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Two-Dimensional *J*-Resolved Nuclear Magnetic Resonance Spectral Study of Two Bromobenzene Glutathione Conjugates

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The application of two-dimensional *J*-resolved nuclear magnetic resonance spectroscopy to determine the structure of two bile metabolites isolated from rats injected interperitoneally with bromobenzene is described. The structures of the two molecules are obtained unambiguously from the proton–proton spin coupling constants. This paper discusses the fundamentals of the technique and demonstrates the resolution of small long-range coupling constants.

Introduction

The recent development of two-dimensional Fourier transform NMR techniques (*1–5*) has greatly extended the applicability of NMR to biological problems. The technique is capable of providing unique information leading to unambiguous spectral assignments for structural elucidation. Proton and carbon spin-spin coupling constants are probably the most informative NMR parameters available to deduce molecular connectivities. One of the better techniques for obtaining those coupling constants is two-dimensional *J*-resolved spectroscopy (*6*). The advantage here is that the resolution of spin multiplets resulting from spin–spin coupling constants is significantly better than for coventional one-dimensional NMR spectroscopy. The purpose of this paper is to demonstrate this feature in a biologically important system and to report the details of the structural determination of two recently isolated bile metabolites.

Injection of bromobenzene (500 mg/kg) into rats led to the presence in bile of two substances not present in the bile of control rats (*7*). The two metabolites could be separated and purified by high pressure liquid chromatography (HPLC). Furthermore, evidence was presented that suggested that both conjugates were formed via a bromobenzene epoxide. In the one-dimensional NMR spectra of these materials, the resolution of the multiplets was inadequate to allow determination of the coupling constants.

J-Resolved Spectroscopy

The free induction decay (FID) following a 90° pulse applied to the spin system decays as exp $[-t/T_2^*]$, where T_2^* is a time constant whose value is usually determined by the inhomogeneity of the static B_O magnetic field. Often T_2^* in not a fundamental property of the nuclear spins. The real spin–spin relaxation time constant T_2 in liquids in much longer than the decay constant T_2^*, as confirmed by refocusing two pulse experiments (*8*).

Hahn first showed in 1950 that spin echoes occur if two radio-frequence pulses, spaced by a delay $t_1/2$, are applied to the spin system (*9*). The sequence, using a 90° pulse followed by a 180° pulse is diagrammed in Figure 1. The vector diagram picture, which depicts the behavior of the magnetization, has been discussed by various authors (*10,11*). The original purpose of this experiment, as described by Carr and Purcell (*10*), was

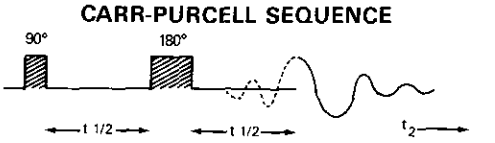

FIGURE 1. Pulse scheme for the Carr-Purcell sequence and for two-dimensional *J*-resolved spectroscopy. The FID is recorded during the interval t_2 for a fixed value of t_1. The t_1 is then incremented and another FID is recorded.

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to measure the true spin–spin relaxation time T_2 by removing the effects of the magnetic field inhomogeneity. Since the effect of the 180° pulse is to refocus the vector component of the magnetization, the value of the magnetization at time t_1 where the echo is maximal, is independent of field inhomogeneity. Thus T_2 can be determined from Eq. (1):

$$M(t_1) = M(0)^* \exp\{-t_1/T_2\} \quad (1)$$

if one has an independent measure of the initial intensity, $M(0)$, from a one pulse experiment. This procedure is appropriate for a single resonance line in the absence of spin coupling. In the presence of homonuclear spin coupling the amplitude of the signal $M(t_1)$ will be modulated by $1/J$, where J is the value of the spin–spin coupling constant. To take this complication into account in a simple spectrum it was pointed out (*12*) that the peaks of the echoes could be recorded as a function of the pulse interval t_1 and the resultant signal Fourier transformed to produce spectra of multiplets whose line widths would then reflect the true T_2 rather than the field inhomogeneity T_2^* where usually

$$T_2^* = \frac{1}{\pi \Delta \nu_{1/2}} \quad (2)$$

This procedure was useful for studying small coupling constants in very simple molecules. However, only one resonance frequency could be investigated in any given experiment, since application of the 180° refocusing pulse, in addition to eliminating the effects of the inhomogeneity of the static magnetic field, also eliminated the chemical shift information at the peak of the echo.

It was nevertheless realized that the remainder of the signal echo, which as usual decays as exp $\{-t/T_2^*\}$, contained both chemical shift information as well as spin coupling constant information. In an elegant paper, Freeman et al. (*4*) pointed out that one could utilize both the information at the echo peak and its ensuing decay by performing a two-dimensional Fourier transform on a data matrix obtained for various values of the interval t_1 where

$$G(F_1, F_2) = \int_0^\infty \int_0^\infty H(t_1, t_2) e^{-2\pi i F_1(t_1)} e^{-2\pi i F_2(t_2)} dt_1 dt_2 \quad (3)$$

Here the spectrum is presented as a function of the two frequency variables, F_1 and F_2. To generate a two-dimensional matrix for Fourier transformation the series of FID are recorded by systematically increasing t_1 over the entire time frame of the FID. A typical time for an FID to decay into the noise is of the order of 1 sec, and a typical two-dimensional data matrix might involve digitizing 1K data points for each value of t_1 and incrementing t_1 over 256 values between 0 and 1 sec. The result of the two-dimensional Fourier transform is a spectrum whose projection in this case along the F_1 axis yields spin-coupled spectra centered at zero frequency with the line widths determined by the natural line width (T_2) rather than by the field inhomogeneity. Projections along the F_2 axis yield the analog of the usual one-dimensional spectrum with line widths determined by T_2^*. Thus by choosing a narrow region of the spectrum and projecting that result along the F_1 axis, one obtains the spectrum associated with a particular chemical shift and with considerably narrower lines. We thus have the ability to resolve very small coupling constants and to solve structural problems that were previously intractable.

Results

A particularly striking result is obtained in the case of two bile metabolites which were isolated from rats injected interperitoneally with bromobenzene. Centrilobular necrosis of the liver is probably mediated by the cytochrome P-450-dependent formation of a bromobenzene epoxide intermediate. It is thought that in the presence of glutathione transferase (*7*) the epoxide is opened to form a glutathione conjugate as depicted in Figure 2. Two metabolites were isolated as described previously (*7*). The purpose of this study is to characterize the metabolites, determine their structure spectroscopically, and to demonstrate the biological application of the powerful 2D Fourier transform NMR method.

The one-dimensional spectra of the metabolites (labeled A and B in order of elution from the column) are presented in Figure 3. Single-proton spin-decoupling experiments readily demonstrated that the resonances at 3.63 ppm and 4.41 ppm for metabolite A are coupled to each other and to the olefinic protons at 5.93 and 6.31, respectively. The resonance at 4.41 ppm is suitable for a carbinol proton, while that at 3.63 ppm is appropriate to a proton on a saturated carbon bearing a thioether substituent. Metabolite A can therefore be formulated as the cyclic dienol anticipated. Exactly analogous observations were made of metabolite B, showing that the carbinol proton at 4.38 is coupled with the olefinic proton at 6.10 ppm, while the aliphatic proton at 3.75 is coupled to the olefinic proton at 6.31 ppm.

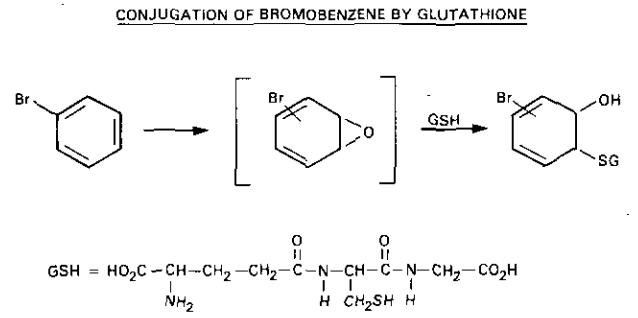

FIGURE 2. Reaction scheme for the formation of the glutathione conjugates through the bromobenzene epoxide intermediate.

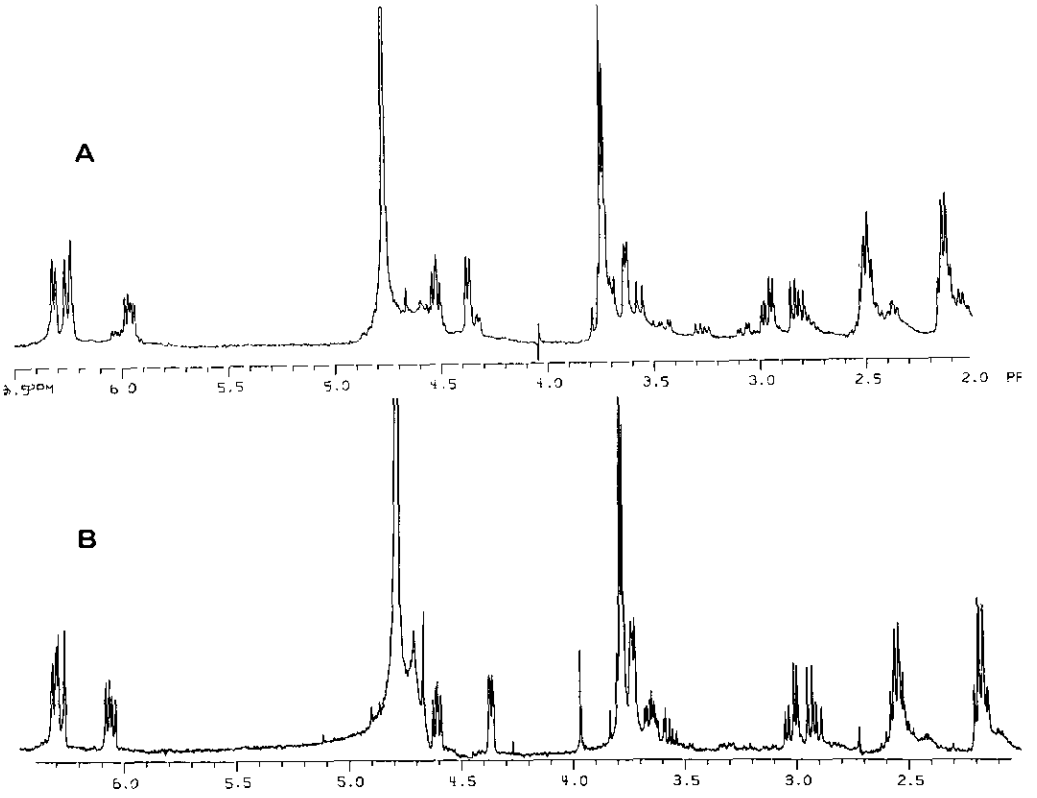

FIGURE 3. One-dimensional 360 MHz proton NMR spectra of the glutathione conjugates of bromobenzene.

A typical result of the expansion of the olefinic region of the one-dimensional spectrum of metabolite B is shown in Figure 4. The high field olefinic proton consists of four lines with some difficulty discernable fine structure. A 2D J resolved spectrum of the olefinic region of the metabolite B is given in Figure 5. Projection onto the F_2, axis will yield the analog of the one dimensional spectrum. This result will generally be of low resolution, since it is still limited by the magnetic field inhomogeneity. However, projecting a cross sectional region along the F_1 axis will yield a spectrum whose resolution is not limited by field inhomogeneities. In the first example, in Figure 6 we show the projection of the high field olefinic resonance of metabolite B. The projection in Figure 6 is to be compared with the one-dimensional expanded spectrum of the same olefinic region in Figure 4. It is clear that a remarkable improvement in the resolution of the fine structure has been achieved. These results typify the amount of resolution enhancement that one may expect using two-dimensional J-resolved spectroscopy. From this projection we measure four coupling constants whose values are 9.8 Hz, 5.5 Hz, 0.9 Hz, and 0.9 Hz. In Figure 7 is a similar projection of the 4.38 ppm region. The procedure applied to metabolite A is identical. Projections of the 5.93 and 4.44 ppm regions of metabolite A are shown in Figures 8 and 9, respectively.

The couplings thus observed from the F_1 projections are collected in Table 1. In metabolite A, the olefinic proton at 5.93 ppm, shown by decoupling to be vicinal to the aliphatic proton at 3.63, shows a large coupling of 9.7 Hz to the proton at 6.25 ppm; the two are therefore vicinal to each other, while that at 6.31 ppm is separated by the bromine atom. The implications of the

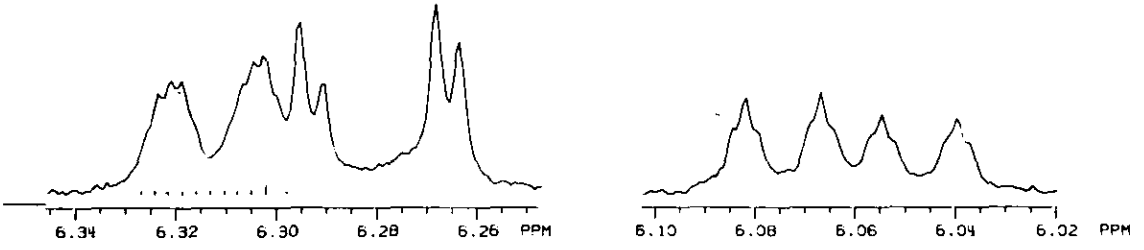

FIGURE 4. Expanded spectrum of the olefinic region of the glutathione conjugate of bromobenzene B.

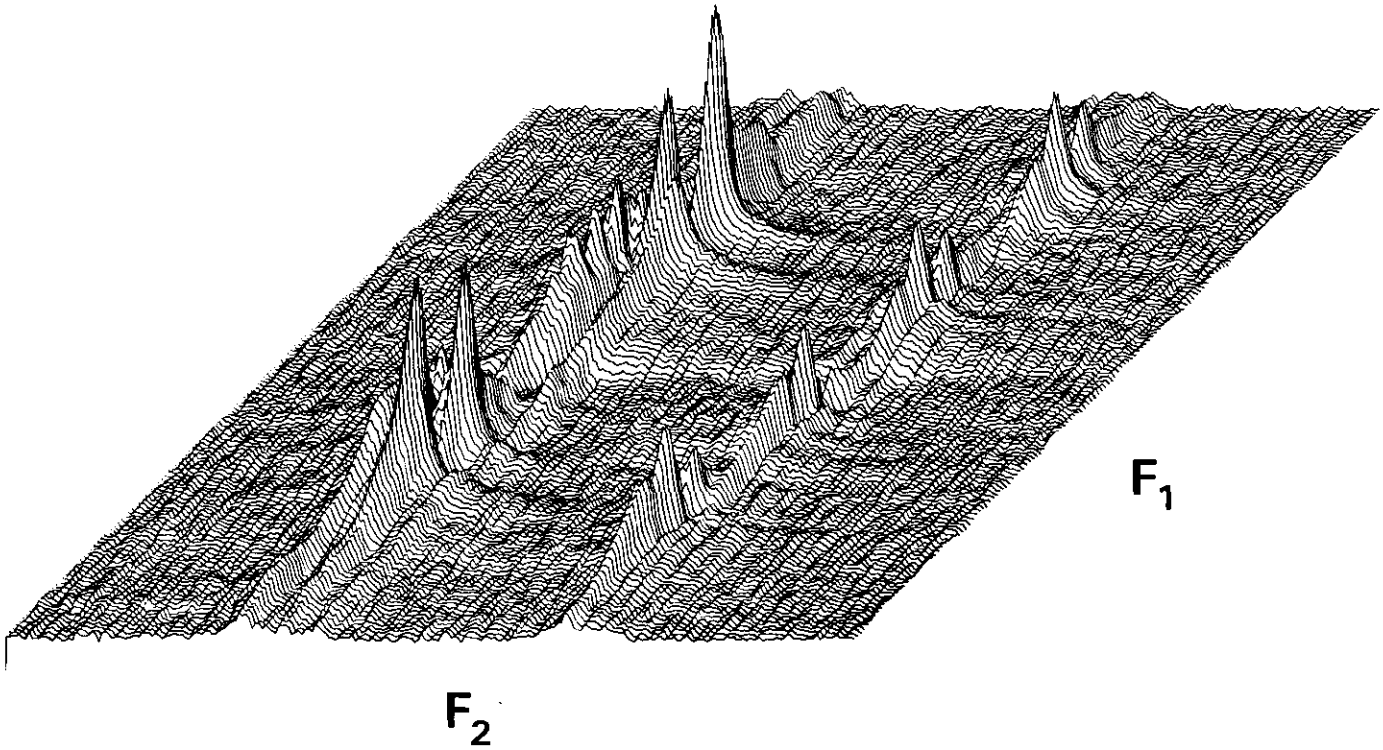

FIGURE 5. Two-dimensional J-resolved 360 MHz proton spectrum of the olefinic region of the glutathione conjugate of bromobenzene B.

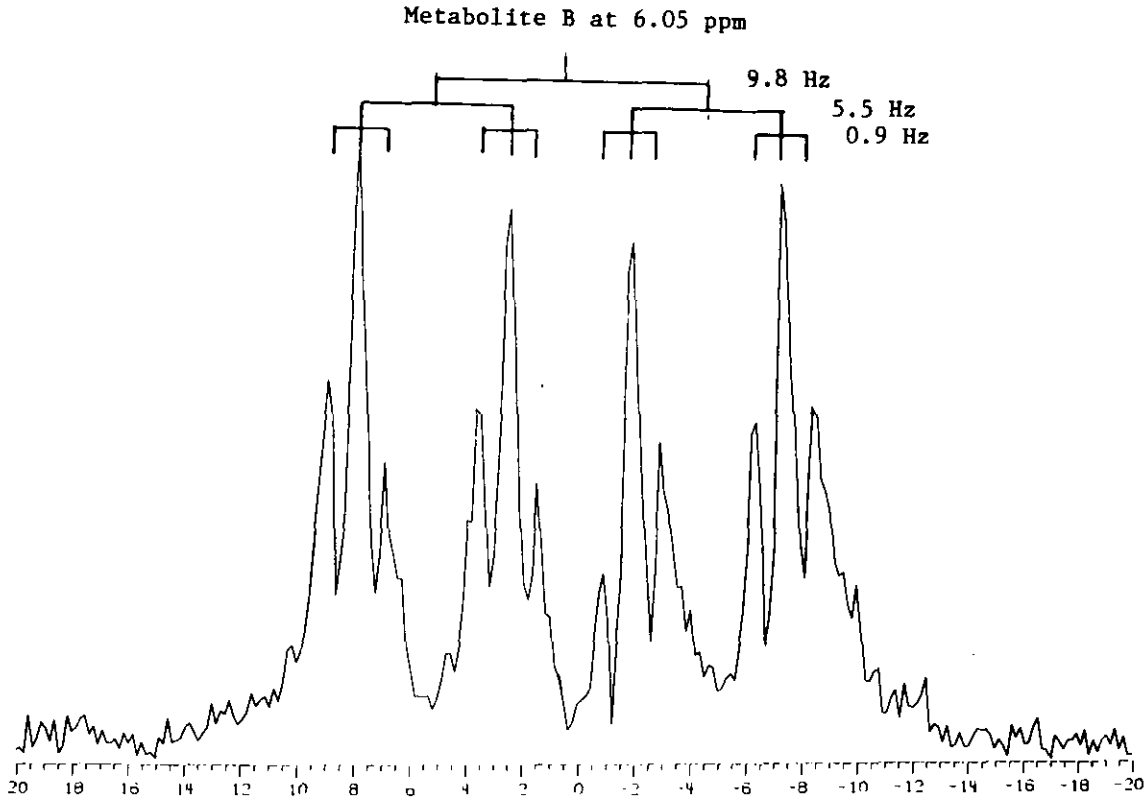

FIGURE 6. Projection of the cross section of the 6.06 ppm region along the F_1 axis of the glutathione conjugate B.

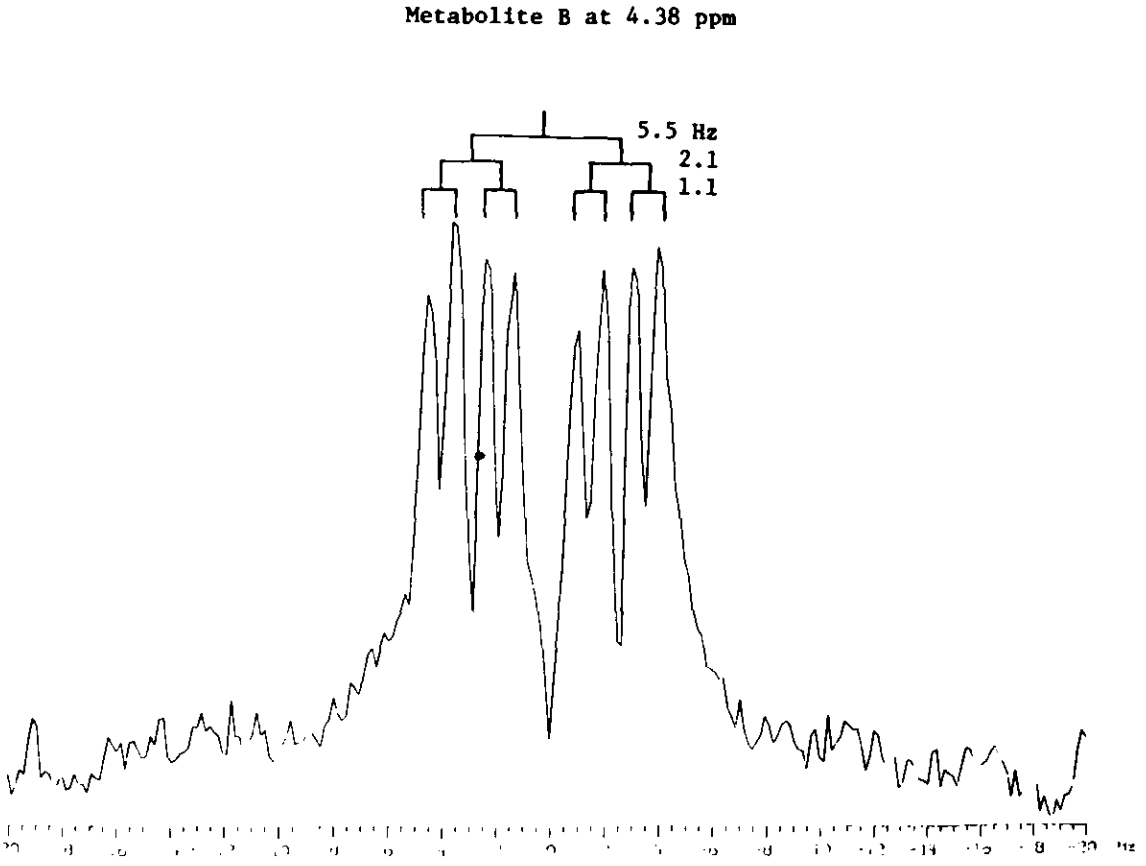

FIGURE 7. Projection of the cross section of the 4.38 ppm region along the F_1 axis of the glutathione conjugate B.

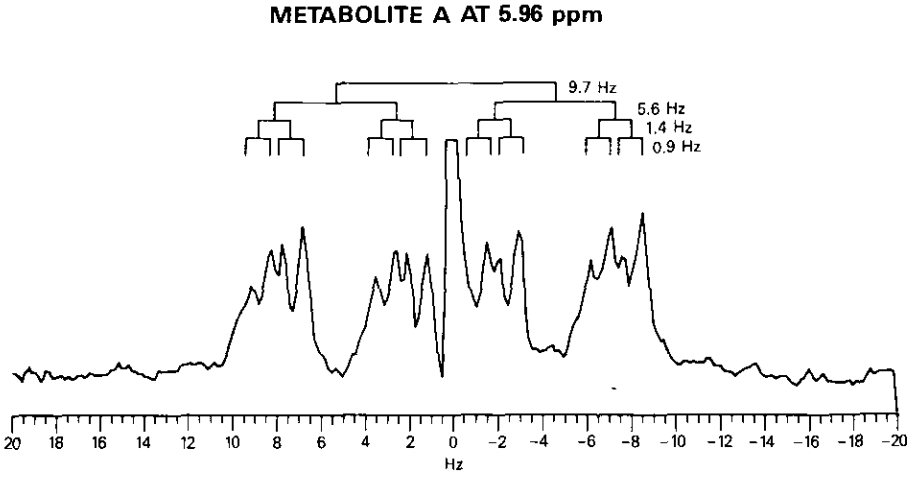

FIGURE 8. Projection of the cross section of the 5.93 ppm olefinic region along the F_1 axis of the glutathione conjugate A.

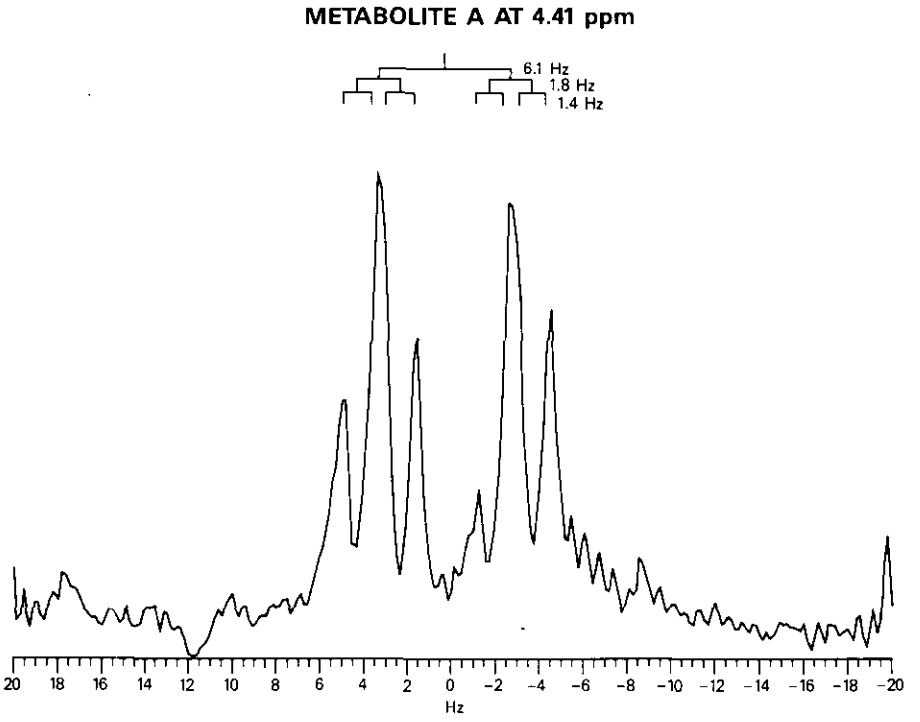

FIGURE 9. Projection of the cross section of the 4.41 ppm aliphatic region along the F_1 axis of the glutathione conjugate A.

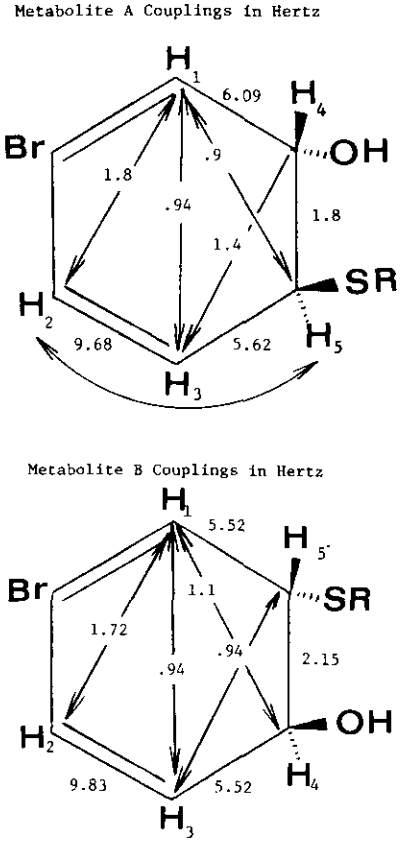

FIGURE 10. Structure and coupling constants (Hz) of the (*a*) metabolite A and (*b*) metabolite B.

Table 1. Observed chemical shifts and coupling constants.

	Proton	Shift, ppm	Observed couplings, Hz
Metabolite A	1	6.31	6.1, 1.8, 0.9, 0.9
	2	6.25	9.7, 1.8, 0.9
	3	5.93	9.7, 5.6, 1.4, 0.9
	4	4.41	6.1, 1.8, 1.4
	5	3.63	5.6, 1.8, 0.9
Metabolite B	1	6.31	5.5, 1.1, 0.9, 1.7
	2	6.28	
	2		9.8, 1.7
	3	6.06	5.5, 9.8, 0.9, 0.9
	4	4.38	5.5, 1.1, 2.2
	5	3.75	5.5, 2.2, 0.9

decoupling experiments are confirmed by the smaller vicinal couplings at 6.1 and 5.6 ppm. Similarly, the couplings observed of metabolite B show the protons at 6.06 and 6.28 ppm to be vicinal to each other, with that at 6.31 ppm isolated from the other olefinic protons by the bromine atom. The structures that result are shown in Figure 10.

Conclusions

We emphasize that considerable effort went into optimization of the magnetic field homogeneity in an effort to improve resolution of the fine structure. While other procedures involving either convolution of the data or delicate multiple resonance experiments are in principle capable of detecting very small coupling constants, the advantage of two-dimensional resolved spectroscopy lies

in its simplicity and ease of operation on a modern NMR spectrometer. It is clearly the method of choice for the present problem.

The application of two-dimensional *J*-resolved spectroscopy to the determination of the structure of biologically interesting molecules is clearly demonstrated. The technique is highly attractive because it permits resolving power to approach the fundamental limit set by the natural linewidths. The two-dimensional technique in general is going to have a major impact on the study of both structural and dynamic properties of molecules, both *in vitro* and *in vivo*.

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