8′)].

The region between δ 1.6 and 1.25 becomes less easy to analyze due to spectral crowding, and the appearance of higher order coupling. However, of particular interest is the region between δ 1.6 and 1.48. An intense singlet due to the methyl group H(31) is prominent at δ 1.51. The multiplets surrounding this singlet are not easily assignable from the ^1H–^{13}C correlation spectrum; however, a signal (appearing as a quartet) is evident in the ^1H–*J*-resolved spectrum at δ 1.60 which is assigned to H(32′). A similar multiplet occurs at δ 1.51 [coincident with H(31)] and is assigned to H(32″) These two assignments are supported by the relevant cross-peaks in the ^1H–^{13}C correlation spectrum, as well as by the COSY spectrum, which shows the coupling of each to the methyl group H(33). The multiplets at δ 1.55 and 1.54 are assigned to H(19″) and H(14″) respectively. The signal for H(19″) is seen to couple with H(20) (COSY spectrum), as well as to H(19′) at δ 2.19, whereas H(14″) couples with H(13) and H(14′), as expected. Immediately upfield of the singlet assigned to H(31) are seen five resonances assigned in order of decreasing chemical shift to H(15″), H(24), H(23′), H(22), and H(23″). Although not all of these signals are evident in the ^1H–*J*-resolved spectrum, the assignments are made with a high degree of confidence based on connectivities established by ^1H–^{13}C and ^1H–^1H couplings (shown in Figures 4 and 6).

No attempt has been made to distinguish between resonances assigned to pairs of protons located at prochiral centres, although this may be possible using n.O.e. difference spectroscopy.

Finally, those protons attached directly to oxygen are revealed through the spectral changes following a shake with deuterium oxide. A broad singlet at δ 9.60 integrating for two proton resonances disappears after this treatment, as does a signal at δ 3.6 which overlaps a doublet of doublets assigned to H(13). The assignment of the exchangeable proton resonating at δ 3.6 is to the hydroxy proton on O(4) since cross peaks in the COSY spectrum indicate coupling to H(8′), and the multiplet assigned to H(8′) simplifies to a doublet of doublets with the deuterium oxide shake. At the same time the exchange leads to a sharpening of peaks at δ 2.20 due to the removal of coupling between this hydroxy proton and H(6). The remaining hydroxy protons at O(9) and O(10), therefore, give rise to the broad exchangeable signal at δ 9.60. A summary of these proton assignments is given in the Table.

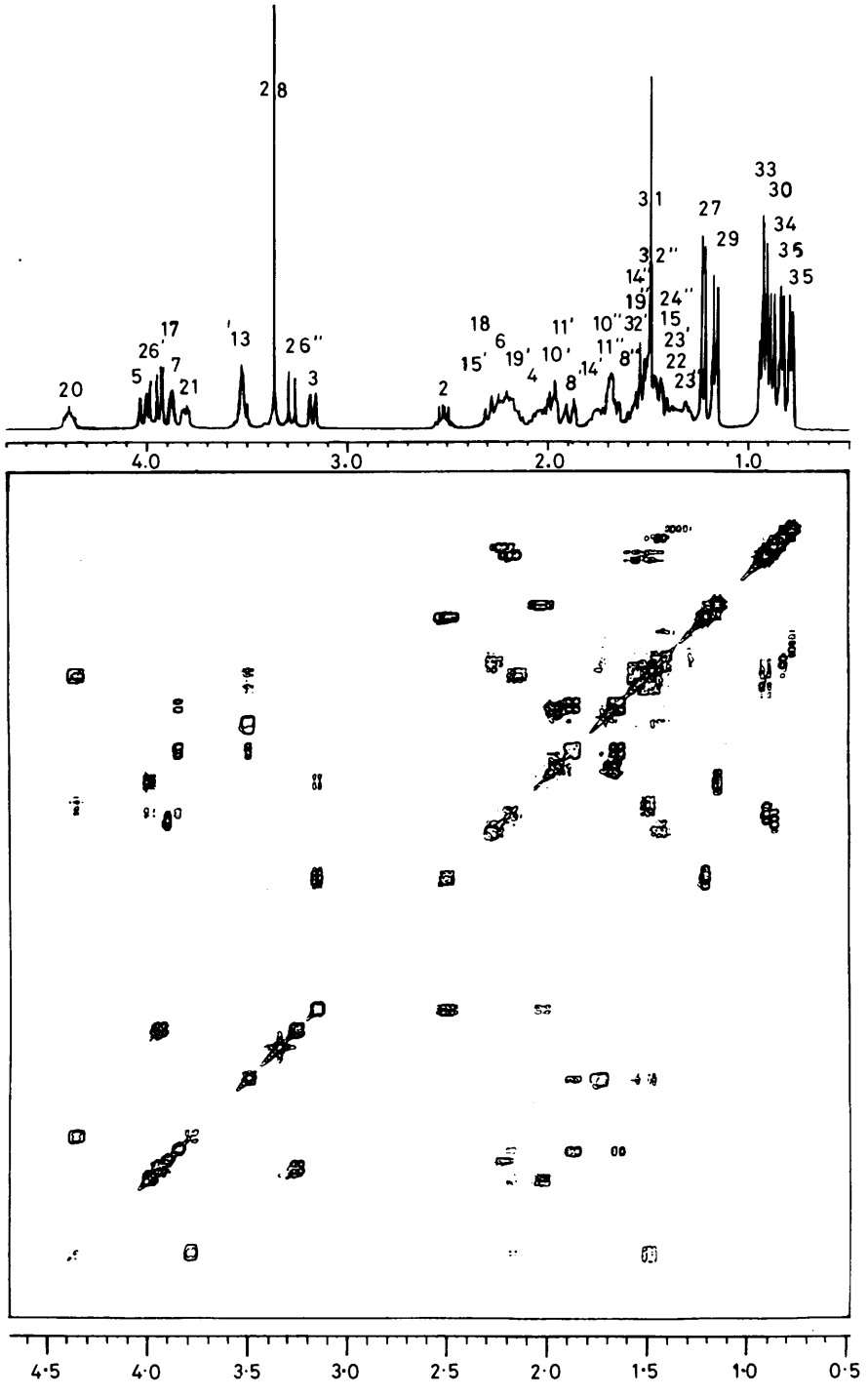
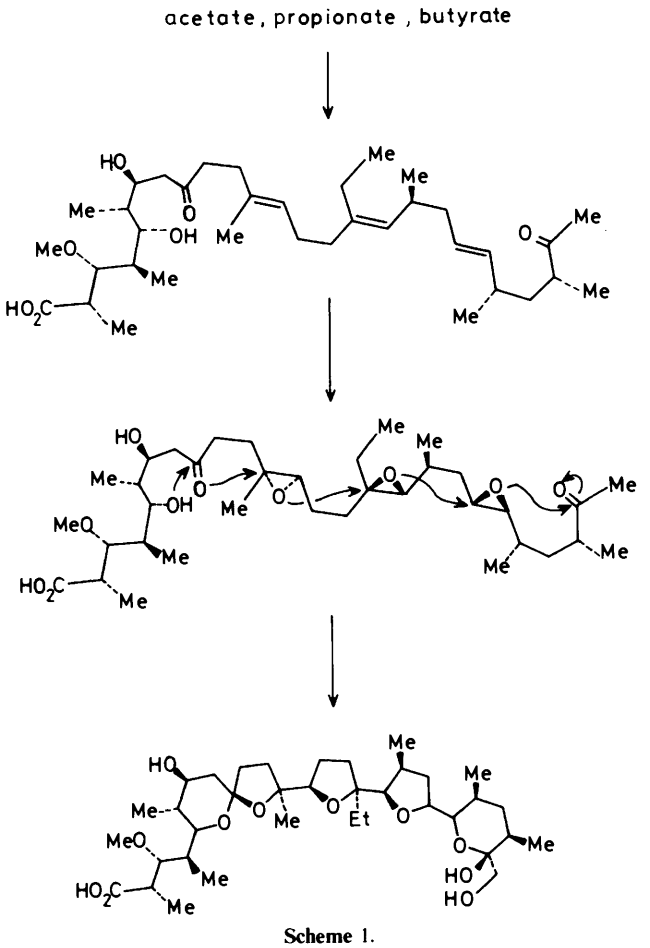


Figure 6. A two-dimensional COSY-90 ^{1}H n.m.r. spectrum. Cross peaks identify nuclei related by scalar coupling. Parameters: $F_2 = 2K$ data points; $F_1 = 512$ W zero-filled to $1K$. Transformed with unshifted sine-bell function and shown symmetrized

The Utilization of Oxygen Atoms from Molecular Oxygen during the Biosynthesis of Monensin A.—Whilst our studies with monensin A were underway, an important advance in the study of its biosynthesis, in cultures of *Streptomyces cinnamonensis*, was reported by Cane and co-workers.[1,2] They described labelling experiments which identified the origins of most of the oxygen atoms attached to the carbon backbone. This information was used to support the biosynthetic route shown in Scheme 1. Although details of the carbon backbone assembly, as well as uncertainties about the involvement of enzyme bound (or enzyme free) intermediates cannot be addressed unambiguously at this level, the elegance of the cyclization-cascade generates a strong *prima-facie* case for its acceptance as a model for understanding how the heterocyclic rings, and the spiro ketal, are generated.

An important role is implicitly given, in Scheme 1, to the involvement of enzymes that catalyze the epoxidation of double bonds using molecular oxygen as a co-substrate. Since early in our work the incorporation of oxygen-18 label from molecular oxygen had not been described[1] we developed a protocol for growing *S. cinnamonensis* under $^{18}O_2$. This proved to be a demanding task since the success of a shake flask ferment-



Scheme 1.

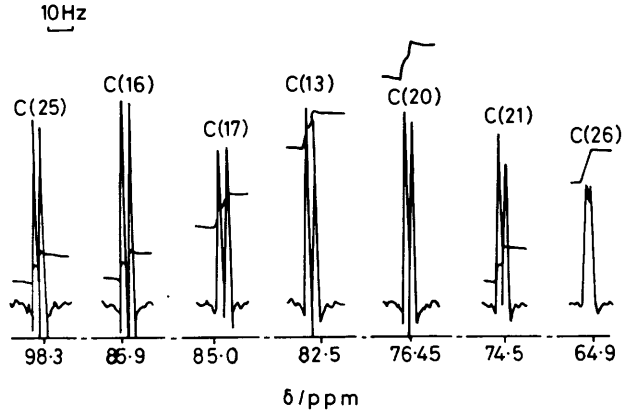
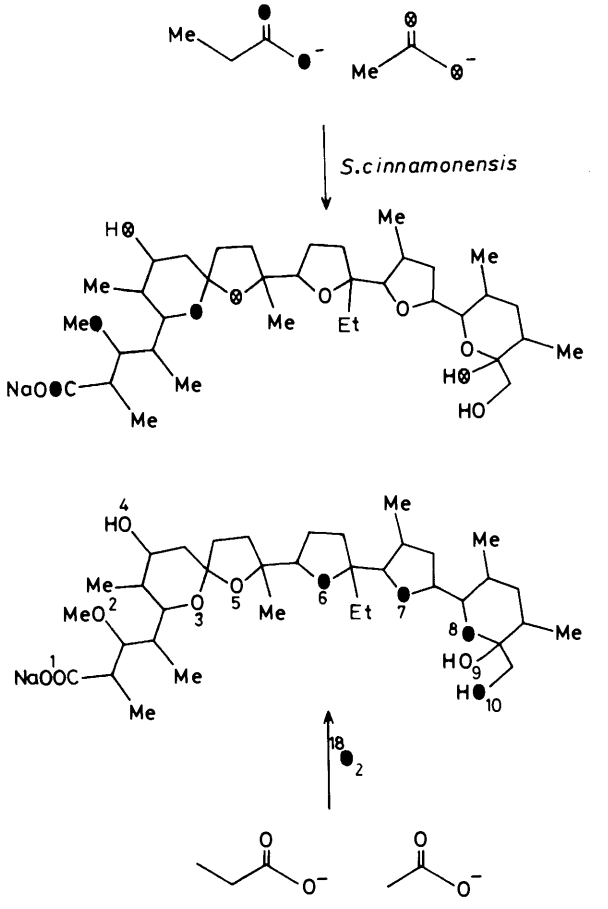


Figure 7. Sections of the 100 MHz ^{13}C-{^{1}H} n.m.r. spectrum of [$^{18}O_4$]Na monensin A (20 mg) + unlabelled Na monensin A (20 mg) in deuteriochloroform. The spectrum is resolution enhanced, spectral width 25 000 Hz, 65° pulse, 6 s relaxation delay, 64*K* data points

ation depends critically upon maintaining the highest possible aeration of the culture, within the constraints imposed by the need to use a closed system containing labelled oxygen.

We eventually adopted a method in which the fermentation vessel was connected to a gas reservoir, and at the same time the gases in the vessel were circulated through a trap containing aqueous potassium hydroxide to remove evolved carbon dioxide. The evolution of CO_2 could be conveniently monitored by mass spectroscopy, and the uptake of oxygen could be followed from the gas reservoir. In practice, a 60 ml shake flask culture was grown for 24 h at 32 °C in a stream of sterile air, and then for a further 50 h under an atmosphere of 99 atom% $^{18}O_2$ and N_2 (50:50). The rate of oxygen consumption remained steady within the range 25—30 ml h^{-1} over the course of the fermentation, and the system was recharged with labelled oxygen at regular intervals to prevent the percentage of oxygen in the atmosphere from falling below 20%. The antibiotic was subsequently isolated and purified by flash chromatography to afford 45 mg of Na monensin A (m.p. 269 °C).

This material was initially characterized by electron impact mass spectroscopy, which revealed clearly that four atoms of oxygen-18 had been incorporated. The mass spectrometry of monensin A has been described by Chamberlin and Agtarap.[30] Thus, in the normal EI mass spectrum an intense peak at *m/z* 617 is seen, which arises from loss of MeO• and CO_2 from the molecular ion at *m/z* 692. With our labelled sample this fragment ion was now displaced to *m/z* 625, indicating that the sample was *ca.* 85 atom% $^{18}O_4$ labelled. The small drop in isotopic enrichment (from 99 atom% in labelled oxygen) is almost certainly due to residual traces of $^{16}O_2$ remaining in the fermentation vessel (*vide infra*). In order to locate unambiguously the specific sites of enrichment we made use of our assignment of the ^{13}C n.m.r. spectrum of Na monensin A, together with the well known ^{18}O-isotope effect[31,32] on ^{13}C chemical shifts. An internal standard was incorporated by diluting the labelled material in a 1:1 ratio with unlabelled Na monensin A. The resolution enhanced ^{13}C n.m.r. spectrum of this mixture at 100 MHz revealed clearly sharp singlets for all peaks except those assigned to C(13), C(16), C(17), C(20), C(21), C(25), and C(26). These peaks appeared as doublets (see Figure 7) due to the presence of ^{13}C–^{18}O resonances upfield by 3.1, 3.4, 3.5, 2.9,



Scheme 2.

2.9, 2.9, and 2.0 Hz, respectively, from the normal ^{13}C–^{16}O signals. The ^{18}O label is, therefore, unambiguously located at position O(6), O(7), O(8), and O(10), shown in Scheme 2.

Related experiments were also reported by Cane and co-workers[2] who, using similar techniques, showed that the oxygens O(6), O(7), and O(8) are derived from molecular oxygen. The low yield of monensin A from their experiment, however, prevented the detection of ^{18}O at position O(10).

The data obtained from these labelling experiments are entirely in accord with the pathway depicted in Scheme 1. The high isotopic enrichments we have seen, which are indicated by the almost equal heights of each ^{13}C–^{18}O resonance compared to the corresponding ^{13}C–^{16}O resonance (Figure 7), are noteworthy. In particular, no ambiguity arises due to the generation, during mono-oxygenase catalyzed processes, of high localized concentrations of $H_2{}^{18}O$, since this labelled water would inevitably be diluted significantly with unlabelled $H_2{}^{16}O$. The timing of these oxygen atom insertions cannot at present be defined. Whereas three inserted oxygens are required for the key cyclization processes, the fourth, that at C(26), together with the hydroxy group at C(25), exert a stabilizing effect in the Na monensin complex (as revealed by *X*-ray crystallography[33,34]) by participating in hydrogen bonding to the C(1) carboxy group. We may speculate that the hydroxy group at C(26) is inserted late in the biosynthesis. Thus, several other structurally related polyether ionophores[12] lack such a hydroxy group at the carbon backbone terminus.

Experimental

Monensin A sodium salt was provided as a gift from Eli-Lilly and Co. N.m.r. spectra were recorded on Bruker CXP-200, AM-300, AM-360, or WH-400 spectrometers. The spectrometer conditions employed during the accumulation and processing of 2D n.m.r. data are included in Figures 1, 4, 5, and 6. All n.m.r. spectra were recorded in $CDCl_3$, with an internal deuterium lock and both ^1H and ^{13}C chemical shifts are referenced to tetramethylsilane as an internal standard (at δ 0.00). Melting points were determined on a Kofler hot-stage melting point apparatus and are uncorrected. Mass spectra were recorded on an MSE-Kratos MS30 instrument, using electron impact ionization (at 70 KeV). Thin layer chromatography was carried out with Machery-Nagel precoated 20 × 20 cm sheets layered with 0.25 mm of silica gel containing a fluorescent indicator, whereas column chromatography was performed on Silica gel 60 of 230—400 mesh. All laboratory grade solvents were distilled before use. Oxygen-18 labelled molecular oxygen (99 atom% ^{18}O) was supplied by BOC Prochem.

Growth of Streptomyces cinnamonensis.—*S. cinnamonensis* A3823.5 was supplied as a lyophilized pellet by Eli-Lilly and Co. To resuscitate this culture, the pellet was resuspended in tryptic soy broth (1 ml) and then added to a sterile vegetative medium having the following composition: D-glucose (0.3 g), potato extract (1.2 g), soybean flour (0.9 g), yeast extract (0.15 g), and calcium carbonate (0.06 g) dissolved in distilled water (60 ml). The medium was dispensed in a 500 ml wide neck Erlenmeyer flask, closed with a cotton wool bung, and sterilized at 121 °C for 20 min. After inoculation, the culture was placed on an orbital shaker rotating at 300 r.p.m. and maintained at 32 °C. After incubation under these conditions for 20 h, this medium was then used to inoculate a fermentation culture having the following composition: soybean grits (1.5 g), D-glucose (1.8 g), calcium carbonate (60 mg), manganous chloride hydrate (12 mg), ferric sulphate hydrate (18 mg), potassium chloride (6 mg), soybean oil (0.9 g), methyl oleate (1.2 g; technical trade), and lard oil (0.3 g), dissolved in distilled water (60 ml). The medium was dispensed in a 500 ml wide neck Erlenmeyer flask, closed with a cotton wool bung and sterilized at 121 °C for 25 min. The medium was inoculated with 5% v/v of broth from the vegetative culture, and incubated on an orbital shaker at 300 r.p.m., 32 °C, for seven days.

The medium was then diluted (1:1) with methanol, homogenized in a high speed blender, filtered through Celite, and the filtrate was extracted with chloroform. The chloroform extracts were dried (Na_2SO_4) and evaporated under reduced pressure to afford a dark coloured oil. This oil was chromatographed on silica gel eluting firstly with methylene chloride and subsequently with methylene chloride–ethyl acetate (1:1). Fractions containing Na monensin A were pooled, evaporated under reduced pressure, and the resulting solid Na monensin A was recrystallized twice from methanol–water to give pure white crystals, m.p. 267—269 °C (lit.,[35] 267—269 °C). Typically, 100 mg of pure Na monensin A was recovered from a single shake flask culture.

Fermentation under $^{18}O_2$.—A vegetative culture of *S. cinnamonensis* (60 ml) was prepared, as described above. This was used to inoculate a fermentation medium (60 ml) dispensed, and sterilized, in a modified 500 ml Quickfit Erlenmeyer flask. After inoculation (5% v/v inoculum) the culture was incubated for 24 h on an orbital shaker at 300 r.p.m. at 32 °C. At this point, the gases in the flask were replaced by a mixture (50:50) of $^{18}O_2$ and N_2, and this was circulated continuously by a peristaltic pump to a trap containing 40% aqueous potassium hydroxide before being returned to the neck of the flask. The flask was also connected to a gas reservoir which contained the same mixture of gases. Gas samples could be removed at intervals through a septum in a side arm for analysis by mass spectroscopy. A water manostat was used to maintain atmospheric pressure in the gas reservoir, as well as to follow the uptake of $^{18}O_2$, which was replenished at regular intervals. The fermentation was continued for a further 5 days, and subsequently the monensin A sodium salt was isolated by the procedure described above, affording white crystals (45 mg), m.p. 269 °C.

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References

1 D. E. Cane, T. C. Liang, and H. Hasler, *J. Am. Chem. Soc.*, 1981, **103**, 5962.
2 D. E. Cane, T. C. Liang, and H. Hasler, *J. Am. Chem. Soc.*, 1982, **104**, 7274.
3 C. R. Hutchinson, M. M. Sherman, J. C. Vederas, and T. T. Nakashima, *J. Am. Chem. Soc.*, 1981, **103**, 5953.
4 C. R. Hutchinson, M. M. Sherman, A. G. McInnes, J. A. Walter, and J. C. Vederas, *J. Am. Chem. Soc.*, 1981, **103**, 5956.
5 J. A. Robinson and D. L. Turner, *J. Chem. Soc., Chem. Commun.*, 1982, 148, corrected on p. 568.
6 A. A. Ajaz and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1983, 679.
7 G. R. Sood, J. A. Robinson, and A. A. Ajaz, *J. Chem. Soc., Chem. Commun.*, 1984, 1421.
8 M. Bulsing, E. D. Laue, F. J. Leeper, J. Staunton, D. H. Davies, G. A. F. Ritchie, A. Davies, A. B. Davies, and R. P. Mabelis, *J. Chem. Soc., Chem. Commun.*, 1984, 1301.
9 H. Tsou, S. Rajan, R. Fiala, P. C. Mowery, M. W. Bullock, D. B. Borders, J. S. James, J. H. Martin, and G. O. Morton, *J. Antibiot.*, 1984, **37**, 1651.

10 D. M. Doddrell, E. D. Laue, F. J. Leeper, J. Staunton, A. Davies, A. B. Davies, and G. A. F. Ritchie, *J. Chem. Soc., Chem. Commun.*, 1984, 1302.
11 A. K. Demetriadou, E. D. Laue, J. Staunton, G. A. F. Ritchie, A. Davies, and A. B. Davies, *J. Chem. Soc., Chem. Commun.*, 1985, 408.
12 D. E. Cane, W. D. Celmer, and J. W. Westley, *J. Am. Chem. Soc.*, 1983, **105**, 3594.
13 See for example: H. Seto, K. Mizoue, H. Nakayama, K. Furihata, N. Otake, and H. Yonehara, *J. Antibiot.*, 1979, **32**, 239; H. Seto, K. Mizoue, and N. Otake, *ibid.*, 1980, **33**, 979.
14 For review see: R. Benn and H. Gunther, *Angew. Chem., Int. Ed. Engl.*, 1983, **22**, 350; D. L. Turner, *Prog. Nucl. Mag. Reson. Spectrosc.*, 1985.
15 A. A. Maudsley, A. Kumar, and R. R. Ernst, *J. Magn. Reson.*, 1977, **28**, 463.
16 C. Le Cocq and J. Y. Lallemand, *J. Chem. Soc., Chem. Commun.*, 1981, 150.
17 D. L. Turner and R. Freeman, *J. Magn. Reson.*, 1978, **29**, 587.
18 A. Neszmelyi and G. Lukacs, *J. Chem. Soc., Chem. Commun.*, 1981, 999.
19 D. L. Turner, *Mol. Phys.*, 1981, **44**, 1051.
20 D. L. Turner, *J. Magn. Reson.*, 1983, **53**, 259.
21 A. Bax, R. Freeman, and T. A. Frenkiel, *J. Am. Chem. Soc.*, 1981, **103**, 2102.
22 A. Bax, R. Freeman, T. A. Frenkiel, and M. H. Levitt, *J. Magn. Reson.*, 1981, **43**, 478.
23 T. H. Mareci and R. Freeman, *J. Magn. Reson.*, 1982, **48**, 158.
24 G. Bodenhausen and C. M. Dobson, *J. Magn. Reson.*, 1981, **44**, 212.
25 M. J. O. Anteunis, *Bull. Soc. Chim. Belg.*, 1977, **86**, 367.
26 A. A. Maudsley, L. Muller, and R. R. Ernst, *J. Magn. Reson.*, 1977, **28**, 463.
27 R. Freeman and G. A. Morris, *J. Chem. Soc., Chem. Commun.*, 1978, 684.
28 W. P. Aue, J. Karhan, and R. R. Ernst, *J. Chem. Phys.*, 1976, **64**, 4226.
29 W. P. Aue, E. Bartholdi, and R. R. Ernst, *J. Chem. Phys.*, 1976, **64**, 2229.
30 J. W. Chamberlin and A. Agtarap, *Org. Mass Spectrom.*, 1970, **3**, 271.
31 J. M. Risley and R. L. Van Etten, *J. Am. Chem. Soc.*, 1980, **102**, 6699.
32 J. C. Vederas, *Can. J. Chem.*, 1982, **60**, 1637.
33 A. Agtarap, J. W. Chamberlin, M. Pinkerton, and L. Steinrauf, *J. Am. Chem. Soc.*, 1967, **89**, 5737.
34 M. Pinkerton and L. K. Steinrauf, *J. Mol. Biol.*, 1970, **49**, 533.
35 M. E. Haney and M. M. Hoehn, *Antimicrob. Agents Chemother.*, 1968, (1967), 349.

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