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Complete Analysis of ^1H NMR Spectra of Complex Natural Products Using a Combination of One- and Two-Dimensional Techniques. 1-Dehydrotestosterone

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Abstract: All the proton resonances of 1-dehydrotestosterone have been resolved and assigned by using a combination of partially relaxed spectra, nuclear Overhauser enhancement (NOE) difference, and decoupling-difference techniques; all the geminal and vicinal coupling constants and several long-range couplings have been measured accurately by using proton two-dimensional *J* spectroscopy. On the basis of these experiments a general, idealized strategy for unraveling the ^1H NMR spectra of complex organic molecules is proposed. The ideal strategy uses a spectrometer which is completely under computer control, but simplified variants which are applicable to less automated instruments are also described. Partially relaxed spectra obtained with a 180°–*t*–90° sequence can be used to separate overlapping methine and methylene protons on the basis of more rapid methylene relaxation. A difference method which allows routine, simultaneous measurement of several NOEs at the 0.5–5% level has been developed and used to resolve hidden protons and assign them on the basis of their spatial relationships; the most important of these relationships is between the 1,3 diaxial protons (and their ring D equivalents) which enables the NOE experiment to "see" both across and between rings. Either steady-state or transient NOEs may be used. Scalar couplings between protons are readily observed by decoupling-difference spectroscopy which also allows detection (and, if necessary, analysis) of otherwise hidden protons. Two-dimensional *J* spectroscopy may be used to separate chemical shifts and coupling constants into different frequency axes and then to measure those parameters.

Introduction

This work was prompted by our belief that ^1H NMR spectroscopy in the past few years has experienced two quite separate revolutions whose potential impact on organic chemistry is enormous but as yet largely unexplored. The first, conceptually new and exciting, involves several variants of the two-dimensional (2D) NMR experiments pioneered by Ernst and by Freeman, for example, 2D *J* spectroscopy, which allows separation of chemical shift and spin coupling information in weakly coupled spin systems,[2] and 2D shift correlation spectroscopy, which enables simultaneous acquisition of *correlated* ^1H–^{13}C spectra.[3,4] The second, superficially less exciting revolution is that highly stable superconducting magnets coupled with modern computer control systems extend into a completely new domain the power of such traditional assignment techniques as the nuclear Overhauser enhancement (NOE) and ^1H–^1H spin decoupling experiments which have previously been thought to be fully developed. As a result there are numerous new opportunities for both spectral "resolution" and assignment; these are the principal remaining problems in the analysis of ^1H NMR spectra of complex natural products such as steroids, the Fourier transform method having essentially eliminated sample size as a serious limitation.

Therefore, we propose a general strategy for the analysis of such spectra, using 2D *J* spectroscopy to resolve and analyze all the resonances, and using relaxation rates, NOE difference, and decoupling-difference techniques to assign them. In this paper we develop and exemplify these techniques with the relatively simple case of 1-dehydrotestosterone (**1**). In a subsequent paper[5,6] we show how they may be applied to the much more challenging 11β-hydroxyprogesterone (**2**) and discuss the chemical implications of some of the derived results. Most of the assignments[6] and 2D *J* results[7] have been briefly reported by us in preliminary form.

Background

In this section we outline the theoretical and experimental background to each of the techniques used in this work, with the emphasis on their potential application in the natural-product area. One assumption implicit in this discussion is that even the apparently intractable methylene envelopes of steroids are actually merely the superposition of many weakly coupled multiplets; given the intrinsic axial–equatorial chemical shift difference of 0.4 ppm

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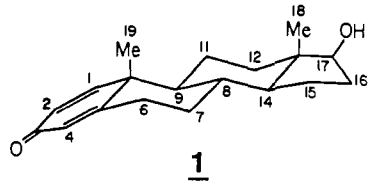

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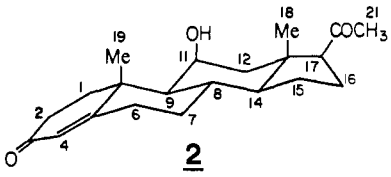

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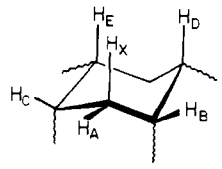
this is at least a reasonable assumption at high fields.[8]

Spin–Lattice Relaxation. It is well established that for organic molecules in solution the dominant proton relaxation mechanism is the dipole–dipole relaxation interaction with other protons.[9,10] The relaxation rate $R_1(I)$ of a proton I as a result of this interaction with its neighboring S protons is given by eq 1 where τ_c is a rotational correlation time and r_{IS} are the distances from I to S:

$$R_1(I) \propto \tau_c \sum r_{IS}^{-6} \quad (1)$$

Proton relaxation rates may therefore be used to obtain direct geometrical information in rigid molecules where τ_c is effectively the same for all protons,[10-12] or to probe the mobility of molecular fragments of known geometry (e.g., methyl groups).[11-13] Where both mobility and geometry are known or can be accounted, then R_1 values can be used to assign resonances to specific protons,[11-15] and it is in this way that they should prove valuable in the present problem: all protons attached to the rigid steroid ring system should have effectively the same τ_c so that methylene protons (having a geminal partner) should relax faster than methine protons. It follows that in an inversion–recovery experiment it should be possible to resolve buried methine signals from the methylene envelope by nulling the methylene resonances.

Nuclear Overhauser Enhancements.[16] The NOE is a manifestation of dipole–dipole relaxation: when more than one S spin relaxes the I spin, the relative enhancements of I when S are separately irradiated are proportional to r_{IS}^{-6}. The maximum combined enhancement is 50% in the proton–proton case. Where a proton has no geminal partner, its relaxation will be determined only by distant protons and therefore those distant protons will induce large NOEs. Thus NOEs have been of great value in studying *methine* protons in the past,[11,12,16] the most spectacular examples being in the polypeptide area.[17] However, since the relaxation of a methylene or methyl proton is dominated by its geminal partner(s), distant protons contribute little relaxation and they induce correspondingly small Overhauser enhancements. This is illustrated for **3** where H_X will be enhanced by up to 32% when H_A is irradiated, but by a maximum of ca. 6% when H_B or H_C is irradiated and by only 3% for saturation of H_D or H_E. Since integrals can rarely be measured with this precision, these small



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NOEs are essentially unobservable on a routine basis in the classical experiment. For the NOE to become a completely general tool for structural studies of, for example, steroids it is necessary that 1,3 diaxial interactions be readily detected and this requires routine measurement of enhancements at the 1% level, or even lower.

In principle NOE difference spectra (NOEDS) provide just such a possibility: since a control spectrum without NOE is subtracted from the enhanced spectrum, only *changes* should appear. The lower limit of detectability should then be determined only by signal-to-noise considerations and instrument stability, and the signal of interest need no longer even be resolved in the normal spectrum. In practice, the enhanced and control spectrum have to be acquired alternately to minimize the effects of long-term drift: the control FIDs may be subtracted from the enhanced FIDs during acquisition so that only the difference spectrum accumulates,[18,19] or the control and enhanced FIDs may be stored separately for later processing.[20-22] With one exception[22] previous NOEDS were obtained with macromolecules where the effects are large and negative. If the same methods are applicable to steroids, allowing routine observation of 1% enhancements, then it should be possible to irradiate, for example, the 18- and 19-methyl groups and "see" their 1,3 diaxial neighbors.

Spin Decoupling. This is of course a standard assignment tool, but given the preceding discussion of NOEDS it seems clear that decoupling difference spectra (DDS) generated by subtracting control spectra from decoupled spectra should reveal buried multiplets rather easily. To our knowledge the experiment in this form has been developed and used independently by only one other group,[20] although it is of course a variant of FT INDOR.[18,23,24] The heteronuclear equivalents of DDS have been used to assign ^{15}N and ^{13}C spectra of polypeptides.[25]

Two-Dimensional (2D) *J* Spectroscopy.[2,26-28] A comprehensive review of 2D NMR covering the period to mid-1978 has been published by Freeman and Morris.[26] An entirely nonmathematical, pictorial description of the proton 2D *J* experiment, and its possibilities and limitations, is forthcoming,[27] while Bodenhausen et al.[28] have given detailed descriptions of the techniques and of the problems of second-order systems. It suffices here to point out that homonuclear 2D *J* spectroscopy is simply a variant of the classical Carr–Purcell spin–echo experiment in which the time delay between a 90 and 180° pulse is systematically varied. The result, after transposition of the individual spin–echo spectra, a second Fourier transformation, and a "tilt" of the frequency

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axes,[29,30] is a spectrum which *in the weakly coupled case* contains only chemical-shift information along one frequency axis (f_2') and homonuclear coupling constant information along the other axis (f_1).[31] It then becomes possible to measure chemical shifts and coupling constants in a region of the spectrum which normally consists of so many overlapping multiplets that analysis is impossible. Thus 2D J spectroscopy has appeared ideal for large molecules which contain many small, isolated spin systems, e.g., polypeptides[29] and oligosaccharides,[32] but it has not previously been applied to complex molecules such as steroids. At the outset of this work it was clear neither to what extent steroids would be weakly coupled nor to what extent the intense methyl singlets would obscure multiplets of interest.

Experimental Section

1-Dehydrotestosterone (**1**) was obtained from the Sigma Chemical Co. and used without further purification. Solutions (0.05–0.06 M) were made up in $CDCl_3$; one sample containing a trace of ethanol was degassed (freeze–pump–thaw) and sealed. As would be expected for a molecule of molecular weight over 300, relaxation rates were sufficiently fast that degassing had little effect.[12,33]

^1H NMR spectra were obtained at room temperature on a home-built spectrometer operating at 270 MHz under the control of a Nicolet 1180/293A′ data system or on a Bruker WH 400 operating at 400 MHz under ASPECT2000 control. The 90° pulse width at 270 MHz was ca. 6 μs; at 400 MHz it was in the range 7–15 μs.

After a few trial experiments one-dimensional spectra at 400 MHz were all run with 16K data points over 2808 Hz, giving an acquisition time of 2.9 s and a digital resolution of 0.34 Hz; quadrature detection and phase cycling were employed. Spin–lattice relaxation rates were obtained by the inversion–recovery sequence, using the null point method. The validity of this method for molecules of this size has been demonstrated elsewhere.[33] Most of the steady-state NOE difference spectra were obtained at 400 MHz by a method designed to maximize spectral quality. Four transients were collected after preirradiation (3–5 s per transient) at a selected frequency. The FID was stored, the irradiation frequency moved, and the irradiation–acquisition cycle repeated for the same number of transients. This second FID was stored separately from the first and the irradiation was moved to a third and fourth frequency in the same way. The entire cycle was repeated 50–400 times under computer control with addition of the new data to that already stored, giving total accumulations of 200–1600 transients per irradiation; in effect data were acquired simultaneously for a control and three NOE experiments. The accumulated FIDs were processed with identical line broadening (usually 1–2 Hz) and phase corrections. Methyl singlets not experiencing enhancements were routinely nulled to better than 99.7%; i.e., their intensity in the difference spectrum was less than 0.3% of that of the normal spectrum, allowing enhancements of 0.5% to be reliably observed. The necessary frequency selectivity in the irradiation was achieved by using subsaturating power levels: inspection of difference spectra after a few accumulations allowed easy determination of the adequacy of frequency selectivity. The low variable level of irradiation generally used leads to observed enhancements which are less than theoretically possible; thus relative enhancements between *different* irradiations cannot be compared.

In a few experiments, transient NOEs were observed at 400 MHz after a selective 180° pulse was applied to the 19-methyl singlet, using a Dante[34] sequence: 31 pulses, each 1 μs long, were applied with a spacing between the pulses of 1.9 ms. Since 31 μs corresponds in this case to a 180° pulse, the sequence generated such a pulse every 526.3 Hz (i.e., the reciprocal of 1.9 ms). The offset and pulse spacing were adjusted so that no proton signals were at 526.3, 1052.6, or 1579 Hz, but the 19-methyl singlet was 2105 Hz from the central pulse and so was inverted. The delay between this selective 180° and the monitoring 90° pulse was varied from 0.1 to 1.5 s and once again control spectra were "simultaneously" acquired to minimize the effects of any long-term drifts.

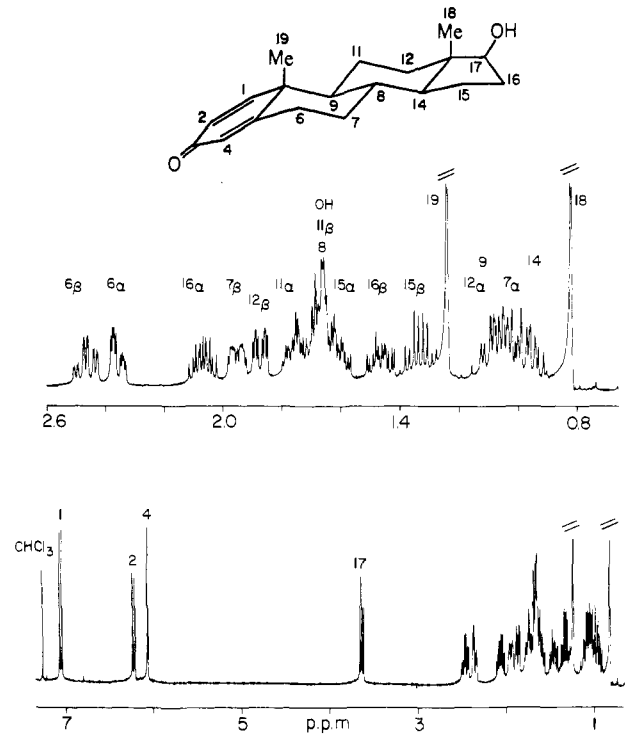

Figure 1. Lower trace: 400-MHz ^1H spectrum of **1**, 0.06 M in $CDCl_3$ (6.5 mg in 0.4 mL), 100 transients, resolution enhanced. Upper trace: expansion of high-field region.

Decoupling difference spectra were obtained at 400 MHz and processed in the same way as the steady-state NOE difference spectra, except that the decoupler was on during acquisitions and no relaxation delay was used. Usually between 5 and 50 transients were collected for each irradiation; the resulting difference spectra contained NOEs at levels too low to be significant. Some NOE- and decoupling difference experiments were carried out at 270 MHz, but as the decoupler of that instrument is not under computer control the various irradiations were done sequentially under manual control.

Spectral simulations were made by using standard NTCFT software on the Nicolet 1180 computer.

The 270-MHz data for 2D J spectra were obtained with a 90°–t–180°–t–acquisition sequence, using phase alternation to suppress flip angle errors. Data processing was carried out with standard Nicolet software except that the 45° tilt routine was written in this laboratory.[30] Data storage and manipulation limitations determined the available digitization: only the high-field region of the spectrum was studied, the f_2 spectral width being 1602.56 Hz (i.e., a nominal 1600 Hz) over 4K data points, giving a digital resolution of 0.78 Hz. Spectrometer offset and filter were adjusted to avoid foldover problems from the olefinic protons;[35] 64 spectra each of 150 transients with t incremented by 9.98 ms each time gave an f_1 width of 50.8 Hz with a digital resolution (after zero filling) of 0.39 Hz. After the first Fourier transformation, only 1K of data points covering the range δ 0.8–3.7 were further processed. For the second transformation data were handled in the absolute value mode both with and without sine bell resolution enhancement in f_1, or in the phase-sensitive mode[27,37] with 0.2-Hz broadening in f_1. In the latter case line shapes in selected cross-section traces were improved by inverse transformation, zero filling once or twice, exponential multiplication, and Fourier transformation. Total acquisition time was 7.77 h and data processing time was about 4 h, of which the tilt required 3 h. Recent modification of the tilt program has cut its execution time by ca. 80%.

The 400-MHz 2D J spectra were obtained with a prototype Bruker automated program which allows use of only absolute value data display. Sweep width and digitization in both f_1 and f_2 were the same (Hz) as in the 270-MHz experiment, and sine bell resolution enhancement was used in the f_1 dimension. Only the high-field portion of the spectrum, above δ 2.2, was processed. Twelve transients were acquired for each spectrum,

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Table I. Proton Chemical Shifts and Spin-Lattice Relaxation Rates in 1

proton	δ, ppm (±0.01)	R_1, s^{-1} (±10%)
1	7.07	1.1
2	6.23	0.7
4	6.08	0.7
6α	2.36	1.5
6β	2.47	1.3
7α	1.01	1.5
7β	1.96	1.5
8	1.67	1.0
9	1.04	1.2
11α	1.77	1.7
11β	1.68	1.5
12α	1.09	1.5
12β	1.87	1.3
14	0.95	1.2
15α	1.61	1.3
15β	1.33	1.3
16α	2.07	1.1
16β	1.47	1.1
17	3.64	0.9
18	0.82	0.9
19	1.24	1.7

giving a total experimental time of 0.87 h and data processing time of 1.5 h; this shorter time required for data acquisition reflects the higher intrinsic sensitivity of a 400-MHz instrument, the fact that it has quadrature detection, and the fact that the phase-sensitive mode used on the 270-MHz spectrometer gives better resolution at the cost of sensitivity.[27]

Results

One-Dimensional Spectroscopy. The 400-MHz spectrum of **1** is rather well dispersed, displaying many first-order multiplets in the region 0.8–2.6 ppm in addition to the readily assigned olefinic protons ($J_{1,2}$ = 10.0, $J_{2,4}$ = 1.7 Hz) and H_{17} (Figure 1). The 18- and 19-methyl groups are also obvious and easily assigned.[38] For ease of further discussion all the chemical-shift assignments are collected in Table I, together with the relaxation rates. Integration reveals the presence of four protons at δ 0.9–1.2 (hereafter the "high-field multiplet") and four carbon-bound protons at δ 1.5–1.8 (the "low-field multiplet"). Irradiation of H_4 removes a 1.5-Hz coupling from the 2.47-ppm signal which must therefore be $H_{6\beta}$ (since it is clearly axial), and so the δ 2.35 resonance must be $H_{6\alpha}$. Similarly H_{17} is coupled to two protons which must be the H_{16} pair. No further simple decoupling experiments were attempted because even at this stage the assignments become stereochemically uncertain. At 270 MHz most resonances above δ 2 overlap, precluding worthwhile integration or irradiation experiments.

Spin–Lattice Relaxation Rates. All the methylene protons of rings B and C have relaxation rates in the range 1.3–1.7 s^{-1}, whereas the methine protons relax more slowly, their rates varying from 1.0 to 1.2 s^{-1}. The high-field multiplet is therefore rather easily resolved into methylene and methine pairs (Figure 2a), allowing the first tentative assessment of coupling and multiplicity in this region. Similar separation is also possible for the low-field multiplet, H_8 relaxing more slowly than the other components.

The relaxation pattern in ring A, as reported previously,[13] indicated that H_1 and H_4 are relaxed largely by protons from outside that ring, a good augury for the intended NOE experiments. In ring D, H_{17} relaxes very slowly as expected for a proton lacking a geminal partner, but the protons attached to C_{15} and C_{16} also relax significantly slower than the methylene protons in the other rings.

Partially relaxed spectra reveal the presence of slowly relaxing, but rather broad, hydroxyl signals around δ 1.6 and 3.5, the exact chemical shifts varying from sample to sample.

Difference Spectra. The method developed for the acquisition of NOE difference spectra allowed "simultaneous" measurement

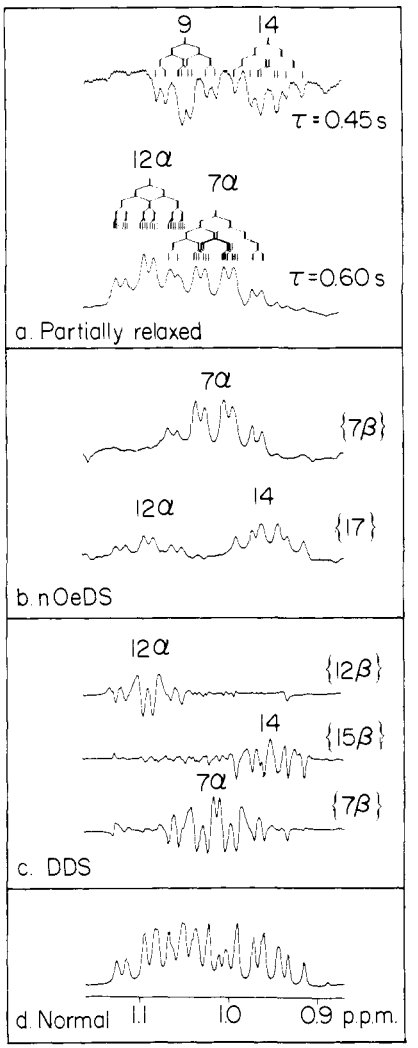

Figure 2. Partial 400-MHz spectra of the high-field multiplet, broadened by exponential multiplication: (a) partially relaxed spectra, demonstrating differential relaxation rates of methylene and methine protons; (b) NOE difference spectra; (c) decoupling difference spectra; (d) the normal spectrum.

of three NOEs and a control: four transients were collected and stored for each of the irradiation frequencies, and the cycle was repeated as many times as necessary, all under computer control. The irradiation time before each acquisition was usually 5 s. The best quality results were obtained by using the maximum available digitization of 16K data points[39] followed by 1–2-Hz exponential line broadening; the latter is necessary, particularly for methyl singlets, as most of the signal is otherwise defined by very few data points, rendering precise subtraction difficult. Indeed, when attempting to detect NOEs on methyl groups, it was helpful to increase the broadening even further. In all applications of difference spectroscopy it proved crucial that the phasing corrections for both spectra were identical. For the NOE spectra where the decoupler was gated off during acquisition no complications arise from spin decoupling or Bloch–Siegert shifts.[40] However, since the decoupler power varied from one experiment to another the absolute values of the observed enhancements have no quantitative significance and accordingly they are generally not reported here; they were in the range 0.5–5%, the subtraction efficiency being greater than 99.7%. Difference spectra were examined and plotted

(38) Bhacca, N. S.; Williams, D. H. "Applications of NMR Spectroscopy in Organic Chemistry: Illustrations from the Steroid Field"; Holden-Day: San Francisco, 1964; pp 13–24.

(39) In the computer memory 32K of data are available, but in the difference routine both spectra are recalled together.

(40) A differential Bloch–Siegert shift of only 2 Hz between two spectra can ruin a difference spectrum and must be avoided as described. Of course the signal in the difference spectrum will then be shifted but this is of little consequence since the accurate chemical shift is known from other experiments.

Table II. Connectivities Established by NOE and Decoupling Difference Experiments[a]

proton irradiated	protons appearing in diff spec	
	NOE	decoupling
1	11α	
4	6α	
6α		7α,7β
6β	7β,8,19	7α,7β
7β	7α	6α,6β,7α,8
11α		9,11β,12α,12β
12β		11α,11β,12α
15β		14,15α,16α,16β
16α	15α,16β	15α,15β,16β
17	12α,14,16α	16α,16β
18	8,11β,12β,15β,16β	
19	6β,8,11β	

[a] Absence of an expected entry is either because of signal proximity or because the experiment was not performed. Difference spectra were only inspected in the 0.8–2.5-ppm region.

only in the δ 0.6–2.6 region as the primary aim was peak assignment. Table II summarizes the NOEs observed, but we will describe the logic associated with some of the assignments.

The first set of NOE difference spectra were obtained by irradiation at the most accessible (and previously assigned) protons, H_4, H_1, and H_{17}. The only signal in the $\{H_4\}$[41] difference spectrum was $H_{6\alpha}$, which evidenced an enhancement of 3.8% (Figure 3b). Since this was already assigned it served as a good test of both the experiment and of our analysis. The $\{H_1\}$ difference spectrum contained only a complex multiplet which was tentatively assigned as $H_{11\alpha}$ and the $\{H_{17}\}$ spectrum (Figure 2b and ref 6) displayed only $H_{12\alpha}$, H_{14}, and $H_{16\alpha}$, the tentative assignment being in each case based on geometrical and multiplicity considerations. For $H_{16\alpha}$ the assignment was firm, given the decoupling results above.

The next irradiations were of the axial family consisting of $H_{6\beta}$ and the angular methyls. The $\{H_{6\beta}\}$ difference spectrum contained a small enhancement of CH_3-19 and a double quartet at 1.65 ppm which was clearly the expected H_8. The {19-methyl} difference spectrum gratifyingly contained $H_{6\beta}$ (Figure 3) and a complicated signal at 1.65 ppm thought to be $H_{11\beta}$ and H_8 together as they are the same distance from that methyl group. The {18-methyl} difference spectrum showed the same 1.65-ppm signal together with $H_{12\beta}$, $H_{16\beta}$, and, near the limits of detectability, $H_{15\beta}$.

These assignments, while all plausible and self-consistent, were neither rigorous nor complete. Both requirements were largely met by decoupling difference spectra in which only protons coupled to the irradiated proton should appear. The necessity for the difference technique is demonstrated in the Discussion and is also illustrated in Figure 2c. In normal spectra, when $H_{7\beta}$ is irradiated, no useful change is apparent. In contrast, the difference spectrum instantly gives the chemical shift of the perturbed $H_{7\alpha}$ resonance. The effects of irradiating $H_{12\beta}$ and $H_{15\beta}$, also shown in Figure 2c, make this point even more forcefully. Bloch–Siegert shifts induced into resonances very close to the site of irradiation sometimes caused problems, and occasionally it was necessary to acquire a blank spectrum for each decoupling, the blank irradiation being as close as possible to the irradiated group.[40] The observed decoupling difference connectivities are given in Table II, and left only one ambiguity in our minds: the stereochemical assignment of the H_{15} protons. The result of the 18-methyl irradiation (δ 0.82) reported above was ambiguous since even the slightest irradiation of the very close H_{14} resonance (δ 0.95) would give enhancement to $H_{15\alpha}$. The problem was resolved by irradiation of $H_{16\alpha}$, which cleanly enhanced $H_{16\beta}$ and $H_{15\alpha}$, confirming thereby the original tentative assignment. At the same time, H_{7b} was irradiated to give an NOE to its geminal partner (Figure 2b). Some of these experiments were also performed with the 270-MHz spectrometer, which lacks computer control of the decoupler, so the data had to be acquired sequentially rather than concurrently. The decoupling difference results, which could be obtained with short accumulation times, where spectral dispersion allowed sufficiently selective irradiation, were of similar quality to those at 400 MHz. The NOE results were much inferior and would not have allowed the successful outcome of this work.

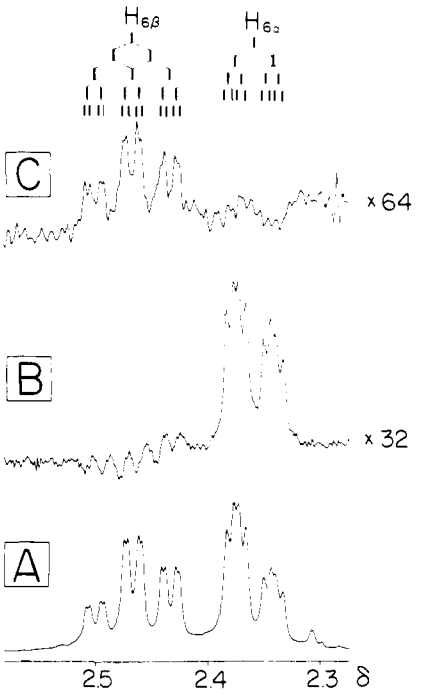

Figure 3. The 400-MHz spectra of the 2.3–2.5-ppm region: (a) normal; (b) NOE difference spectrum obtained by preirradiation of H_4; (c) NOE difference spectrum obtained by preirradiation of H_{19}.

In one experiment at 400 MHz selective inversion of the 19-methyl group induced transient NOEs in the same protons as in the steady-state experiment, i.e., $H_{6\beta}$, H_8, and $H_{11\beta}$. The enhancements (which were observed with only 200 transients) built up during the first second after the inversion and then died away, although the signal-to-noise level was not good enough for worthwhile quantitative measurements. In the difference spectrum there was a negligible perturbation of the signals near to the inverted peak, demonstrating the potentially excellent frequency selectivity. In several of the steady-state and transient NOE experiments the hydroxyl resonance evidenced intensity changes as a result of NOE or saturation transfer but no particular pattern was apparent.

Two-Dimensional Spectroscopy. The absolute value spectra at 270 and 400 MHz gave rather similar information, whereas the phase-sensitive display gave different information, and these will therefore be considered separately.[42]

Absolute Value Display. The 270-MHz tilted 2D J spectrum (not shown) completely resolves and separates all the proton resonances of **1** except those of H_8, $H_{11\alpha}$, and $H_{11\beta}$. The geminal pair are rather second order (Δ/J = 1.8), and H_8 is virtually coincident with $H_{11\beta}$. The f_2 "tails" of the methyl singlets extend into several nearby multiplets, appearing as prominent central lines and obscuring important details unless sine bell resolution enhancement is used. The f_1 tails appear off center and, fortuitously, do not interfere in this particular case. Figure 4 shows the tilted 400-MHz 2D J spectrum of the high-field region in stacked form, as the f_2' sum, and as individual partial J spectra, in addition to the normal one-dimensional spectrum. The f_2' sum gives the "proton decoupled" spectrum in which each proton appears at its chemical shift as a singlet. In addition there is a broad hump at δ 1.7 arising from the expected[28] second-order transitions of the H_{11} pair. Much of the width of the δ 1.67 peak arises from the

(41) The proton being irradiated is indicated by { }: Baldeschweiler, J. D.; Randall, E. W. *Chem. Rev.* **1963**, *63*, 81.

(42) A detailed description of the phase-sensitive method and of the distortions inherent in tilting phase sensitive spectra will be given elsewhere: Hall, L. D.; Sukumar, S., in preparation.

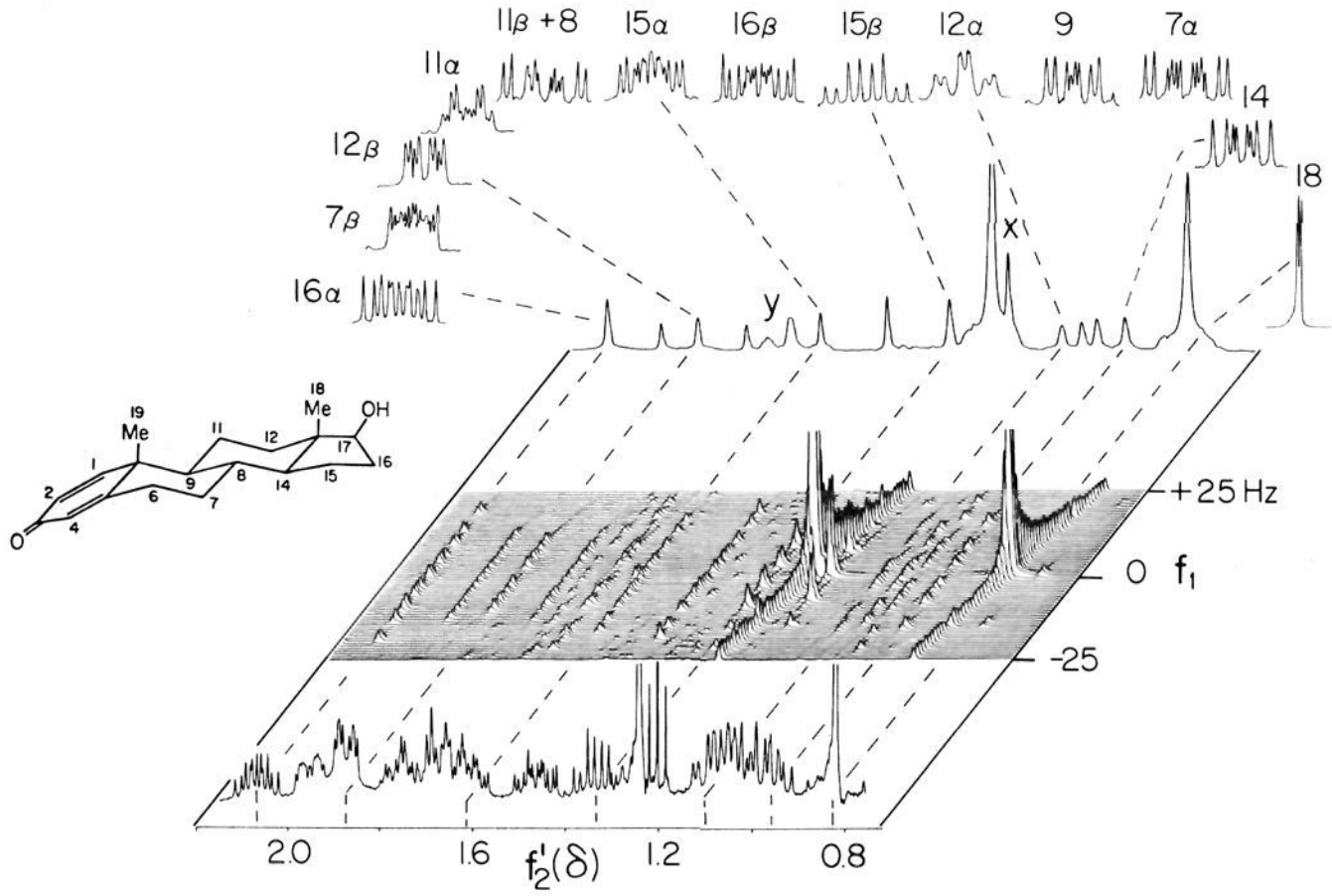

Figure 4. Partial 400-MHz spectra of **1** showing (from bottom to top) the normal spectrum, the two-dimensional *J* spectrum (for clarity, only 64 traces are shown in the stack plot), the "proton decoupled" spectrum derived from the 2D *J* spectrum, and the partial *J* spectra for CH_3(18) and the multiplets. X denotes CH_3 group of added ethanol; Y denotes second-order transitions of the 11α,11β geminal pair.

near but incomplete coincidence of $H_{11\beta}$ and H_8. The signal at δ 1.18 is from a trace of added ethanol.

The stack plot is dominated by the angular methyls and their f_1' tails which in tilted spectra run at 45° to the axes when the latter are plotted on the same scale. Also visible are several spinning sidebands from the methyls in the expected position (some folded over)[43] and with intensities ca. 1.5% of the main peaks. All the remaining significant signals are from spin-coupled multiplets in the compound, and since some of these are split into 16 resolved lines their intensity is comparable with methyl spinning sidebands. All the multiplet intensity falls on lines parallel to the f_1' axis except for the second-order transitions noted previously.

Individual cross sections from each proton are shown in Figure 4; all appear at first order multiplets. The f_2' tails create no problems at all when sine bell resolution enhancement is used, even for $H_{15\beta}$, which is only 0.09 ppm from the 19-methyl. Despite the use of sine bell the line width of the slowly relaxing ethanol methyl group and of most of the steroid signals is ca. 1 Hz, precluding the measurement of most long-range coupling constants. The only long-range couplings found in the absolute value spectra at either frequency were $J_{12\alpha,18}$ = 1.0 Hz and the obvious $J_{4,6\beta}$ = 1.5 Hz.

Phase-Sensitive Display. These were obtained at 270 MHz as described in the Experimental Section. Wiggles are created as a result of the inverse transformation of a truncated FID but they may be suppressed by use of a line broadening equivalent to the digital resolution. Tilted phase sensitive cross sections of **1** display line widths down to 0.4 Hz in the absence of long-range couplings, close to the limit imposed by T_2; these line widths not only permit accurate measurements of geminal and vicinal coupling constants (Tables III and IV) but they also reveal numerous long-range couplings of 0.3–0.6 Hz which as yet are unassigned.

Table III. Geminal and Vicinal Coupling Constants in Rings B and C[a]

proton pair	$\lvert^2J\rvert$	^3J ax–ax	^3J ax–eq	^3J eq–eq
6α,6β	13.4			
6α,7α			4.3	
6α,7β				2.6
6β,7α		13.4		
6β,7β			4.8	
7α,7β	12.8			
7α,8		11.5		
7β,8			4.0	
8,9		12.3		
8,14		11.0		
9,11α			4.4	
9,11β		10.5		
11α,11β	13.3			
11α,12α			4.6	
11α,12β				3.0
11β,12α		12.8		
11β,12β			3.9	
12α,12β	12.7			

[a] In Hz (±0.3).

Table IV. Vicinal and Geminal Couplings in Ring D[a]

proton	14	15α	15β	16α	16β	17
15α	7.4		|12.1|	9.4	3.2	
15β	12.1			5.6	11.8	
16α					|13.2|	9.0
16β						7.8

[a] Hz (±0.3).

Discussion

One-Dimensional Spectroscopy. Initially some assignments were made by simple spin decoupling, and for this particular molecule that approach could have been used more extensively. However, we chose not to pursue this approach for two reasons. First, as Figures 2c and 5 show, it is a futile exercise when signals overlap severely (and this is the most common situation for molecules such as steroids); second, because, although we could probably have assigned the stereochemistry of, for example, the H_{16} pair with some confidence, the chemical literature abounds with "confident" assignments which turn out to be wrong.

Spin–Lattice Relaxation. It is known that H_1 relaxes much faster than H_2; assuming that the relaxation of H_2 is dominated by H_1, then this implies that H_1 is being relaxed also by the 19-methyl or ring B protons (or both). H_4 lacks a vicinal partner, yet it relaxes at the same rate as H_2, and so H_4 is also being relaxed rather efficiently by protons of another ring. The reciprocal nature

(43) Bodenhausen, G.; Kempsell, S. P.; Freeman, R.; Hill, H. D. W. *J. Magn. Reson.* **1979**, *35*, 337.

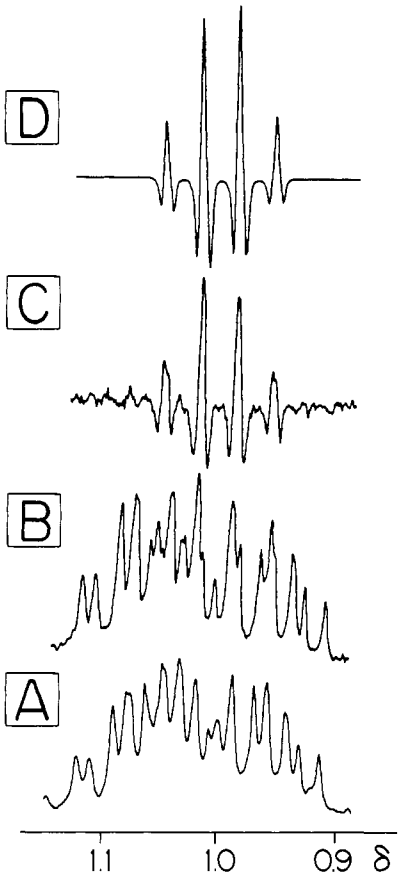

Figure 5. (a) Partial 400-MHz spectrum of the high-field multiplet; (b) the same region with $H_{6\alpha}$ decoupled; (c) difference spectrum, (b) − (a); (d) computer simulation of (c).

of relaxation therefore demands that H_1 and H_4 each relax those protons in the other rings. These results reinforce the expectations derived from inspection of molecular models that inter-ring NOEs should be observable in this part of the molecule (see next section).

The separation of methylene and methine protons in the partially relaxed spectra of the high- and low-field multiplets on the basis of their differing relaxation rates is a simple and pleasing result. It is a natural consequence of the fact that the ring B and C interproton vectors are all likely to have essentially the same rotational correlation time but only the methylene protons have very close neighbors. The methylene protons of ring D relax rather slowly (R_1 in the range 1.1–1.3 s^{-1}) in comparison with the other rings where only $H_{6\beta}$ and $H_{12\beta}$ relax more slowly than 1.4 s^{-1}. Presumably these slower rates reflect the lack of nearby protons: $H_{6\beta}$ lacks relaxation from ring A, $H_{12\beta}$ points toward the hydroxyl group, and the ring D protons mostly lack axial neighbors. In this molecule the slow relaxation of H_{17} is of little consequence but in **2** the buried H_{17} methine proton is readily located in partially relaxed spectra.[5] This approach should be of general utility. Indeed, when successful, this type of resolution at such an early stage is invaluable for defining the multiplicity and chemical shifts of individual protons and, in favorable cases, assigning them.

Methyl relaxation in this molecule has been discussed elsewhere.[13]

NOE Difference Spectroscopy. We discuss separately the generation, detection, and interpretation of Overhauser enhancements.

Enhancements may be generated by steady-state irradiation or, transiently, by selective 180° pulses.[44] For this assignment work we have used the former as it is the experiment most familiar to chemists. In order to generate maximum enhancements, the irradiation time before acquisition was chosen to be roughly $5T_1$; it may be that the optimum experiment in terms of total measurement time would be to use a shorter irradiation time but more transients. The great disadvantage of the steady-state method is that high-frequency selectivity, which is rather important in these experiments, can only be achieved by use of low irradiation power which leads to loss of enhancement intensity. The importance of high selectivity is that even slight saturation of a neighboring proton would give a significant enhancement to its geminal partner, which can cause confusion when the NOEs of interest are themselves small.[45] In cases where irradiation of neighboring resonances is unlikely to affect the same protons, selectivity may be sacrificed and the decoupling frequency stepped in closely spaced increments: a plot of the resulting enhancements vs. irradiation frequency then gives the desired assignment.[12] Selectivity is also less crucial where the expected NOEs are large.

The long-term solution to the selectivity problem appears, on the basis of our few experiments, to lie with transient enhancements generated by selective 180° pulses.[5] These can be produced with a predetermined frequency selectivity by gated decoupling,[24] tailored excitation,[46] or Dante[34] and have the additional advantage that at the optimum delay between perturbing and monitoring pulses the enhancements will be much larger than in a subsaturating steady state experiment. Dante techniques have an appealing conceptual elegance but in complex molecules the sideband problem becomes severe and gated decoupling may well be the most versatile approach to transient NOEs.

We turn now to the detection of enhancements by difference spectroscopy. The very small effects used in this work (down to ca. 0.5%) can, in our experience, only be easily observed if the irradiated and control spectra are acquired simultaneously. Even in a superconducting magnet field, shimming and phase drifts over a period of hours create problems in sequential acquisition experiments. Thus at this level of enhancement (and lower!) computer control of the decoupler seems mandatory. Difference spectra may then be directly acquired by adding a few FIDs with NOE, then subtracting the same number without NOE, and repeating the cycle as long as necessary.[18,19] However, since only the difference spectrum accumulates there is no direct record of the normal spectrum. Several groups have shown that, if the FIDs corresponding to the two irradiations are stored separately, then the normal spectrum is available and the difference spectrum may be obtained from either the FIDs or transformed spectra.[20-22] The method used in our work is a minor but useful improvement on that just described in that one control spectrum serves three separate NOE experiments, all acquired simultaneously. Thus, if N transients are needed per irradiation, only $4N$ are needed for three NOEs rather than $6N$ as in previous work.[47]

Finally, what do these enhancements tell us? Most importantly they have established spatial connections across and between rings by using protons whose relaxation is actually dominated by neighbors which are much nearer through space; moreover, many of these protons are not even resolved in the normal spectrum. There are many possible geminal and vicinal enhancements, some of which we have used here, but scalar couplings often provide essentially the same connectivity information more readily. The new class of NOEs, and hence assignment, which we hoped to find and have indeed successfully exploited involved the 1,3 diaxial interaction (and its ring D equivalent). The importance of this class of assignment is that it provides a direct entry into a new, remote part of the molecule which can in favorable cases be extended by further enhancement or decoupling experiments. Thus $H_{6\beta}$, H_8, $H_{11\beta}$, and the angular methyls can be located and assigned

(44) Also by chemical exchange from molecules which themselves show enhancements through irradiation or CIDNP: Bargon, J.; Gardini, G. P. *J. Am. Chem. Soc.* **1979**, *101*, 7732.

(45) Actually, it need not matter that irradiations of degenerate protons are involved: the logic in disentangling results is just much more complicated (ref 5).

(46) Hill, H. D. W.; Tomlinson, B. L. *J. Chem. Phys.* **1973**, *59*, 1775.

(47) We now routinely carry out 12 "simultaneous" irradiations under computer control, i.e., 11 NOEs serviced by just one blank. The only limitations are data storage space and the need to acquire adequate signal-to-noise for each irradiation frequency.

through NOEs by virtue of their diaxial relationships. Subject only to the requirement of adequate spectral dispersion, this should be an entirely general method for all steroids with similar ring junction stereochemistry. In ring D, irradiation of H_{17} does not lead to enhancements of $H_{15\alpha}$ but it does enhance $H_{12\alpha}$, H_{14}, and $H_{16\alpha}$. This would appear to indicate that the conformation of ring D is approximately as shown at the beginning of this paper, which is in remarkably good agreement with the crystal structure.[48] If that is so, then the fact that the 18-methyl induces a greater enhancement on $H_{16\beta}$ than on the spatially closer $H_{15\beta}$ presumably reflects the lack of neighbors around $H_{16\beta}$ (in agreement with the R_1 values).

The enhancements from the protons of ring A to those of B are much as expected, foreshadowed as they were by R_1 measurements, but it is noteworthy that in each case they were on protons with both vicinal and geminal partners. Even more noteworthy is observation of an enhancement at the 19-methyl when $H_{6\beta}$ is irradiated. We know of few other reported NOEs *into* a methyl group,[19] apart from our experience with **2**,[5] but the diagnostic utility of such enhancements is obvious. The enhancements and saturation transfers associated with the hydroxyl resonances could have created problems, although they were hardly unexpected in $CDCl_3$ solution.[49] It would be prudent in general to avoid these difficulties by prior deuterium exchange when working in $CDCl_3$.

We have been aware throughout this work of the possibility of "three-spin" effects which could lead to misleading absences of enhancements or even to negative effects.[16] With the possible (but unlikely) exceptions noted two paragraphs above with $H_{15\beta}$ we have noted no such problems. They may simply be lost in the experimental noise or in fact they may become insignificant in systems with so many mutually relaxing spins. Reliable judgment must await many more such experiments.

Decoupling Difference Spectroscopy. Since the intensity of individual resolved lines may change by up to 100% during a decoupling experiment, the sensitivity of decoupling difference spectra is comparable with normal spectroscopy and is one to two orders of magnitude higher than FT INDOR or NOE difference spectroscopy. As a result we found in this study that a set of five decoupling difference spectra could be obtained in less than 10 min under computer control. This experiment therefore places much less stringent demands on magnet stability and can easily be carried out sequentially; thus our 270-MHz decoupling difference spectra are of comparable quality to those at 400 MHz.

In principle, "pure" decoupling difference spectra free of NOEs require relaxation delays between acquisitions, but in practice we have noticed no substantial enhancements. This is partly because so few transients were collected, partly because the vertical display gain is roughly ten times smaller than that in the NOE experiment, and partly because the protons experiencing the largest enhancements are likely also to be coupled and so any effect would be partly hidden in the difference peaks. Gibbons has, however, reported simultaneous effects in gramicidins.[20]

As Figure 5 shows, it is possible to simulate decoupling difference spectra and thereby analyze the multiplet structure. In this work such a course of action was not strictly necessary, but while carrying out simulations we found that large coupling constants were rarely completely removed in our experiments. This is irrelevant for our purposes, and is a result of the low decoupling power levels used in the cause of frequency selectivity, but it does suggest an intriguing homonuclear equivalent of the noise modulated decoupling experiment which can be used to selectively broaden carbons which are coupled to protons[50] or protons coupled to fluorine.[51] Irradiation of a proton with a weak noise modulated frequency should broaden the multiplets coupled to it. The difference spectrum would then show only those coupled multiplets but in a directly analyzable form.

The only problem associated with generation of decoupling difference spectra is due to the induction of Block–Siegert shifts; this can usually be dealt with successfully as described earlier. It is worth noting that these difference spectra are a particularly good way of assigning small couplings even when no decoupling effect is apparent in the normal spectrum; for example, $J_{2\alpha,4}$ = 0.6 Hz in **2**.

Two-Dimensional Spectroscopy. The power of the 2D J technique is forcefully demonstrated by the fact that one tilted[52] 270-MHz 2D J spectrum gave almost complete separation, and very high resolution, of signals which otherwise required several NOE and decoupling difference experiments at 400 MHz. The 400-MHz 2D J spectrum is even clearer and easier to interpret: with a combined data acquisition and processing time of less than 3 h it enabled measurement of all chemical shifts and vicinal and geminal coupling constants. The only second-order peaks—the hump midway between $H_{11\alpha}$ and $H_{11\beta}$ in the f_2' sum—are recognizable as such because they do not fall on a line parallel to the other multiplets and also through prior knowledge of the chemical shifts and constants involved.[28] If necessary the second-order spectrum can be simulated,[26] but it would seem preferable to change the spectrum to remove its second-order character.[53]

It is clear from inspection of Figure 4 that the absolute value display gives multiplets close in appearance to "conventional" spectra, although all components in the multiplet have the same intensity. However, in these particular experiments the associated line broadening negates the resolution benefit inherent in the spin–echo experiment and precludes the measurement of most of the long-range couplings in the system. Digital resolution could be improved by collecting more spectra, but at the expense of much greater experimental and processing time and data storage space. The phase-sensitive display is accompanied (in tilted spectra) by both resolution enhancement and distortions which are inherent in the experiment.[27,42] There is the added advantage that selected traces can be inverse transformed to give FIDs which can in turn be manipulated in a variety of ways to further improve resolution. The resulting line widths should then be at or below[42] the T_2 limit and the only problem might be that so many long-range couplings are resolved that they lose their diagnostic value.

It is encouraging that the dominant methyl resonances actually interfere so little with neighboring signals in this particular molecule, but they could be a major problem in more complex steroids. This point has been only briefly addressed in previous 2D J work,[54] and it is appropriate to discuss it at greater length here. The problems associated with such intense resonances are (i) dynamic range, the FID being dominated by the least interesting signals; (ii) f_1 and f_2' tails, which can obscure important detail in both dimensions; (iii) spinning sidebands which can be more intense than the multiplet structure of interest. These problems will be most severe for methyl groups at the end of extended side chains or which are part of $OCOCH_3$ or CO_2CH_3 groups since these are the methyls with the longest T_1 values and the minimum of long-range coupling, both factors leading to very sharp resonances.[12] Fortunately these are also the resonances most easily "removed" by WEFT techniques; i.e., the pulse sequence is modified to $180°-\tau-90°-t-180°$, where τ is adjusted to null the slowly relaxing component.[54] Methyl groups directly attached to rigid frameworks, for example, in steroids[13] or porphyrins,[12] relax at similar rates to ring methylenes and so cannot be treated in this way, although they should create less problem being much broader. Another route for nulling methyl signals is of course preirradiation, provided that the associated NOEs create no problem!

(48) Duax, W. L.; Norton, D. A.; Pokrywiecki, S.; Eger, C. *Steroids* **1971**, *18*, 525.
(49) Feeney, J.; Heinrich, A. *Chem. Commun.* **1966**, 295.
(50) Opella, S. J.; Cross, T. A. *J. Magn. Reson.* **1980**, *37*, 171, and references cited therein.
(51) Evelyn, L.; Hall, L. D. *Carbohydr. Res.* **1976**, *47*, 285.
(52) Note that, as the chemical-shift separation (Hz) between multiplets is so much smaller than their width, it would not have been possible to *conveniently* extract coupling data from the untilted spectrum.
(53) Using, for example, solvents or lanthanide-induced shifts in combination with the 2D J experiment: Hall, L. D.; Sanders, J. K. M., in preparation.
(54) Hall, L. D.; Sukumar, S. *Carbohydr. Res.* **1979**, 74, C1.

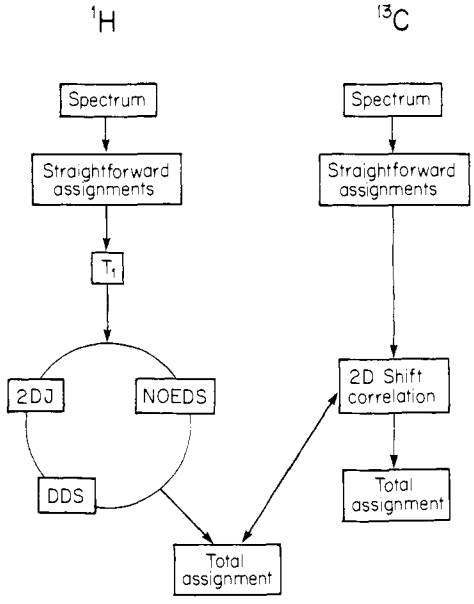

Figure 6. Schematic representation of the proposed strategy.

Both of the above solutions to the dynamic range problem require a flexibility in pulse programming which is not yet generally available in commercial 2D J software, but there are solutions to (ii) and (iii) above which require no sophisticated equipment. f_2' tails arise simply from experimental noise and are suppressed by collecting more transients. The improved signal to noise also allows stronger resolution enhancement in f_1 and f_2, thereby suppressing tails in both dimensions at the same time. Sidebands may be suppressed by continuously varying spin rate, either manually[55] (for very short runs) or by putting a peristaltic pump in the spinner air line.[56] We defer until a later paper detailed discussion of the chemical implications of the coupling constants derived in this work but will comment on one specific finding here. Couplings of ca. 1 Hz between $H_{12\alpha}$ and the 18-methyl protons are well known in 11-keto steroids, and a few similar couplings have been reported between 1α and 19.[57] Our results indicate that these may indeed be entirely general and independent of functional group. It is noteworthy that in **2** $J_{1\alpha,19}$ is ca. 0.4 Hz,[5] and that in neither **1** nor **2** is any other four-bond coupling even hinted at.[58]

Strategic Implications. At the outset of this work it was our intention to see how far one could take the NMR analysis of complex natural products. 1-Dehydrotestosterone was chosen as a simple example of that class and succumbed rather easily to the combined attack we have described here. One-dimensional experiments yielded all assignments and much multiplicity information, but fell short of providing subtle scalar couplings. Two-dimensional experiments gave a formidable array of coupling constants but little guidance as to assignment or chemical implications. In the more challenging problem of assigning the resonances of **2**, which has few resolved signals even at 400 MHz, neither one- nor two-dimensional techniques alone provided the above information, but in combination essentially total analysis was possible. Based on our experience we suggest a general strategy for the complete analysis of complex natural products and this is summarized in Figure 6.[61]

The starting point must be the normal ^1H NMR spectrum measured at the highest possible dispersion, and with as many obvious assignments as possible. The next step is determination of spin–lattice rates, in part as a necessary preliminary to various irradiation and 2D experiments, but also in the hope of separating and characterizing some resonances on the basis of their differential spin–lattice relaxation. The next step is a 2D J spectrum to resolve, count, and analyze as many signals as possible.[59] Finally, continuing cycles of NOE difference and decoupling difference experiments are carried out until assignment is complete. A ^1H–^{13}C 2D shift correlation experiment[3,4] would then give all the ^{13}C assignments also, and in those cases where some parts of the ^{13}C spectrum are more readily assigned than the proton spectrum then the assignment may be made in the reverse order through the correlation experiment.[32] In an extension of this strategy detailed analysis of R_1 values and the kinetics of transient NOEs[60] could lead to determination of the complete solution geometries of steroids and similar molecules.

The above is an idealized strategy dependent on extended access to the most modern currently available equipment, making severe demands on both hardware and software, but it does suggest a variety of useful experiments which can be usefully performed on almost all FT spectrometers at any field strength. In ascending order of difficulty they are (a) measurement of partially relaxed T_1 spectra for the separation of backbone methylene from methine proton resonances; (b) measurement of decoupling difference spectra (such experiments are easily carried out in the sequential mode under manual control); (c) measurement of NOE difference spectra (for many vicinal, and especially geminal, enhancements the sequential mode will be adequate); (d) measurement of 2D J spectra. Although most FT spectrometers have the requisite hardware, in many cases it will be easiest to transfer the raw FIDs to another computer for data processing. As the value of 2D J spectroscopy is severely compromised with tightly coupled spin systems, it is advantageous to work at the highest possible field strength or to use a lanthanide shift reagent (or both!).[53]

Acknowledgments. We are indebted to S. Sukumar and Dr. B. K. Hunter for many valuable and stimulating discussions on the 2D aspects of this work. This work was carried out by J.K.M.S. during the tenure of an MRC (UK) Travelling Fellowship, and was supported by an operating grant to L.D.H. from NSERC of Canada.

(55) Sanders, J. K. M., unpublished experiments.
(56) German Patent Application P.28 16 225.6, April 14, 1978.
(57) Reference 38, pp 115–121.
(58) We find that these very small couplings are most readily revealed in simple spin–echo spectra: Freeman, R.; Hill, H. D. W. In "Dynamic Nuclear Magnetic Resonance Spectroscopy", Jackman, L., Cotton, F. A., Eds.; Academic Press: New York, 1975; pp 131–162.
(59) It will rarely be necessary to acquire or process a whole 2D J spectrum. In our experience only small portions of the spectrum will actually require 2D analysis; the time saving involved is considerable.
(60) Hall, L. D.; Wong, K. F.; Hill, H. D. W. *J. Chem. Soc., Chem. Commun.* **1979**, 951. Wong, K. F. Ph.D. Thesis, University of British Columbia, 1979.
(61) **Note Added in Proof**: We have now successfully used this strategy to analyze completely the proton spectra of vinblastine alkaloids (L.D.H., B. K. Hunter, and J.K.M.S., in preparation) and of a modified enkephalin (M. J. Gidley, L.D.H., J.K.M.S., and M. C. Summers, in preparation).