3.17 Two-dimensional NMR

We have seen in Sec. 3.12 that a full set of proton decoupling experiments would give much information about the connectivity of the various kinds of CH_3, CH_2, and CH groups in a molecule and in Sec. 3.13 that a full set of NOE experiments would define many spatial relationships within a molecule. These are two key types of experiment in the determination of structure, and it would therefore be of enormous advantage to determine (a) all the coupling information in a single experiment and (b) all the spatial information (NOEs) in a single experiment. Both of these extremely powerful single experiments can in fact be carried out by means of *two-dimensional NMR spectroscopy*.

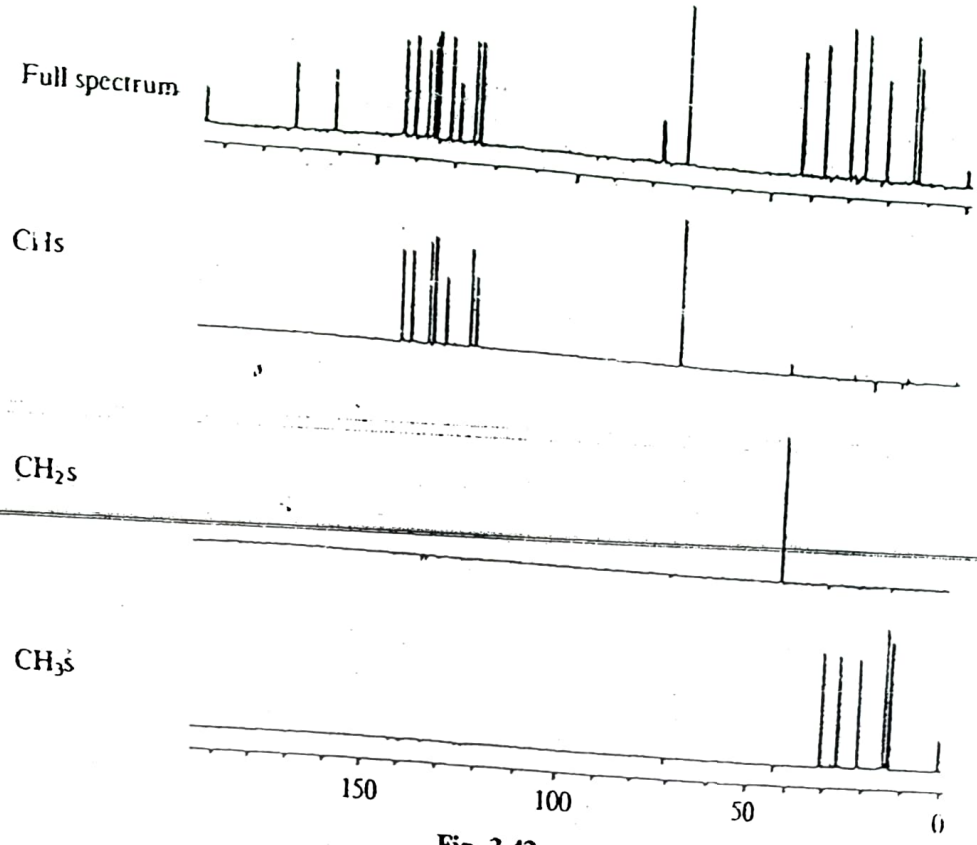

Fig. 3.42

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In Sec. 3.15 we introduced the concept of plotting the coupling constant on an axis orthogonal to the chemical shift, thus generating a two-dimensional spectrum (ignoring the dimension of intensity). In an analogous way, two-dimensional spectra can be generated where the chemical shift is plotted on two orthogonal axes. When these spectra have been obtained, using multi-pulse sequences, they can be displayed as stacked plots (cf. Fig. 3.40a), but it has proved most useful to plot the view that would be obtained if the eye looked down on such a stacked plot, along the peak intensity axis, so that the spectrum appears as a 'board' in which the sides of the board are constituted from the two orthogonal chemical shift axes. The intensities of the peaks are then indicated by contours, exactly as are the heights of mountains on maps.

In all of the two-dimensional spectra that we shall describe, multi-pulse sequences are applied, such that in the first part of the experiment the magnetization is changed from its equilibrium state in a specific manner ('prepared'). It is then allowed to evolve as a function of time ('evolution'), and the spins allowed to affect each other's behaviour ('mixing', e.g. according to whether they are spin–spin coupled or relax each other through space). In the last part of the multi-pulse experiment, the resulting magnetization is detected by collecting FIDs ('acquired'). The experiments can be arranged so that in one kind of pulse sequence (COSY) magnetization is transferred between nuclei that are spin–spin coupled, or in another (NOESY) magnetization is transferred between nuclei that undergo mutual dipolar relaxation.

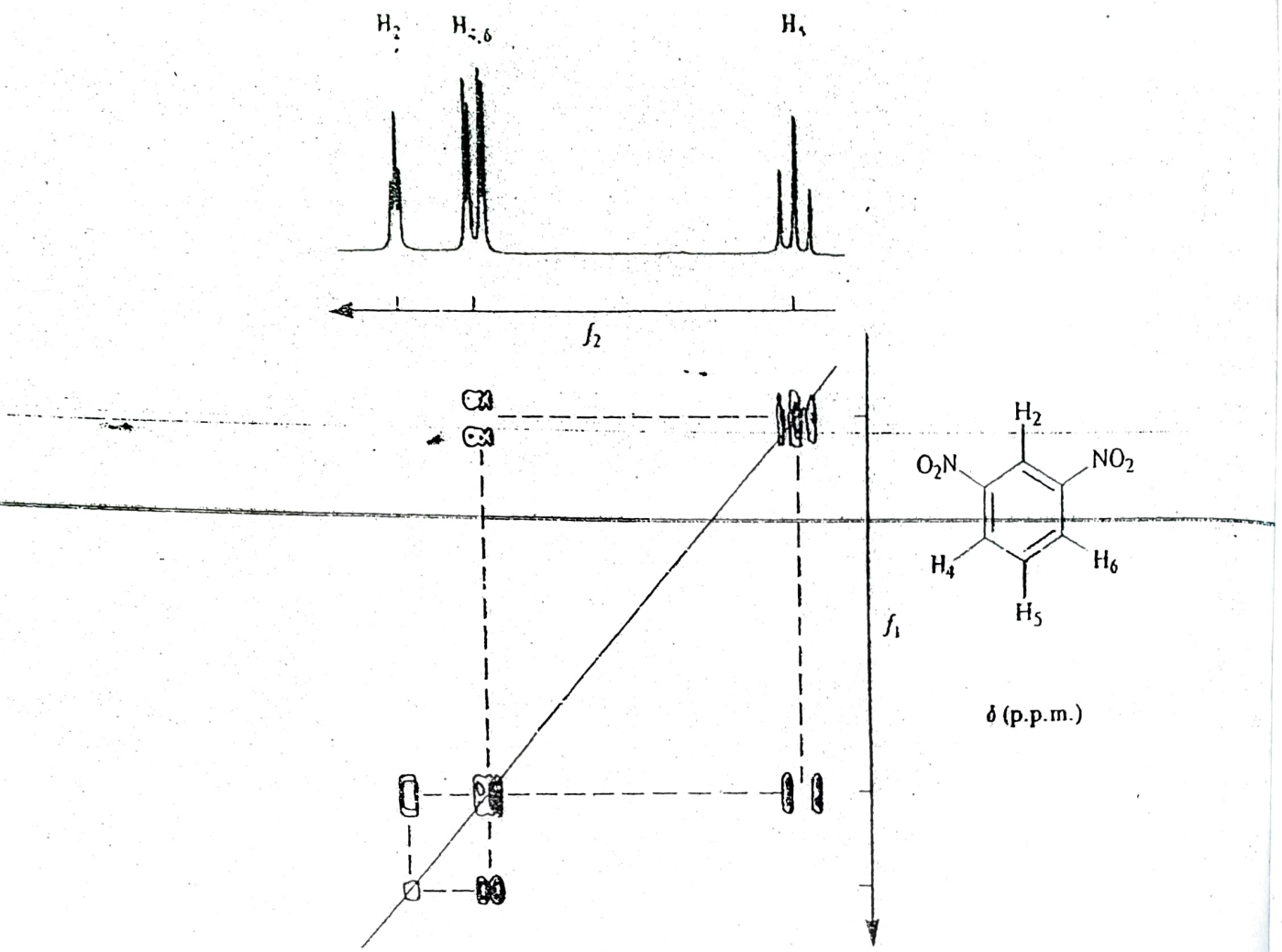

Fig. 3.43

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3.18 COSY spectra

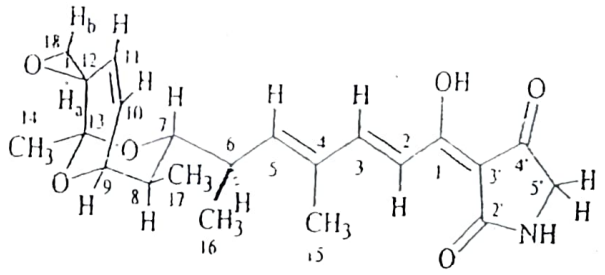
A two-dimensional experiment which indicates all the spin–spin coupled protons in one spectrum is called a COSY spectrum (COrrelated SpectroscopY). The COSY spectrum of *m*-dinitrobenzene is reproduced in Fig. 3.43.

In the COSY spectrum, two essentially identical chemical shift axes are plotted orthogonally (although the resolution on each chemical shift axis is normally different). The one-dimensional spectrum which we have been accustomed to seeing appears on the diagonal of this plot, but for convenience of interpretation it is extremely helpful to plot independently a one-dimensional spectrum on one of the orthogonal chemical shift axes (frequently labelled f_1 and f_2) (the f_2 axis is usually better resolved, and the one-dimensional spectrum is best displayed on this axis; see Fig. 3.43). All peaks that are mutually spin–spin coupled are shown by *cross-peaks* which are symmetrically placed about the diagonal. Thus, by virtue of H_2 being coupled to H_4 (H_6) (Fig. 3.43), there are cross-peaks that occur at the chemical shift of H_2 in one dimension and at the chemical shift of H_4 (H_6) in the other dimension (see the dashed lines in Fig. 3.43). Similarly, the signal from H_4 (H_6) is further connected (dashed lines) by cross-peaks to the signal from H_5.

The cross-peaks themselves contain the coupling constants, but not the multiplicity. Whereas the signal of H_5 on the diagonal is a triplet, viewed both fr the abscissa looking up or, less clearly, from the ordinate looking to the right, t cross-peaks do not have the centre-line of the triplet in either direction. The same lc of the centre-line occurs with quintets, but doublets and quartets remain as double and quartets in the cross-peaks. By introducing extra delays in the pulse sequenc COSY spectra can be obtained to emphasize long-range couplings. In such *long-rang COSY* or *delayed COSY* spectra, which may be used to uncover couplings (J) of th order of 1 Hz and thereby give connectivities over 4 or 5 bonds, the extra delay should be of the order of $\frac{1}{2}J$. The planes used to define the contours have to b chosen carefully if all the cross-peaks are to be displayed.

In the case of *m*-nitrobenzene, the couplings are all evident and analysable in the conventional spectrum, but in more complicated cases it is very valuable to be able to see where all the couplings are without carrying out separate decoupling experiments for all the possible connections. Furthermore, when two (or more) signals have very similar chemical shifts, irradiation of one of the signals inevitably hits the other at the same time, and we are then unable to tell from the decoupling observed what is coupled to what. COSY spectra are not as limited in this way: as long as the signals are resolved, the cross-peaks can be associated reliably with a specific pair of coupled protons.

To illustrate the power of the technique, the much more complex *COSY* spectrum of the secondary metabolite tirandalydigin (55) in CH_3OD as solvent is reproduced and interpreted in Fig. 3.44. The spectrum is actually a composite. The cross-peaks from a normal COSY, to uncover vicinal couplings, and geminal couplings between any non-equivalent protons of methylene groups are reproduced and connected by full lines below the diagonal. The cross-peaks from a delayed COSY, designed to uncover long-range couplings, are reproduced and connected by dotted lines above the diagonal. The spectrum should be studied as an exercise in interpretation in the light of the provided structure (55).



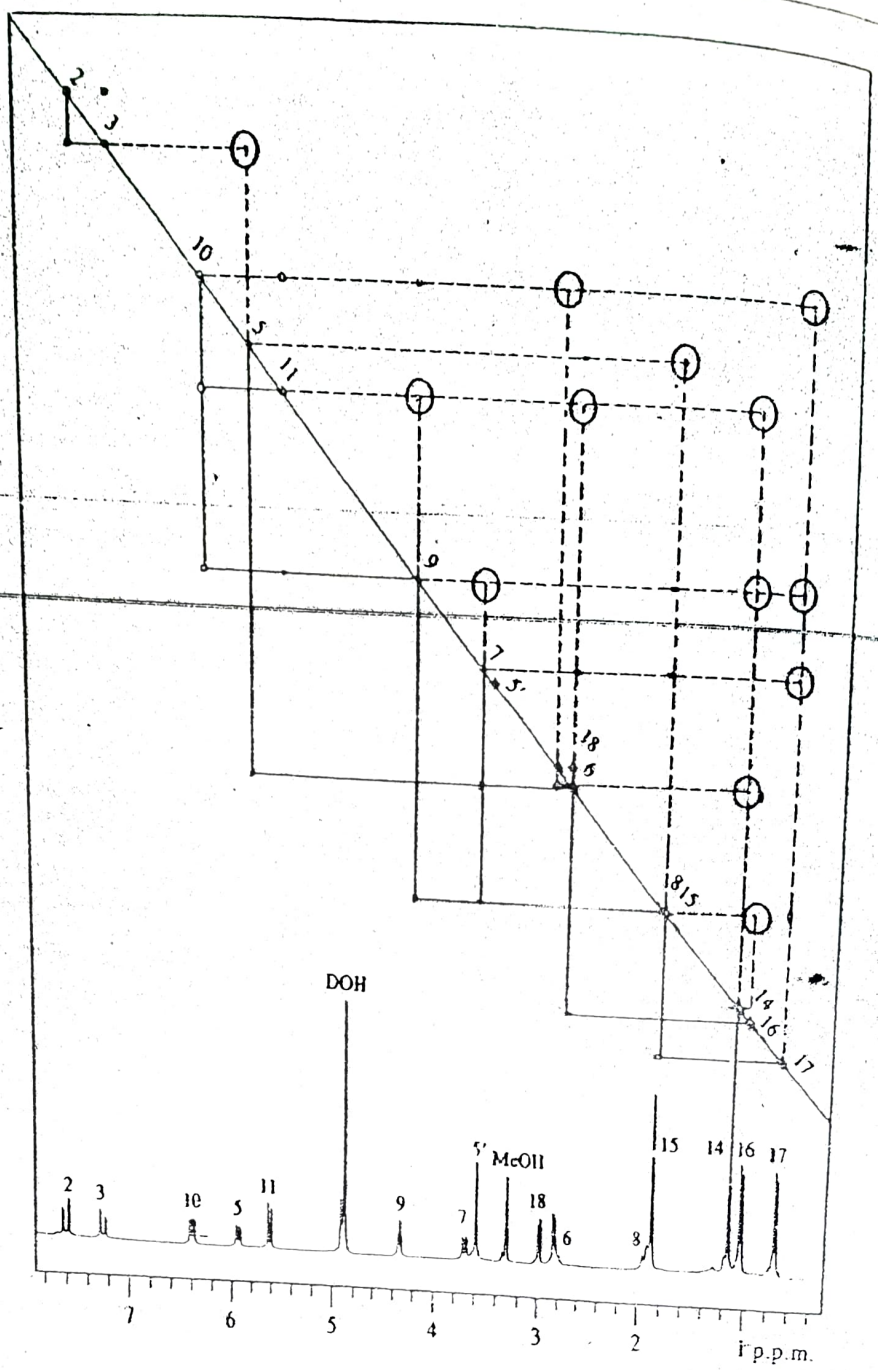


Fig. 3.44

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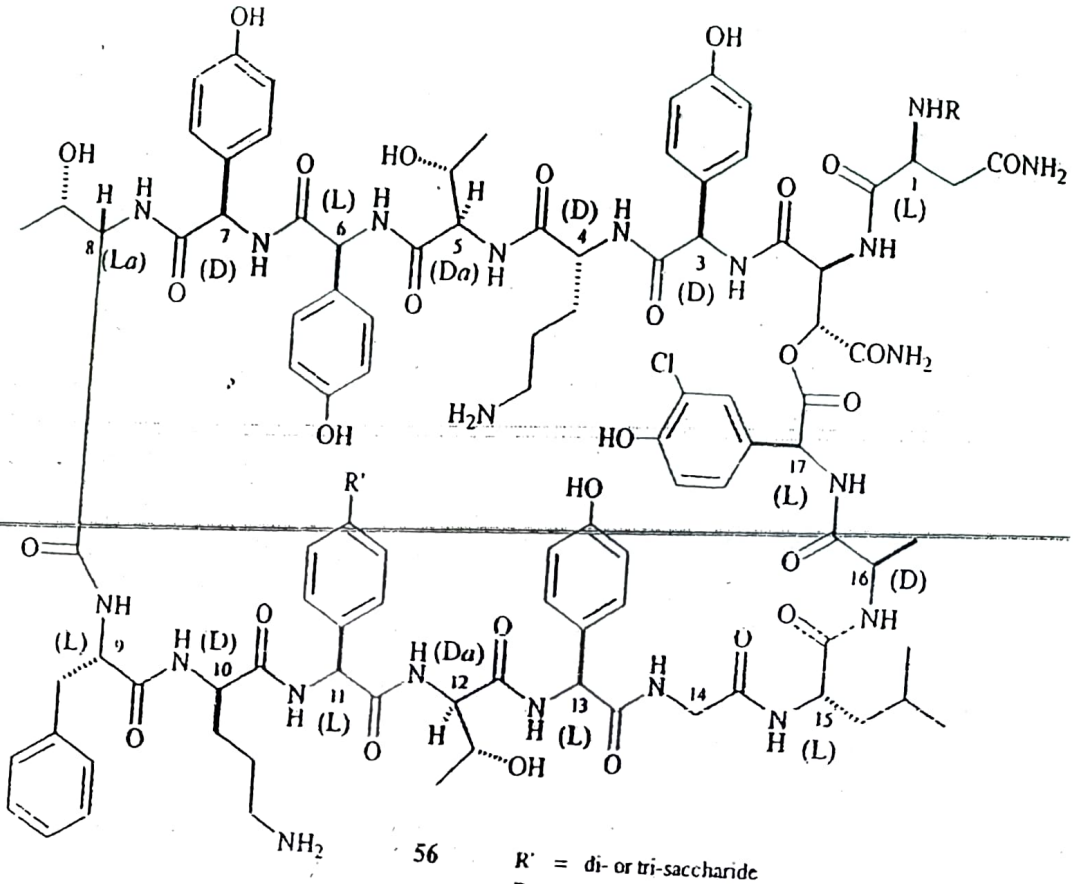
3.19 TOtal Correlation SpectroscopY (TOCSY)

In a TOCSY spectrum, cross-peaks are found for all protons that are part of the same spin system. Thus, whereas in a COSY spectrum, for an AMX system of three coupled spins, cross-peaks are found only for A to M and for M to X, in the TOCSY spectrum a cross-peak between A and X also occurs (even if a direct coupling of A to X is not discernible). The advantage of this technique therefore lies in the interpretation of spectra where there are overlapping resonances. For example, consider the hypothetical case where the M resonance of an AMX system overlaps with the M′ resonance of an A′M′X′ system (Fig. 3.45). From a COSY spectrum, the cross-peaks displayed above the diagonal would be seen, and it would be impossible to know if A was part of the same spin system as X or X′. In the



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R' = di- or tri-saccharide
R = fatty acid side-chain

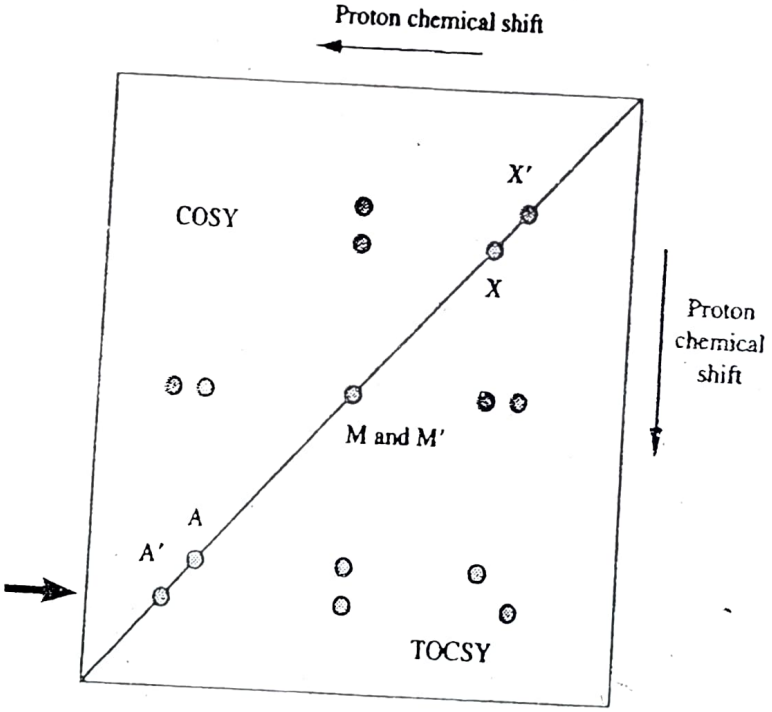


Fig. 3.45

corresponding TOCSY spectrum, the peaks shown below the diagonal would be seen, and it is directly indicated that A is part of the same spin system as X and A′ is part of the same spin system as X′.

This kind of information from a TOCSY spectrum can be very important in structural studies of peptides and proteins by NMR. In the proton spectra of these

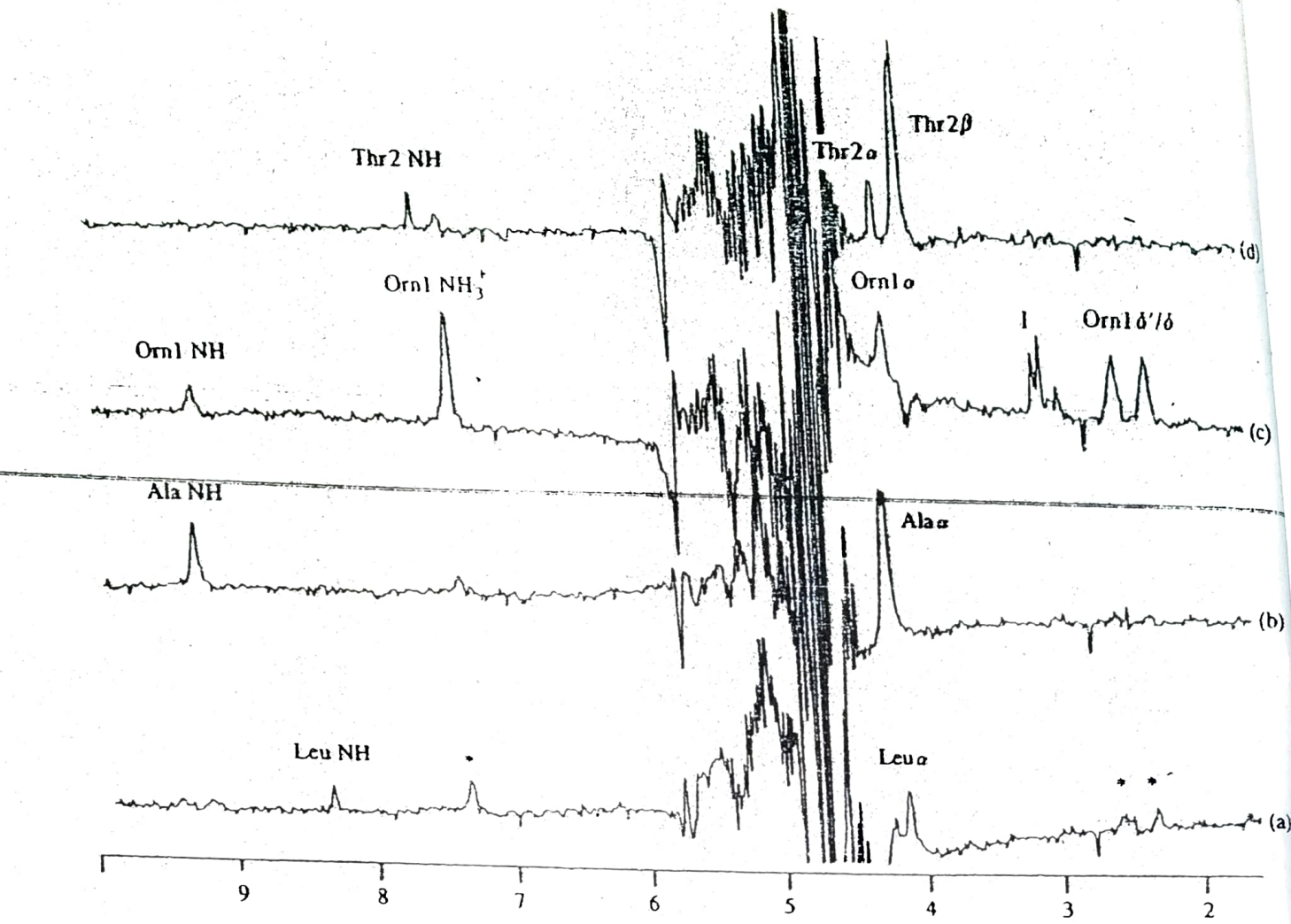
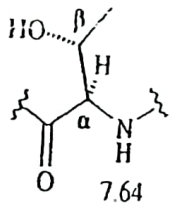


Fig. 3.46

substances, it is crucial to be able to assign the amide NH protons to a particular amino acid, since further studies of the behaviour of these resonances can then show how they are involved in hydrogen bond formation, and hence the hydrogen bonds formed by each residue can be characterized. A useful way to do this is to take a 'slice' through a TOCSY spectrum at the chemical shift of an identifiable resonance. For example, in Fig. 3.45 the chosen chemical shift might be that of A′, and the slice would then contain the cross-peak signals due to M′ and X′ (see direction of heavy arrow in Fig. 3.45). In Fig. 3.46, such sections from the TOCSY spectrum to the secondary metabolite 56 are reproduced; the sections correspond to the frequency of (a) leucine β-protons (see 60), (b) alanine methyl group protons (see 59), (c) ornithine γ-protons (see 58), and (d) threonine methyl group protons (see 57). (Where the signals due to ornithine side-chain protons are incompletely separated out from the section at the chemical shift of the leucine β-protons, these peaks are indicated by asterisks.) In this way, the NHs of a threonine (57), an ornithine (58), an alanine (59) and a leucine (60) residue are identified. The chemical shifts of these identified protons are given in the structures.

The COSY spectrum would not have given this information because of the very large number of overlapping α-CH resonances near the 4–5 p.p.m. region, and the further obscuring of this region by the resonance of H_2O (which is the necessary solvent if the secondary structure of 56 is to be determined in aqueous solution, the



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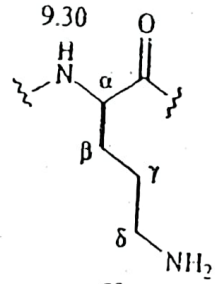

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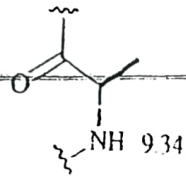

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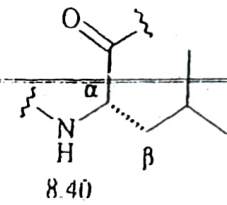

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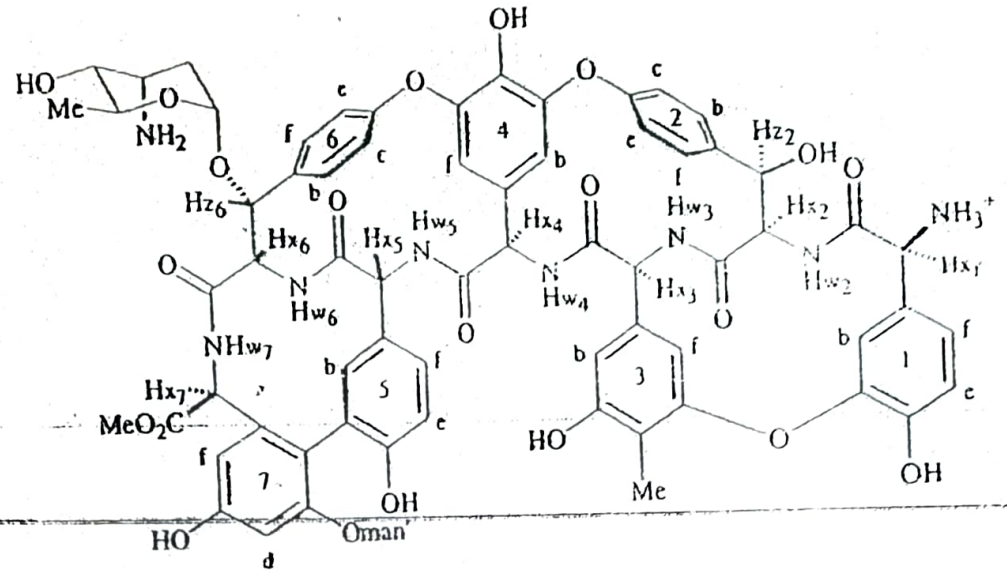
use of D_2O also being excluded when the NHs need to be seen). Although the unwanted resonance of the H_2O has been largely suppressed by presaturation of its resonance before acquisition of each TOCSY spectrum, some residual H_2O signal nevertheless remains. The suppression of the H_2O signal causes distortion of the proton intensities observed in the 4–5 p.p.m. region. Since **56** contains three threonine and two ornithine residues, further experiments are necessary for a full assignment.

3.20 NOESY spectra

A two-dimensional spectrum which records all the proton–proton NOEs occurring in a molecule in a single experiment is called a NOESY spectrum. Superficially, it appears like a proton–proton COSY spectrum, insofar as each orthogonal axis is of the proton chemical shifts and the normal spectrum appears on the diagonal. However, it differs crucially in that the cross-peaks now indicate those protons that are close in space, i.e. they are evidence of through-space, rather than through-bond, interactions. Thus, a NOESY spectrum provides crucial information about the geometry of molecules.

The positive NOE enhancements from small molecules (*ca.* 100–400 Daltons) are rather weak, and these are often most conveniently examined by NOE difference spectroscopy (Sec. 3.13). Larger molecules (1000 Daltons or more, in non-viscous solvents, or even greater than 500 Daltons in a more viscous solvent such as d_6-DMSO) normally give much more intense NOE cross-peaks and can give a wealth of information with regard to their geometry. The NOE is due to mutual dipolar relaxation of protons (Secs 3.10 and 3.13) and, since this effect falls off with $1/r^{-6}$ (where r is the distance between the protons giving rise to the cross-peak), the intensity of NOE cross-peaks falls off rapidly with increasing internuclear separation of the



61

protons (but is also affected by other variables). A useful guide is: large cross-peaks, $r = 2.0$–2.5 Å; medium cross-peaks, $r = 2.0$–3.0 Å; small cross-peaks, $r = 2.0$–5.0 Å.

Figure 3.47 is a portion of the NOESY spectrum of a derivative of the antibiotic ristocetin A (**61**). The spectrum was taken in CD_3CN/D_2O as solvent, so that the NH and OH resonances are removed from the spectrum. Since the contour plot along the 45° diagonal (which represents the normal one-dimensional spectrum) is not the clearest way to follow the proton resonances, the one-dimensional spectrum is frequently reproduced (as in Fig. 3.47) along one axis of the two-dimensional contour plot. In Fig. 3.47, nine cross-peaks appear which are symmetrically placed with respect to the diagonal. The interpretation of any particular spectrum may be complicated by overlapping resonances (such as the six overlapping resonances at ~7.2 p.p.m. and the four at ~5.4 p.p.m.), but these overlaps can often be removed by running the spectrum again at different temperatures, pH, or in a modified solvent.

The data in Fig. 3.47, together with data obtained from spectra measured at other temperatures, show that the following pairs of protons are close to each other in space: 2f ↔ 2e, 6b ↔ 6c, 6b ↔ z_6, 1f ↔ 1e, 1f ↔ x_1, 2c ↔ 4b, 2b ↔ z_2, 1b ↔ 3f, and 3b ↔ x_3. The proximities of the pairs 2f ↔ 2e, 6b ↔ 6c, and 1f ↔ 1e were, of course, already available from spin decoupling and COSY experiments. However, the NOESY experiment, by communicating information through space, allows connections to be made between the various spin systems already defined by the spin decoupling and COSY experiments. Thus the x_6,z_6 spin-coupled pair must be close in space to the aromatic ring 6, because of the existence of the NOE 6b ↔ z_6; similarly, the x_2,z_2 spin-coupled pair must be close to the aromatic ring 2, because of the NOE 2b ↔ z_2. Additionally, this partial spectrum allows some of the α-CH protons (x_1, x_2, ... , x_7) of the peptide backbone to be correlated with the appropriate aromatic ring (for example, 1f ↔ x_1 and 3b ↔ x_3). In a similar way, the proximity of aromatic rings in the structure is indicated (2c ↔ 4b, 1b ↔ 3f). Indeed, with only limited chemical information and a molecular weight from FAB mass spectrometry (Chapter 4), it is possible from complete COSY and NOESY spectra to solve structures as complex as **61** with complete stereochemical detail. The NOESY experiment is one of enormous power. Although it does not give quite the wealth of data and precision available

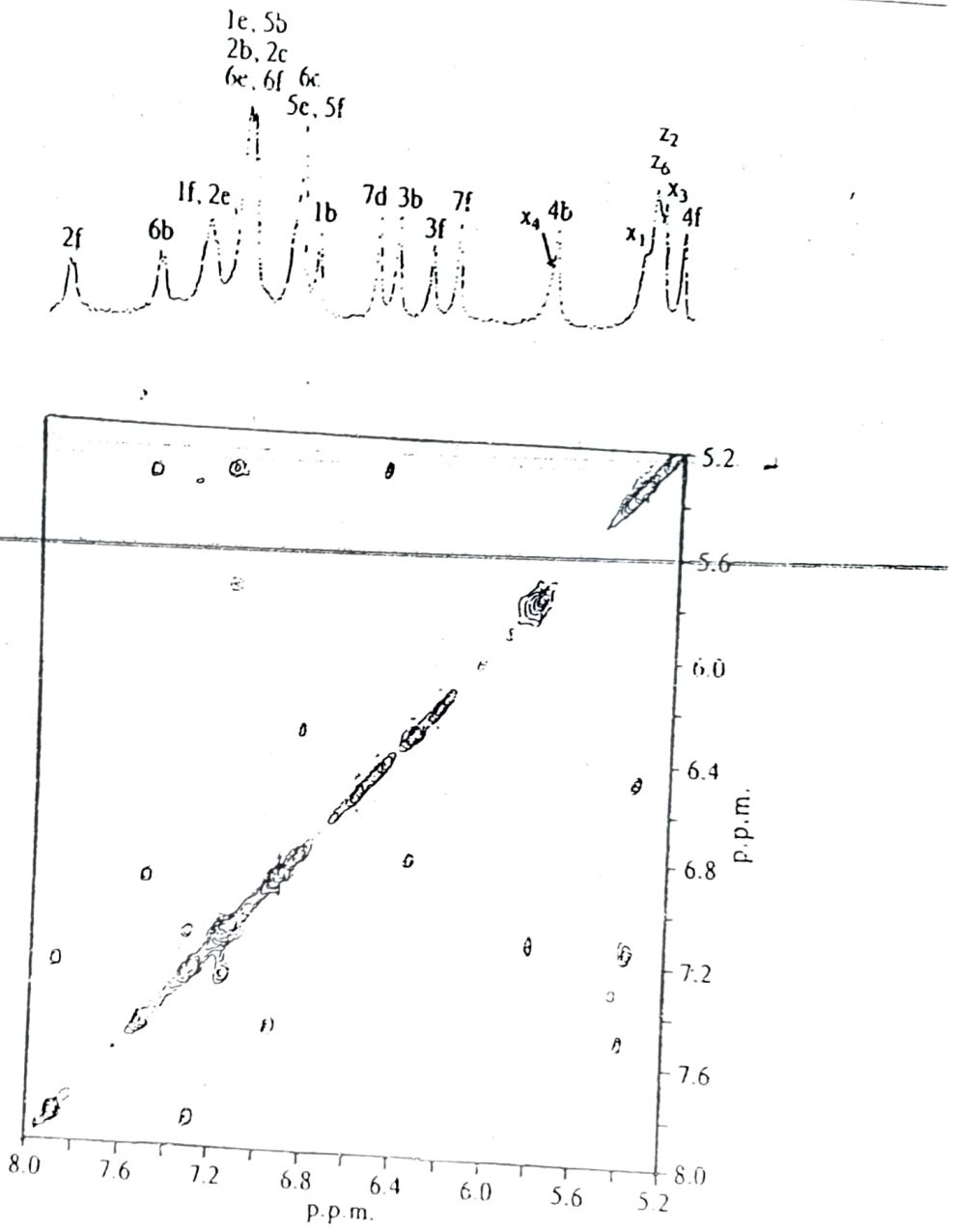
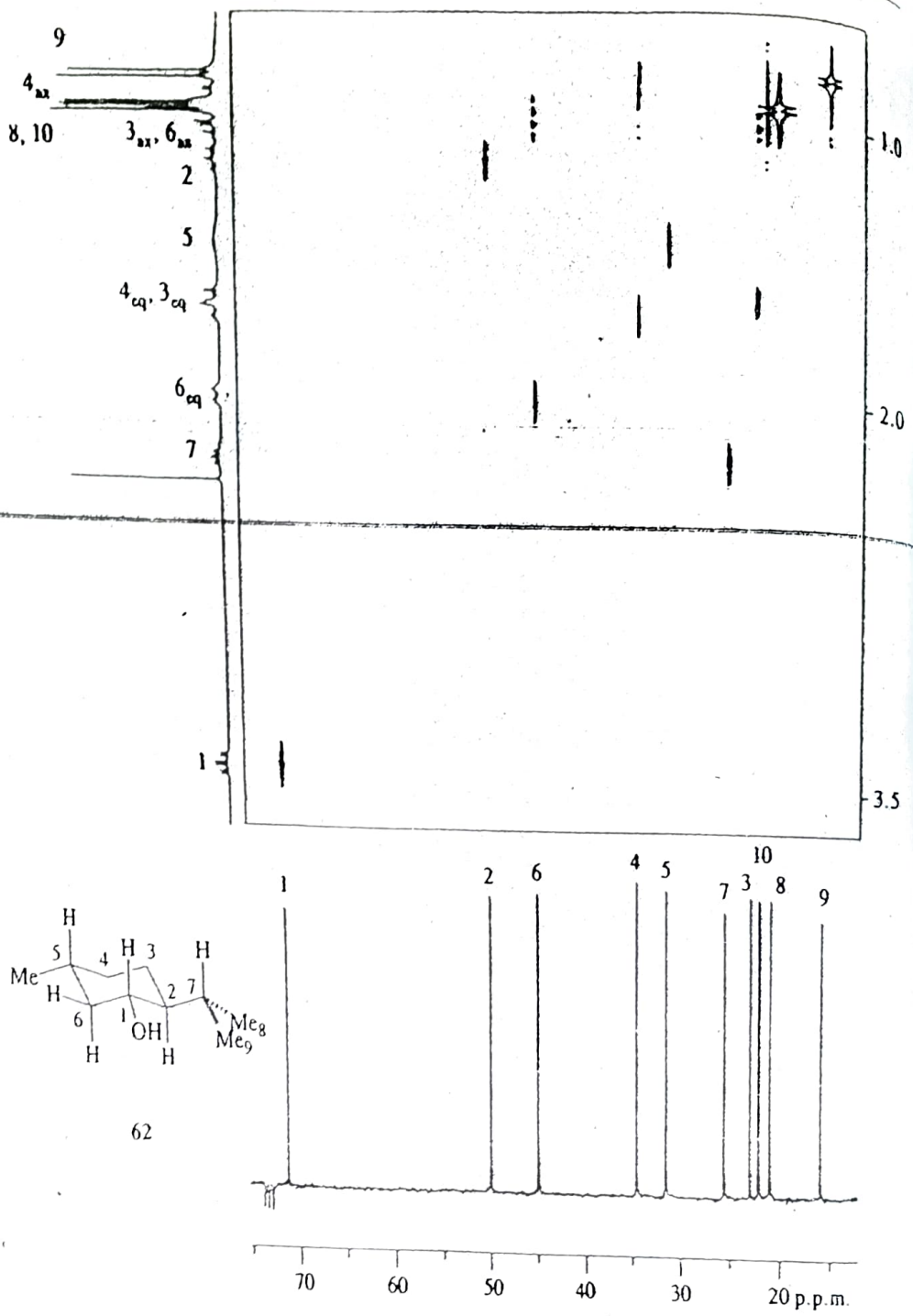


Fig. 3.47

from X-ray crystallography, it has the compensating advantages that it applies to molecules in solution and the compound does not have to be crystalline.

3.21 ^{1}H–^{13}C COSY spectra [also known as Heteronuclear Multiple Quantum Coherence (HMQC) spectra]

If the proton spectrum can be correlated with the carbon spectrum, then the complete assignment of both, and hence structure elucidation, may be facilitated. Additionally, the carbon dimension can be used to resolve the (often severely overlapping) proton dimension. The ^{1}H–^{13}C COSY spectrum allows this correlation to be made by using a pulse sequence in which, following the 'preparation' of the nuclear spins, a delay time in the pulse sequence is set to $\frac{1}{2}J$, where J is the value of the one-bond ^{13}C–^{1}H coupling constant (usually in the range 100–200 Hz; see Table 3.16). In this way, a



62

Fig. 3.48

correlation is achieved between ^{13}C and the proton to which it is directly attached (i.e. a one-bond correlation).

The ^{1}H–^{13}C COSY spectrum of menthol **(62)** is reproduced in Fig. 3.48. The calculated chemical shifts of the carbon atoms of menthol (assuming the preferred conformation shown in the structure), estimated through the use of Eq. 3.15 and Tables 3.8–3.10 (with observed values to the nearest p.p.m. indicated in parentheses) are: C-1, 76 (72); C-2, 50 (50); C-3, 27 (23); C-4, 38 (35); C-5, 32 (32); C-6, 43 (45); C-7, 32 (26); C-8, 22 (21); C-9, 22 (16); C-10, 25 (22). [In calculating the shift for C-2, which has five substituents other than H on the atoms directly bonded to the observed ^{13}C, it is assumed that the 'steric' correction is −20.5 (cf. the tertiary row in Table 3.9).] The estimations are within 4 p.p.m. of the observed values, with the exception of those for C-7 and C-9. This is because the data of Table 3.10 express

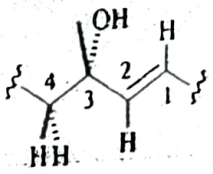
inadequately the effects of conformational preferences of functional groups at the γ-position, and ignore the effects of conformational preferences at the δ-position. Since the equatorial OH functionality of menthol is held proximate to both C-7 and C-9, an important effect on both is quite feasible. The above chemical shift estimations suggest that in each case this corresponds to a shielding effect. The important point in the present context is that a proton COSY spectrum clearly indicates the assignment of H-7 (coupled to two secondary methyl groups), and the ^{1}H–^{13}C COSY spectrum therefore unambiguously leads to the assignment of C-7. Indeed, using coupling constants and NOEs, it is so easy to assign fully the proton spectrum that the hetero-correlation was used to assign the carbon spectrum. Other noteworthy features of Fig. 3.48 are:

1. Methylene carbons (C-3, C-4, and C-6) correlate with pairs of protons where the methylene protons are chemically non-equivalent, whereas methine and methyl carbons correlate with only a single proton (these protons have a single chemical shift).
2. In the cases of methylene groups, the equatorial protons are to low field of their axial counterparts, as is normally the case. Each of the C-3, C-4, and C-6 equatorial protons is coupled to its non-equivalent geminal (axial) partner by *ca.* 12 Hz, but only by a vicinal coupling in the range 2–6 Hz to the axial or equatorial protons on neighbouring carbons. Therefore, each equatorial proton appears as a 'broad doublet', where several smaller couplings (2–6 Hz) are not clearly resolved in the presentation and appear as broadening, but the large geminal couplings (12 Hz) are resolved. Since the high-field half of the C-4 equatorial proton signal overlaps with the low-field half of the C-3 equatorial proton signal (as clearly shown by the hetero-correlation), the combined signal appears rather like a 1 : 2 : 1 triplet (Fig. 3.48).

3.22 Long-range ^{1}H–^{13}C COSY spectra [also known as Heteronuclear Multiple Bond Connectivity (HMBC)]

The long-range ^{1}H–^{13}C COSY pulse sequence also gives a two-dimensional spectrum with ^{13}C chemical shifts on one axis and ^{1}H chemical shifts on the other. However, in this sequence, after the 'preparation' of the nuclei, a time delay in the pulse sequence is set to correspond to $\frac{1}{2}J$ where J is in the region of 10 Hz, i.e. a delay of about 50 ms. Since many ^{1}H–C–^{13}C (two-bond) and ^{1}H–C–C–^{13}C (three-bond) coupling constants are rather similar in value and lie in the range 2–20 Hz, then ^{13}C chemical shifts are now correlated with the chemical shifts of those protons separated from them by two and three bonds. It is unfortunate that the values of the two- and three-bond couplings overlap, since the two sets of information cannot therefore be separated in this spectrum. The technique is nevertheless one of great importance since it is now possible to connect together units in a way that has not hitherto been possible. For example, using proton–proton coupling information, the presence in a structure of a methylene group and a *trans* double bond might be inferred. However, if, as in the case of the unit 63, they are separated by a quaternary carbon atom, then H–H coupling information will not normally extend from H-2 to H-4 and the location of carbons that do not carry protons (e.g. C-3) may be unknown. The long-

range ^{1}H–^{13}C COSY spectrum overcomes this problem by the possibility of correlating H-2 to C-3 and also H-4 to C-3. In a similar way, long-range ^{1}H–^{13}C COSY allows us to 'talk' across carbonyl groups (e.g. **64**), which would terminate the chain of ^{1}H–^{1}H coupling information which exists to both right and left.



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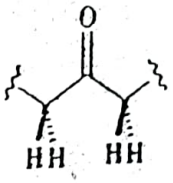

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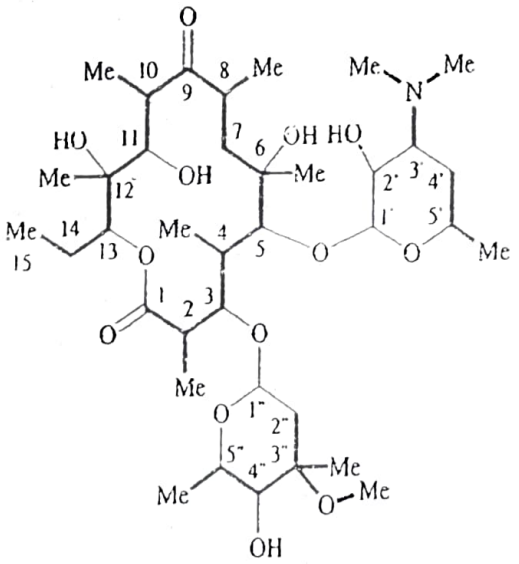
An illustration of the power of the technique is given by part of the long-range ^{1}H–^{13}C COSY spectrum (Fig. 3.49) of erythromycin A (**65**). This spectrum was recorded by detecting the magnetization on protons (which is a more sensitive way of carrying out the experiment than by recording the magnetization on carbons—as was done in Fig. 3.48). Rather unconventionally in Fig. 3.49, the f_2 proton dimension is plotted vertically and the f_1 carbon dimension horizontally.



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In order to get the full amount of information which is available from such a spectrum, a detailed analysis of the proton and ^{13}C spectra, using a number of the methods that we have already covered, is necessary. As an aid to follow the arguments, the full assignment of the 36 non-equivalent carbons of erythromycin A is given in Table 3.4.

The proton spectrum in the region 0.8–2.0 p.p.m. shows the presence of 11 non-equivalent methyl groups: four are singlets, six are doublets, and one is a triplet. In a COSY spectrum (data not shown), it is very easy to correlate the doublet and

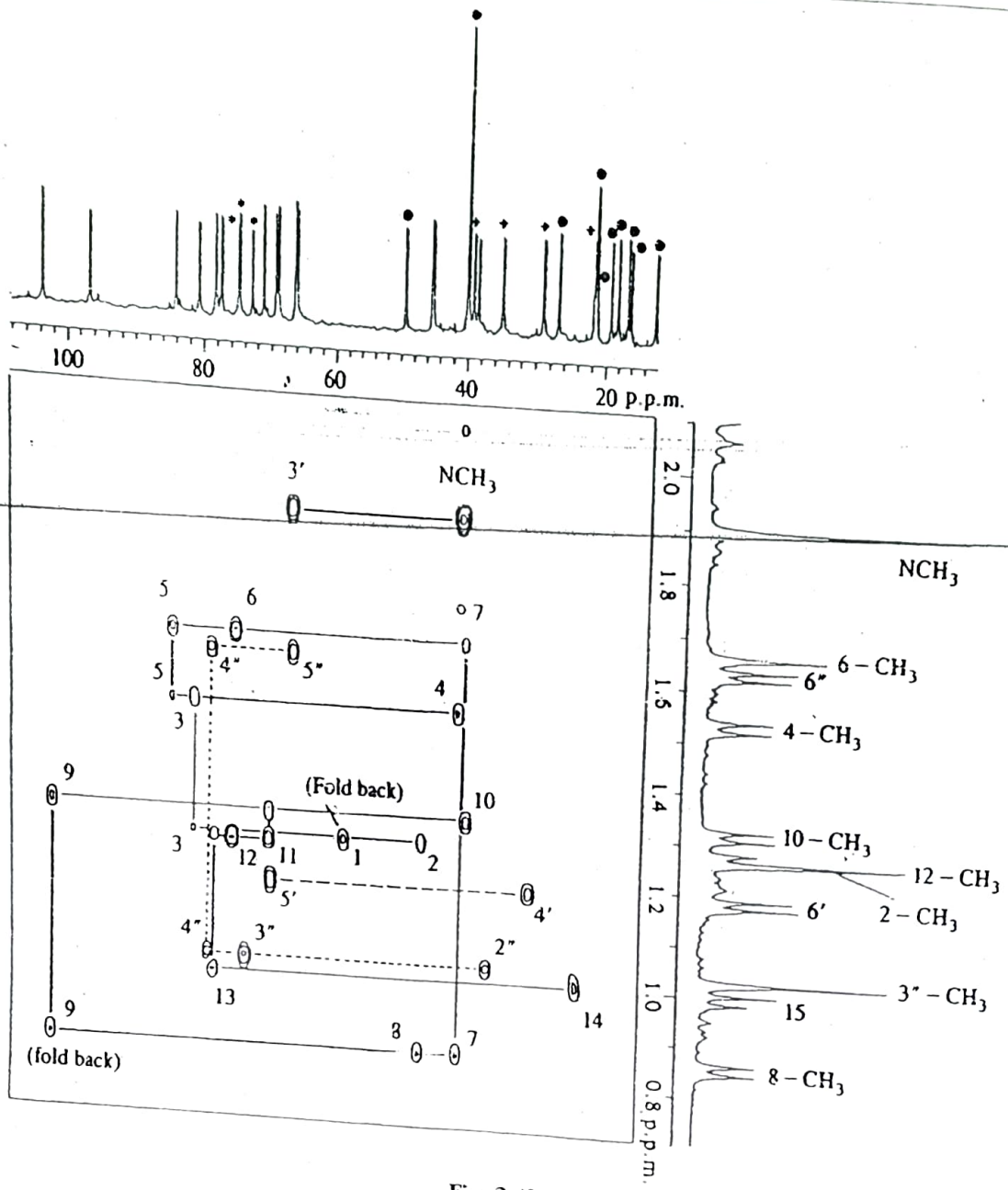

Fig. 3.49

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triplet signals with the corresponding signals due to the protons from which their multiplicity arises (CH_3—CH and CH_3—CH_2). A ^{13}C–^1H COSY (HMQC) spectrum is then used (data not shown) to correlate the thus identified protons with the carbons to which they are directly attached (CH_3—CH and CH_3—CH_2). In this way, the methyl protons are already correlated with the carbon atoms which are separated from them by two bonds (CH_3—CH and CH_3—CH_2). Thus, one of the correlations (two-bond) which would be shown by the long-range ^1H–^{13}C COSY (HMBC) correlation is already known, and any further correlations in this spectrum (Fig. 3.49) must therefore correspond to carbon atoms separated from the methyl protons by three bonds. This latter information helps in the building of extended pieces of molecular skeleton, which is of course crucial in the structure elucidation of an unknown.

Table 3.4 ^{13}C chemical shifts of erythromycin

Carbon	δ_C	*Carbon*	δ_C
C-1 (COO)	175.9	C-1′ (O–CH–O)	103.8
C-2 (CH)	45.4	C-2′ (CH–O)	71.3
C-3 (CH–O)	80.8	C-3′ (CH–N)	66.3
C-4 (CH)	40.1	C-4′ (CH_2)	29.1
C-5 (CH–O)	84.2	C-5′ (CH–O)	69.0
C-6 (C–O)	74.9	C-6′ (CH_3)	21.6
C-7 (CH_2)	39.2	N–CH_3	40.1
C-8 (CH)	45.2	C-1″ (O–CH–O)	96.8
C-9 (C=O)	220.6	C-2″ (CH_2)	35.0
C-10 (CH)	38.5	C-3″ (C–O)	72.9
C-11 (CH–O)	69.4	C-4″ (CH–O)	78.3
C-12 (C–O)	75.0	C-5″ (CH–O)	66.0
C-13 (CH–O)	77.5	C-6″ (CH_3)	19.3
C-14 (CH_2)	21.9	3″-CH_3	21.5
C-15 (CH_3)	11.1	O CH_3	49.4
2-CH_3	16.3		
4-CH_3	9.6		
6-CH_3	26.9		
8-CH_3	18.2		
10-CH_3	12.4		
12-CH_3	16.8		

Taken in C_6D_6.

In an initial analysis of the ^{13}C spectrum as complex as that of erythromycin A, a DEPT spectrum would be run (Sec. 3.16) to allow the differentiation of signals due to the carbons of CH_3, CH_2, CH, and quaternary carbons. This establishes the presence of:

1. Twelve methyl carbons, 10 of which are in the 12–104 p.p.m. region plotted in Fig. 3.49 and indicated by ◆ over the peaks (the two not plotted appear at 9.6 and at 11.1 p.p.m.).
2. Four CH_2 carbons, indicated by + over the peaks.
3. Five quaternary carbons (three indicated by * over the peaks, plus the two carbonyl carbons that come in the 160–230 p.p.m. region).
4. Fifteen CH carbons.

[Two carbons at *ca.* 75 p.p.m., two at 66 p.p.m., and two at *ca.* 45 p.p.m. are just resolved, whereas the C-4 methine carbon is coincident with the $(CH_3)N$ carbons at 40.1 p.p.m. Thus all 36 non-equivalent carbons of erythromycin are accounted for.]

It is useful to divide the ^{13}C spectrum into four regions:

1. The carbonyl region, containing one ketone carbon (220.6 p.p.m.) and one lactone carbon (175.9 p.p.m.).
2. The region near 100 p.p.m., which characteristically contains the anomeric carbons (each attached to *two* oxygen atoms and therefore at relatively low field) of the two sugar units.
3. The region from 60 to 90 p.p.m., which contains the quaternary and methine carbons that are attached to oxygen atoms. This region also contains a methine carbon attached to nitrogen which, all other things being equal, would resonate at

ca. 50 p.p.m. to high field of a corresponding CH_2O resonance (Table 3.8). However, this particular carbon (C-5 in 65) has no fewer than four β substituents, each causing a downfield shift of ca. 9 p.p.m. (Table 3.8). (In principle, carbons attached to basic nitrogen atoms can be distinguished from others by running the spectrum in a relatively polar solvent, such as D_2O or d_6-DMSO, at various pHs, and noting the downfield shift caused by protonation of the nitrogen.)

4. The region from 0 to 60 p.p.m. which contains the methyl resonances (whether attached to C, N or O), and the CH_2 and CH carbons which are not attached to electronegative atoms.

Before extracting information from the long-range ^1H–^{13}C COSY spectrum, we note that since the spectral width for the ^{13}C frequencies has been set to accommodate only the signals shown in the 1D spectrum of Fig. 3.49, the carbonyl signals are 'folded back' into the 2D spectrum. Thus, the cross-peak associated with the C_9 ketone carbonyl and the cross-peak associated with the C_1 lactone carbonyl both appear at 118 p.p.m. higher than their true chemical shifts. Given this information and that in the preceding discussion, many atom-to-atom connections can now be deduced from the long-range ^1H–^{13}C COSY spectrum. In making the following connections, it should be kept in mind that a combination of ^1H–^1H COSY and ^{13}C–^1H COSY (HMQC) spectra has already established which one of the two or three cross-peaks occurring to each methyl group in the proton spectrum (Fig. 3.49) is due to a two-bond coupling (see above). This cross-peak is indicated in each case discussed below. By exclusion, we therefore know that the remaining cross-peaks must be due to three-bond couplings. Arguing from:

1. *The protons of the methyl group centred at 0.86 (cross-peak at 45.2 is due to two-bond coupling, and as a starting point this carbon at 45.2 is given the nomenclature 'C-8'):* the protons of this methyl group are three bonds from the carbon at 39.2 (a CH_2) and from the ketone carbonyl carbon. These can therefore be numbered 7 and 9, and the connectivities indicated by the top three bold bonds in the lactone ring of 65 are established.
2. *The protons of the methyl group centred at 1.30 (cross-peak at 38.5 is due to two-bond coupling):* the ketone carbonyl carbon is three bonds from these protons, which are in turn three bonds from the carbon at 69.4. The methyl group is therefore attached to C-10 and the carbon at 69.4 must be C-11 (a CH–O group).
3. *The protons of the singlet methyl group at 1.27 [cross-peak at 75.0 (a quaternary carbon from DEPT) is due to two-bond coupling]:* these protons are also separated by three bonds from C-11, and additionally by three bonds from the carbon at 77.5 p.p.m. The singlet methyl group must therefore be attached to C-12 and the carbon at 77.5 (a CH–O group) must be C-13.
4. *The protons of the triplet methyl group at 1.01 [part of which is obscured under the singlet at 1.03 p.p.m; the cross-peak at 21.9 (a CH_2 carbon from DEPT) is due to two-bond coupling]:* these protons are separated by three bonds from the 77.5 ^{13}C already assigned as C-13. Therefore, an ethyl group, constituting C-14 and C-15 of the skeleton, is attached to C-13.
5. *The protons of the singlet methyl group at 1.67 [the cross-peak at 74.9 (a quaternary carbon from DEPT) is due to two-bond coupling]:* these protons are separated by three bonds from the carbon at 39.2 (already defined as the C-7 CH_2 group under

argument 1) and from the carbon at 84.2. This methyl group must therefore be attached to C-6 and the CH–O carbon at 84.2 must therefore constitute C-5 of the skeleton.

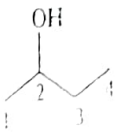
6. *The protons of the doublet methyl group centred at 1.53 [the cross-peak at 40.1 (a methine carbon from DEPT) is due to two-bond coupling]*: these protons are separated by three bonds from the carbon at 84.2 (already assigned as C-5 in argument 5) and from the carbon at 80.8. This methyl group must therefore be attached to C-4 and the CH–O carbon at 80.8 must be C-3 of the skeleton.
7. *The protons of the doublet methyl group centred at 1.27 (half of which is obscured under the singlet at 1.26; the cross-peak at 45.4 is due to two-bond coupling)*: these protons are separated by three bonds from the carbon at 80.8 (already assigned as C-3 in argument 6) and from the carbon at 58.0. The latter is the 'folded-in' lactone carbonyl group. Therefore, this methyl group is attached to C-2 of the skeleton and the lactone carbonyl constitutes C-1.

The above arguments illustrate the enormous power of NMR spectroscopy, allowing in this case the derivation of all the bond connections indicated by bold lines in the macrocyclic portion of erythromycin A (65). In an analogous manner, the data allow the connectivities indicated by bold lines in the two sugars of erythromycin A to be deduced. Bearing in mind that ^{1}C–^{1}H COSY spectra would allow the completion of the 1′-2′-3′-4′ and 1″-2″ connections of the sugars (even with stereochemical data with application of the Karplus equation and NOEs), a complete determination of the whole covalent structure could then be approached.

3.23 Identifying ^{13}C–^{13}C connections (INADEQUATE spectra)

The spectra discussed in Secs 3.21 and 3.22 show us how to make connections from ^{13}C to ^{1}H through one to three bonds, but it would also be useful to have a technique to show direct ^{13}C–^{13}C connections. A pulse sequence called INADEQUATE does this, but there is a problem of sensitivity: since the natural abundance of ^{13}C is only *ca.* 1 per cent, the probability of finding one ^{13}C attached to another is only 1 in 10^4. INADEQUATE therefore finds applications only where (a) extremely concentrated solutions of sample can be prepared or (b) the molecule is enriched in the ^{13}C isotope.

Figure 3.50 shows the INADEQUATE spectrum of 2-butanol (66) in contour form, together with the conventional ^{13}C spectrum. It is best to start with an unambiguously assignable signal, which in this case we can take to be the signal of C-2, downfield because it carries a hydroxyl group. The cross-peaks labelled *a* and *b* identify the connections between C-2 and C-3 and between C-2 and C-1, respectively. The cross-peaks *c* then identify the remaining connection between C-3 and C-4. The dashed line bisects the midpoint between each of the pairs of cross-peaks, and is useful in picking the cross-peaks out from noise. In interpreting these spectra, a connectivity is initially established by making a horizontal correlation between cross-peaks symmetrically



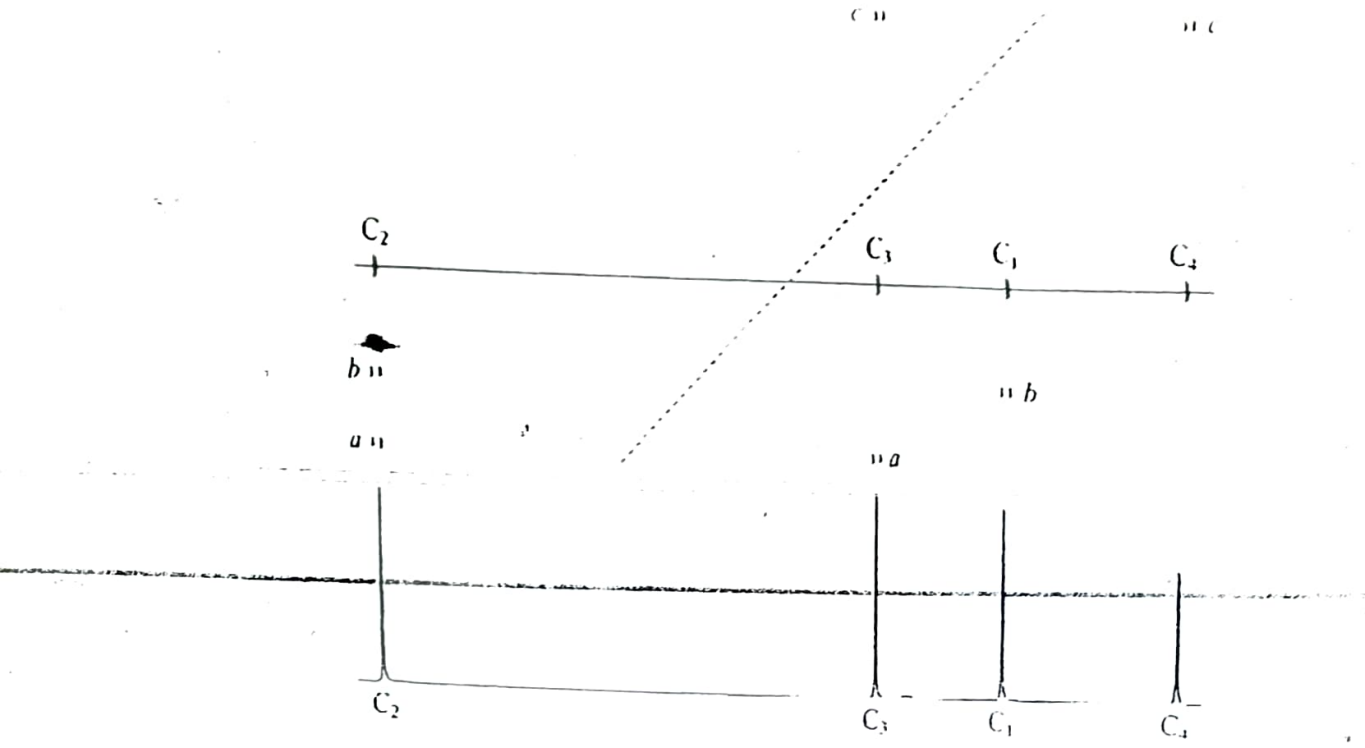
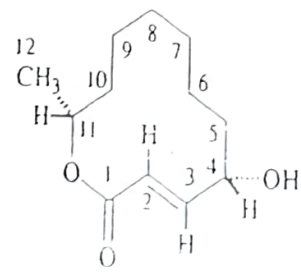


Fig. 3.50

(Reproduced with permission from J. K. M. Sanders and B. K. Hunter, *Modern NMR Spectroscopy*, OUP, Oxford, 1987.)

placed with respect to the dashed line, and further connectivity is established by vertical correlation at either end to other cross-peaks. Hence the termini of carbon chains are readily identified, because they only have vertical correlations at one end. Thus the upfield *b* and *c* cross-peaks have no cross-peaks above or below them, showing that these atoms are bonded only to one carbon atom each.

The power of the INADEQUATE pulse sequence in the study of the biosynthesis of secondary metabolites is illustrated by a study of the biosynthesis of patulolide C (67). The techniques and principles which have been discussed in previous sections readily allow the unambiguous assignment of C-1, C-2, C-3, and C-4 at one end of the molecule and of C-11 and C-12 at the other. The one-dimensional ^{13}C spectrum obtained when patulolide C was produced under conditions where it could incorporate 2-^{13}C-(99%)-enriched acetate (68) into its skeleton during its biosynthesis is reproduced in the top part of Fig. 3.51; the two-dimensional INADEQUATE spectrum of the same material is reproduced below this trace. It is obvious from the 1D trace that 6 of the carbons of patulolide C can be derived from C-2 of the 2-^{13}C-



67

$^{13}CH_3CO_2^-$

68

enriched acetate (68) and that 6 (the tiny peaks in the 1D trace) are not significantly derived from this carbon atom.

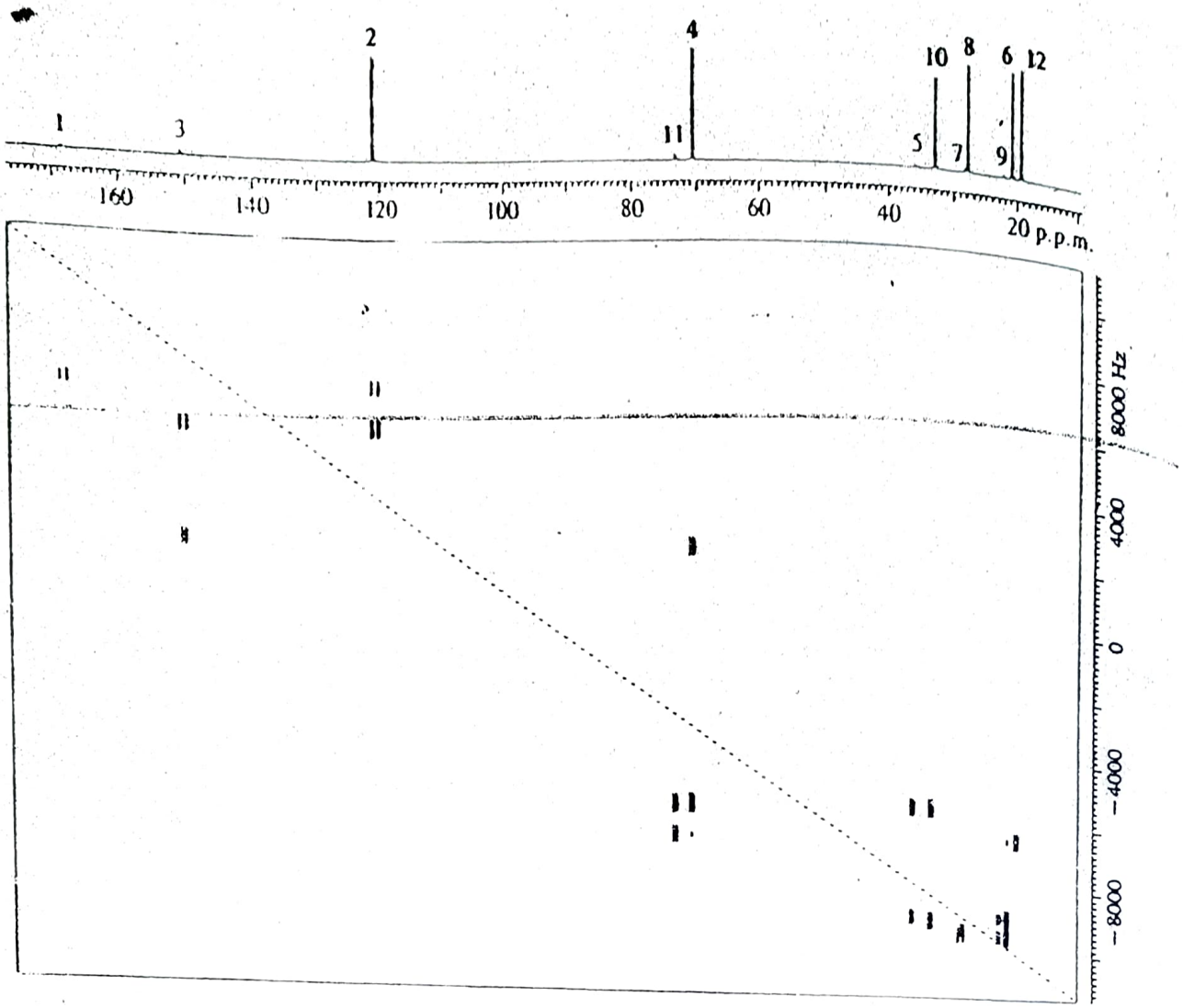


Fig. 3.51

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A straight line, drawn diagonally from the top left hand to the bottom right hand of the INADEQUATE spectrum (see dashed line in Fig. 3.51), must bisect the mid-point (along the ^{13}C axis) between each of the pairs of cross-peaks. The connectivity 168.3 p.p.m. (C-1) → 121.4 (C-2) → 150.3 (C-3) → 70.8 (C-4) is obvious. Also 70.8 (C-4) must be connected to the signal at 35.8 (C-5), and the further connectivity 35.8 (C-5) → 20.8 (C-6) → 28.3 (C-7) is evident. In a similar manner, we can start the analysis from the other end of the molecule. The carbon (19.4) known to be associated with a methyl group (e.g. from DEPT, Sec. 3.16) is seen to be connected to 73.3 (C-11) → 32.9 (C-10) → 22.2 (C-9) → 27.8 (C-8).

There are subtle points that aid the relative assignments of the signals due to C-7 and C-8, which initially appears to be based on no more than wishful thinking! The spectrum is taken on a 270 MHz spectrometer (for protons), and so, for ^{13}C, 1 p.p.m. corresponds to 68 Hz. The doublet nature of the cross-peaks in the spectrum is due to ^{13}C–^{13}C coupling which, applying Eq. 3.7, should have values of 64 Hz (sp^2–sp^2), 44 Hz (sp^2–sp^3), and 29 Hz (sp^3–sp^3). This gradual reduction in coupling is clearly

seen; the top two left pairs of cross-peaks are more widely separated than the third pair, which is in turn more widely spaced than the remaining pairs. An expanded version of the high-field cross-peaks, *in which all the cross-peak doublets must have essentially constant splittings* (*ca.* 29 Hz), is reproduced in Fig. 3.52. The apparently three-line cross-peak centred at *ca.* 28 p.p.m. must therefore be made up of two cross-peaks, with the higher-field line of the one at larger chemical shift (28.3) overlapping with the lower-field line of the one at smaller chemical shift (27.8). Hence, because of the symmetrical arrangement of peak pairs relative to the dashed line bisecting the midpoint between each pair of cross-peaks, the cross-peaks appearing near −9.400 Hz on the vertical frequency axis must be paired to connect the peak 28.3 with that at 20.8 p.p.m., and that at 27.8 with that at 22.2 p.p.m.

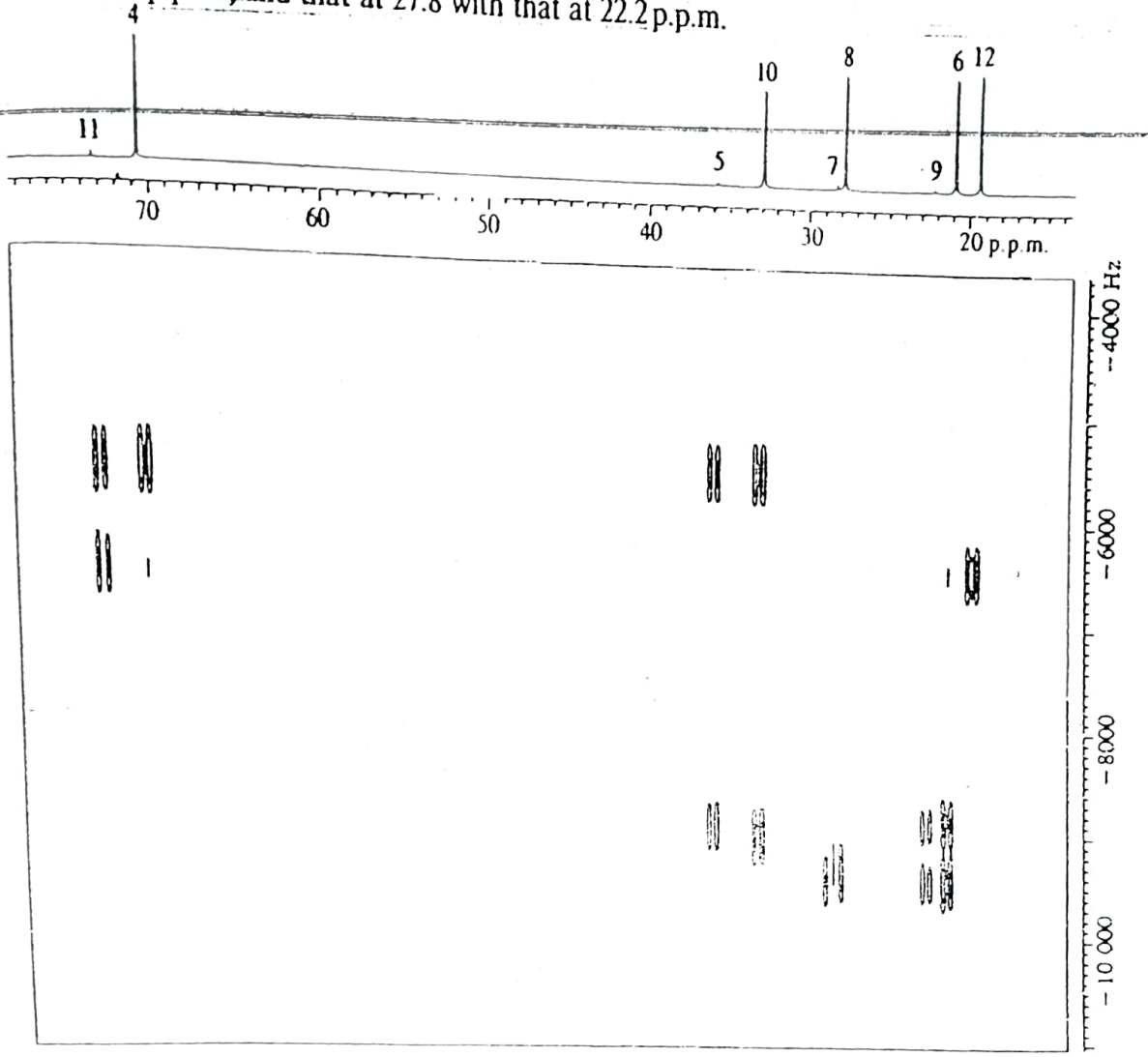

Fig. 3.52

(Reproduced with permission of *J. Antibiotics*, 1988, 41, 1649.)

In summary, the assignments of the carbon signals of patulolide C are as in Table 3.5. Given these assignments, it is evident that ^{13}C-enrichment of C-2, C-4, C-6, C-8, C-10 and C-12 occurs upon feeding with [2-^{13}C]-acetate (Fig. 3.51). Conversely, the experiment of feeding [1-^{13}C]-acetate (data not shown) led to ^{13}C-enrichment at C-1, C-3, C-5, C-7, C-9 and C-11. These data are consistent with the hypothesis that patulolide C is biosynthesized from six acetate units which are coupled together in a head-to-tail manner (69 and 70). During the course of the biosynthesis, the oxygen