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BIOSYNTHESIS OF MALONOMICIN-II

PROTON AND DEUTERON NMR SPECTRA

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Abstract: The ^{1}H and ^{2}H NMR spectra of malonomicin were analysed for use in biosynthetic incorporation experiments with ^{2}H labelled precursors.

Extensive chemical degradation studies have been the basis of the structure elucidation[1] of the antibiotic malonomicin (1). However, a suitable and reliable degradative chemistry to determine in an unambiguous way the site and quantity of biosynthetic labelling is lacking. Mainly for that reason, the origin of the carbon atoms of the malonomicin skeleton was established[2] by means of ^{13}C labelled precursors and ^{13}C NMR spectroscopy. For the same reason, it was decided to use ^{2}H labelling in combination with high-field ^{2}H NMR spectroscopy to provide the necessary sensitivity for tracing the fate of hydrogen atoms[3] in our following studies[4] on the sequence and mechanistic steps leading from the established basic precursors and hypothetical intermediates to malonomicin. As permeability barriers were expected and found to cause low incorporation of the very polar intermediates, an accurate analysis of the ^{1}H and ^{2}H NMR spectra of malonomicin was carried out in order to determine optimum NMR-measuring conditions in the biosynthetic experiments.

(1)

A priori, the proton NMR spectra of malonomicin seem easy to obtain and to interpret because the non-exchangeable hydrogen atoms are represented by two CH_2CH-groups and one CH_2-group only. Malonomicin, however, has several characteristics which make the interpretation of deuteron spectra particularly difficult:

- it is a very polar compound which is only soluble in aqueous acid or base. These solvents are relatively viscous which will lead to considerable broadening of the resonances; moreover, because of the relatively large quantity of HDO present in these solvents, its resonance shows severe overlap with almost all deuteron resonances of malonomicin;
- it is not stable at temperatures higher that 50°C which might have been applied to narrow the resonances;
- the chemical shift dispersion in the proton spectra is very small; at pH 8, six protons resonate between ca. δ 4.0-3.6 and two protons resonate at ca. δ 3.3 ppm. In the deuteron spectra, the differences in chemical shifts (in Hz) are further reduced by a factor of 6.5 (at constant field) so that the resonances will show even more serious overlap;

- poor solubility in the pH region 2-6 and undesirable exchange of the carbon bound hydrogens at lower pH and at pH>12 demand that spectra be taken in the pH region 6-12 (preferably in the region 7-10). However, two of the pK_A values of malonomicin fall in this region: pK_A = 7.8 of the serine amino group and pK_A = 9.2 of the aminomethyl group. Therefore, all chemical shifts are strongly pH-dependent, so that the proton spectra of solutions with only a small difference in pH show a marked difference in appearance (cf. Fig. 1).

Most of these problems which interfere with the quantitative interpretation of ^2H NMR spectra of biosynthetically enriched malonomicin can be partly solved by measuring the deuteron spectra of solutions in deuteron-depleted water at high field strength and at a temperature of 323 K (a compromise between line-narrowing and decomposition). Even then, the line-widths are too large with regard to the very small chemical shift dispersion to discern single resonances for all deuterons. However, as deuteron chemical shifts are essentially the same as proton chemical shifts under the same conditions, a precise analysis of the proton spectrum of malonomicin gives accurate information on the chemical shifts of the single deuteron resonances which constitute the largely unresolved ^2H NMR spectrum.

Proton Spectra

Proton spectra of malonomicin (250 MHz) were measured in D_2O at different pD's in the region 6-12 and analysed by simulation. In Fig. 1, the measured and simulated spectra at pD 8.37 and at pD 10.39 are given.

Depending on the pD, three spin systems can be distinguished:

- an ABX or A_2X system which is assigned to the methylene protons of the aminomethyl group (C(5)) and the proton at C(4);
- an ABX, ABM or ABC system formed by the protons of the serine moiety (C(12) and C(13));
- an AB or A_2 system which stems from the protons at C(7).

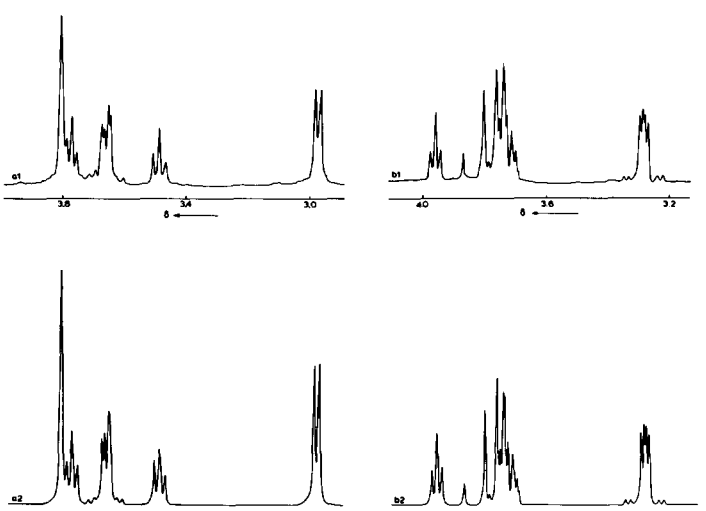

Fig. 1

Proton spectra of malonomicin (250 Hz) at pD 10.39 (a) and at pD 8.37 (b); measured (1) and simulated (2).

These assignments were made on the basis of the chemical shifts and decoupling experiments, and agreed very well with the expected behaviour of the chemical shifts on changing the pD. In Fig.2, the chemical shifts of all resonances are plotted as a function of the pD. From these data it is

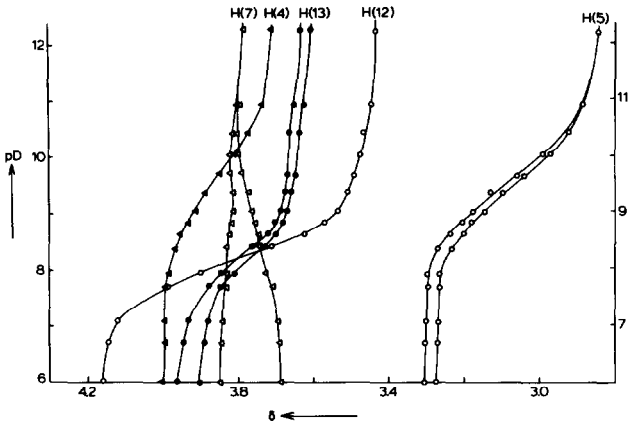

Fig. 2. Proton chemical shifts of malonomicin as a function of the pD.

Table

Coupling constants (absolute values) observed in the proton spectrum of malonomicin at pD 8.5 (the different protons bonded to one carbon atom are designated by a and b)

$n_{J(H-H)}$	Hz	$n_{J(H-H)}$	Hz
4 -5a	4.2	12 -13a	4.7
4 -5b	4.7	12 -13b	5.8
5a-5b	13.3	13a-13b	12.2
7a-7b	17.4		

evident that nearly all shifts are strongly pH dependent. On the contrary, the coupling constants only show a small variation with change of the pH. In the Table, the coupling constants of the proton resonances at pD 8.5 are given.

The non-equivalence of the aminomethyl protons at C(5) (see Fig. 2) at pD<10.5 is probably due to the formation of a hydrogen bridge between the protonated amino group at C(5) and the oxygen at C(3). The same bridge formation has been invoked[1b] to explain the remarkable acidity of malonomicin (pK_A 0.8). The reason for the increasing difference in chemical shift of the C(7) protons at lower pD's is not clear at the moment.

Deuteron Spectra

Natural abundance ^2H NMR spectra of malonomicin were measured of 0.5 M solutions in deuteron-depleted H_2O at 9.3 Tesla (61.42 MHz) and at a temperature of 323 K. In Fig. 3a, a deuteron spectrum at pH 8.0 is given. Except for the solvent peak at δ4.5 ppm, only two deuteron resonances are visible: the C(5) deuterons at high field (δ3.25 ppm) and the other deuterons as a broad signal with a maximum at δ3.82 ppm. Line-widths of the contributing resonances could be determined by measuring ^2H NMR spectra of malonomicin specifically labelled[2] by exchange with ^2H_2O at C(4), C(7) or C(12), respectively. They appeared to be in the range of 14-18 Hz (at 323 K).

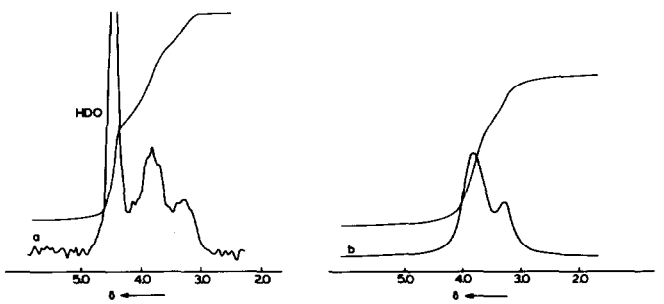

Fig. 3

Natural abundance ^2H spectrum of malonomicin (61.42 MHz) at pH 8.0 measured (a) and simulated (b).

By using the chemical shift data for protons summarized in Fig. 2, the natural abundance deuteron spectrum at any pH between 6 and 12 can be simulated accurately. This was done by calculating the proton-stick spectrum and adding a Lorenztian linewidth of 19 Hz (16 Hz linewidth + 3 Hz line-broadening). In Fig. 3b, a simulated natural abundance ^2H NMR spectrum at pH 8.0 is shown. Similarly, the shape as well as the integral ratio of both ^2H peaks can be computed exactly if the intensity of one or several of the contributing resonances is enhanced with any arbitrary chosen amount, thereby simulating an enrichment with ^2H.

The NMR analysis described permits to draw reliable conclusions from the results of biosynthetic incorporation experiments with ^2H labelled precursors. For this purpose, the position of ^2H label(s) in the required precursor and the NMR-measuring conditions (especially pH) can now be selected such that the expected ^2H resonances of biosynthetically enriched malonomicin are distanced as far from each other as possible. For instance (cf. Fig. 2):

- to distinguish between a specific enrichment either of $^2H(7a)$ or of $^2H(7b)$, the ^2H NMR spectrum should be measured at low pH;
- to verify whether a precursor is incorporated intact or not, the precursor can be double labelled in such a way that enrichment can be expected at C(5) and at C(7) which can be determined at high pH.

Low level incorporation of ^2H label in malonomicin (enrichment <0.1%) can be positively indentified and quantified with an error range of ± 20% by comparison of the ^2H NMR spectrum and its integral (obtained under optimum conditions) with the natural abundance spectrum and corresponding simulated spectra and their integrals.

Given the good signal-to-noise ratio obtainable with modern high-field NMR spectrometers (in the present study of ^2H spectra, a Bruker WH 400 spectrometer was used; S:N ratio 25:1; transients 40.000; repetition time 0.13 sec), we estimate a specific enrichment of 0.01% of deuterium (which gives an increase of the natural abundance signal with ca. 50%) to be at present the lower limit of unequivocal detection.

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