18-Substituted Steroids, Part 14.[1] The High-field ^1H and ^{13}C Nuclear Magnetic Resonance Spectra of Aldosterone; Full Assignments for the Main Equilibrating Forms in Solution

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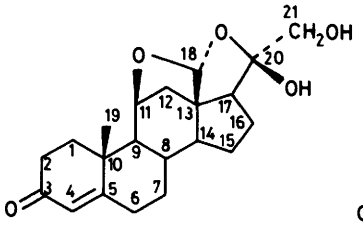
Aldosterone exists in solution predominantly as an equilibrating mixture of the 20-oxo-11,18-hemiacetal (**2**) and the 11,18-epoxy-18,20-hemiacetal (**1**), resulting in distinct but superimposed n.m.r. spectra. Full assignments of the ^1H and ^{13}C spectra have been achieved by carrying out a ^{13}C–^{13}C connectivity experiment (INADEQUATE) to identify the set of ^{13}C signals for each of the two forms, followed by the use of ^1H–^{13}C heteronuclear shift-correlated spectroscopy, aided by a phase-sensitive double quantum filtered COSY and 2D *J*-resolved spectra (^1H), with a few selective nuclear Overhauser difference measurements. This permitted assignments of all the ^1H and ^{13}C chemical shifts and almost all of the two- and three-bond interproton coupling constants for each tautomer.

High-field ^1H n.m.r. spectra have been fully assigned during the last few years for several steroids, including 1,2-didehydrotestosterone,[2] 11β-hydroxyprogesterone,[3] 17α-acetoxy-6α-methylprogesterone,[4] progesterone,[5] 17α-ethynyl-17β-hydroxyoestr-4-en-3-one (19-norethisterone)[6] and its 5α- and 5β-dihydro derivatives,[6] 17α-ethynylestradiol 3-methyl ether,[6] and the most common bile acids.[7]

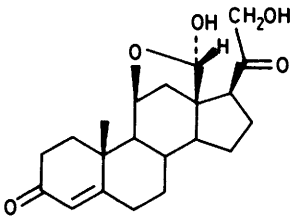
These inherently difficult assignments, for spectra which have many overlapping features even at high fields, have become possible through the development of complex multi-pulse and double-irradiation experiments, with computer analysis of data. Two-dimensional *J*-resolved (2D-*J*) spectra, decoupling-difference, and nuclear Overhauser difference (NOEDS) methods were used in the earlier of the analyses;[2–4] more recently 2D spin-echo-correlated spectroscopy (SECSY)[6,8] and ^1H–^{13}C heteronuclear shift-correlated spectroscopy[5–9] have been shown to offer advantages in some cases. Nevertheless such studies continue to be demanding on instrument time and interpretive skill. The diversity of structures for which complete assignments have been achieved is not yet sufficient to permit reliable conclusions to be reached for other steroids by inspection and comparison of spectra with published data. No extensive correlation of ^1H spectral features yet exists, in contrast to the case for ^{13}C n.m.r. spectra.[10]

We needed a full interpretation of the ^1H spectrum of aldosterone as the basis for structural studies on a series of unidentified polar metabolites.[11,12] The spectral analysis is no trivial problem because aldosterone, unlike the steroids already mentioned, exists in solution as an equilibrating mixture of structural isomers. The two principal forms are the 18,20-hemiacetal (**1**), which corresponds to the structure of aldosterone hemihydrate in the crystal,[13] and the 20-oxo form (**2**). The precise ratio of (**1**) and (**2**) is solvent-dependent, but approximates to 4:3. Equilibration is slow on the n.m.r. time-scale, resulting in distinct but superimposed spectra of the two forms, but is too rapid to permit their chromatographic separation and individual study. High-field n.m.r. spectra of aldosterone and some of its derivatives exhibit small additional peaks due to minor contributing structures, believed to be the C-20 and C-18 epimers of (**1**) and (**2**), respectively.[1,14]

Our first attempts to obtain a ^1H spectral assignment were based upon conversion of aldosterone into derivatives in which structures of types (**1**) and (**2**) were effectively locked, namely, the 20,21-phenylboronate for the 18,20-hemiacetal (**1**), and



(**1**)



(**2**)

the 18,21-diacetate for the 20-oxo form (**2**). We then hoped to use the assignments for these derivatives, and especially the distinctive multiplet profiles for individual proton signals, as a way of recognising signals from the corresponding protons in aldosterone itself, albeit expected at appreciably different chemical shifts for those protons effected by derivative formation. However, we abandoned this indirect approach in favour of one based upon determining ^{13}C–^{13}C connectivities (INADEQUATE)[15–22] within each of the distinct structural forms of free aldosterone, followed by the use of ^1H–^{13}C heteronuclear shift-correlated spectroscopy to locate signals from the proton or protons attached to each carbon atom for both isomers. With the aid of ^1H 2D *J*-resolved spectra in two solvents, a phase-sensitive double quantum filtered ^1H COSY spectrum,[23] and a small number of NOEDS experiments to confirm particular features, we were then able to complete the assignments of chemical shifts and determine almost all the geminal and vicinal ^1H–^1H coupling constants for both the major isomers of aldosterone in solution.

Genard[24] has reported an analysis of the ^{13}C spectrum of aldosterone, but at 22.63 MHz and without the benefits of INADEQUATE and other 2D methods. The broad-band proton-decoupled spectrum (in $CDCl_3$) was reported to show only 32 lines (*cf.* later), and some of the resulting assignments, notably for carbon atoms 8, 9, 15, and 17, differ from our results (in CD_3OD) to an extent which cannot be ascribed solely to the change of solvent.

Results and Discussion

The 100 MHz 1D broad-band-decoupled ^{13}C n.m.r. spectrum of aldosterone (Figure 1) showed a total of 40 lines. As the

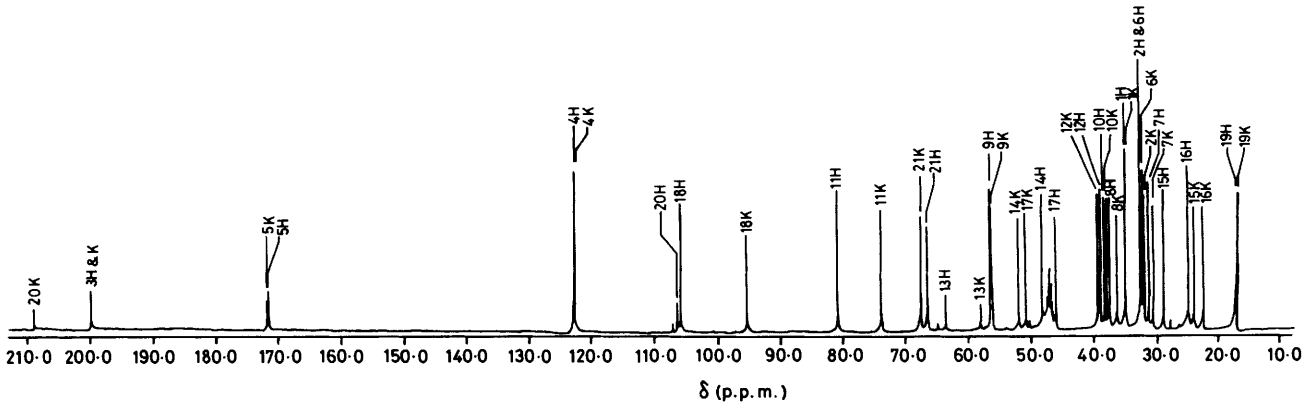


Figure 1. 100 MHz Broad-band-decoupled ^{13}C n.m.r. spectrum of aldosterone (in CD_3OD): H, peaks due to the 18,20-hemiacetal form (**1**); K, peaks due to the 20-oxo form (**2**)

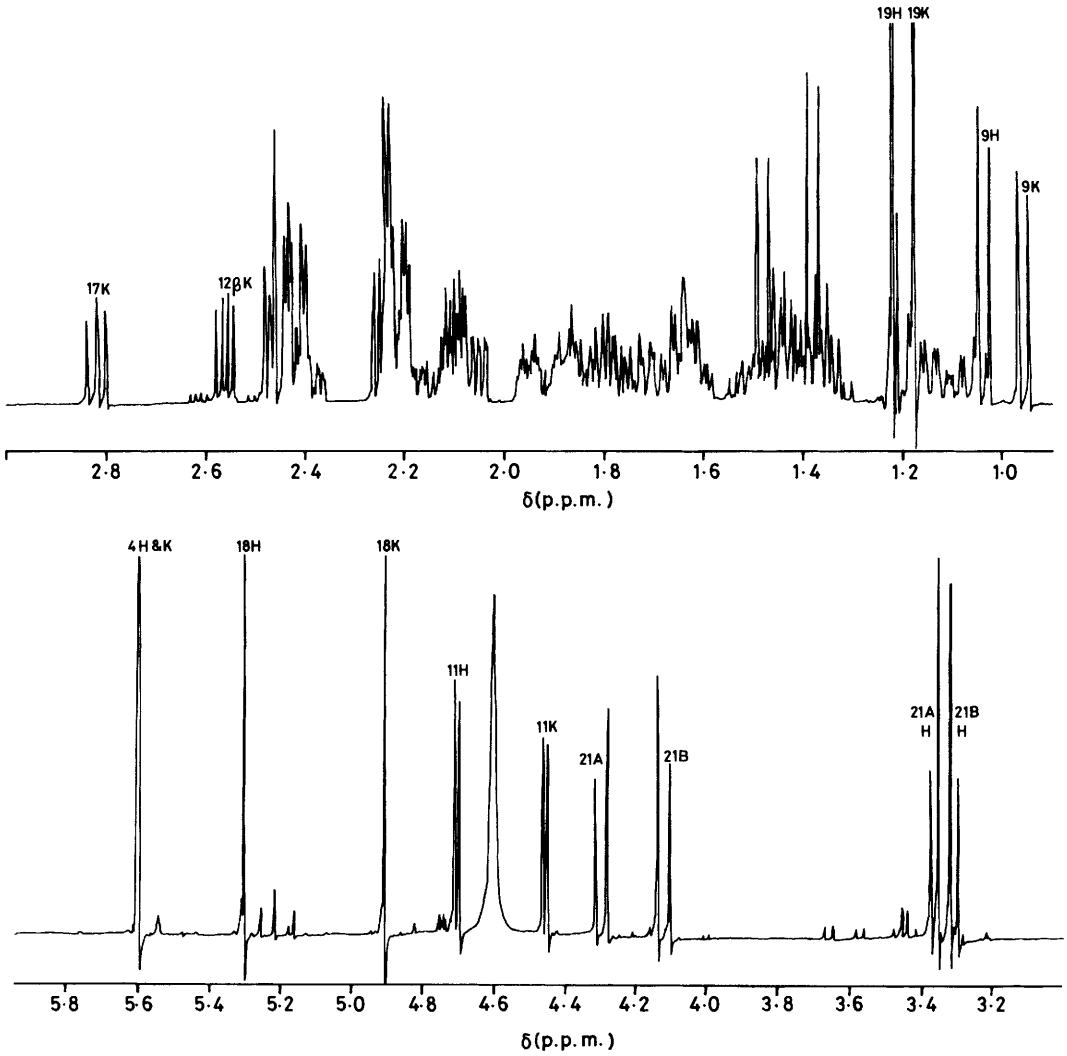


Figure 2. Resolution-enhanced 400 MHz ^1H n.m.r. spectrum of aldosterone (in CD_3OD) (GB = 0.3, LB-2)

molecule contains 21 carbon atoms, this implies that almost all the lines for the two isomers of aldosterone had been resolved at 100 MHz. The lines from the minor C-18 and C-20 epimers were relatively very weak and were not considered.

The 400 MHz ^1H n.m.r. spectrum of aldosterone (Figure 2) showed a number of well resolved and separated signals above δ 2.5. However, below δ 2.5 the spectrum consisted of a large number of overlapping multiplets leading to a number of small envelopes which made it difficult to make assignments in this region. Even the 2D *J*-resolved spectrum did not entirely separate all the signals for the protons of the two isomers.

As the ^{13}C spectrum was well resolved, it was decided to

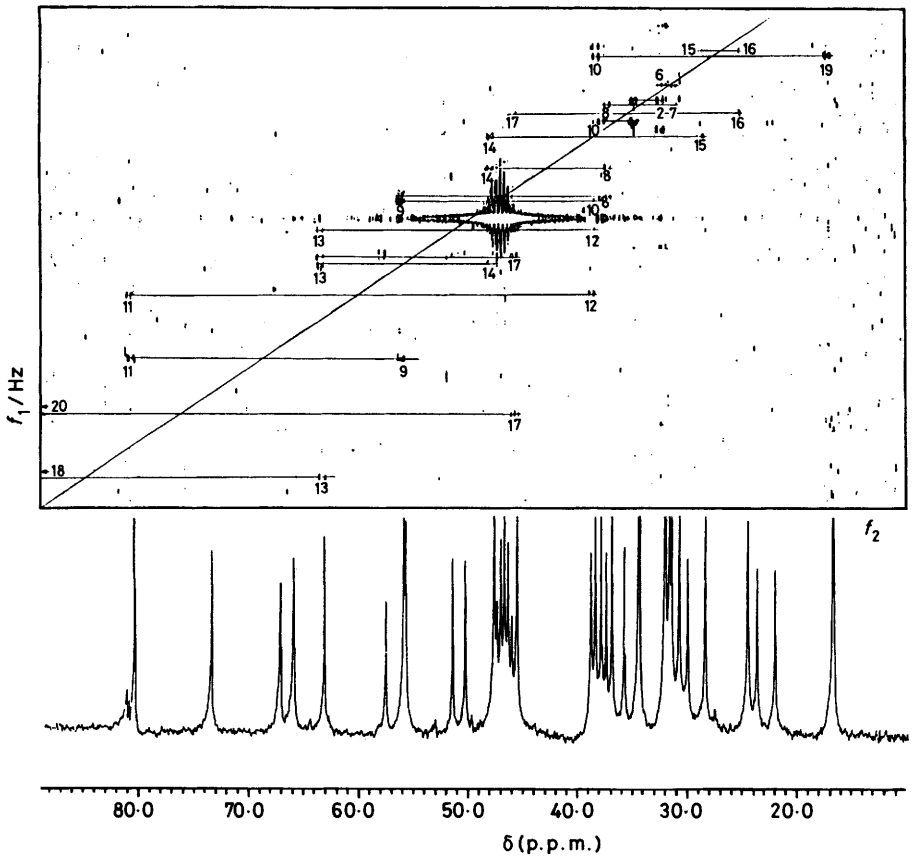


Figure 3. Two-dimensional 62.5 MHz INADEQUATE ^{13}C n.m.r. spectrum of aldosterone for the chemical shift region δ 10—90. The f_2 dimension corresponds to the conventional ^{13}C chemical-shift axis except that the strong lines from isolated nuclei (one-dimensional spectrum included below) are suppressed, leaving only the weak satellite signals in the form of four line AX- or AB-type spectra. [The connectivities for the carbons in the 18,20-hemiacetal form (**1**) are shown.] The f_1 dimension separates these spectra according to their individual double quantum frequencies (the sum of the chemical shifts relative to the r.f. pulse frequency). The signals are numbered according to the standard steroid numbering system

assign this first and then use a heteronuclear correlation experiment to identify the protons attached to each of the carbon atoms. One of the easiest methods available for the assignment of carbon spectra is the 2D (INADEQUATE) experiment.[15-22] This method is not often used as it requires a large quantity of material (*ca.* 400 mg for a C_{21} compound) if the time taken for data accumulation is not to be prohibitively long. In the case of aldosterone the quantity required was doubled as it is in effect the simultaneous study of the connectivity of 42 carbon atoms spread over two molecules. A solution was made by dissolving 1.1 g of aldosterone in 2 ml of CD_3OD, which gave an approximately 0.8M-solution for each of the isomers. This solution was used for the measurement of the 1D ^{13}C n.m.r. spectrum, the 2D (INADEQUATE) experiment, and the first heteronuclear correlation experiment.

Two separate 2D (INADEQUATE) experiments were carried out (both at 62.5 MHz), using different sweep widths. The first was done on the region between 10 and 90 p.p.m.; the full contour plot is given in Figure 3. The second experiment used the region between 10 and 126 p.p.m.; part of the contour plot (with increased vertical scale to reveal weaker signals) is given in Figure 4. From the contour plot in Figure 3 it was possible to trace out the strongest signals from the up-field carbon atoms that constitute the backbone of the predominant hemiacetal form (**1**). The remainder were identified by their characteristic chemical shifts[10] and intensities in the 1D spectrum.

By increasing the vertical scale of the contour plots (*e.g.* Figure 4), it became possible to identify the correlations belonging to carbon atoms that form the backbone of the lesser component, the 20-oxo isomer (**2**). The fact that correlations appeared as a pair of doublets equidistant from the diagonal $f_1/f_2 = 2$ made it possible to differentiate between genuine correlation signals and the rather high level of 'noise'. For both experiments a delay of 6 ms was used, which corresponds to a value of 41.7 Hz for the one-bond carbon–carbon coupling constant, and which gives maximum signal intensity.[17] The chemical shifts of the carbon atoms for the hemiacetal and oxo forms are given in Table 1.

Using the heteronuclear correlation experiment it then became possible to assign many of the ^1H resonances to individual protons in one or other of the two isomers. However, a number of the protons had very close or virtually identical chemical shifts, and even the 2D *J*-resolved spectrum of aldosterone in CD_3OD did not resolve many of the closely separated multiplets. This also made it difficult to obtain all the coupling constants for rings *A*, *B*, and *C* for both the hemiacetal and oxo forms. Ring *D* showed second-order characteristics in the form of the ^1H multiplets, and was not analysed. Some of the experiments were therefore rerun using a solution of aldosterone in pentadeuteriopyridine (C_5D_5N) in the hope that some of the overlapped resonances would shift sufficiently to allow the interpretation of their multiplet structure in a 2D *J*-resolved experiment. The ^{13}C spectrum in C_5D_5N was almost identical, both in terms of shifts and relative intensities, with that obtained for the CD_3OD solution. Therefore the carbon assignments that were made for the CD_3OD solution (determined by the two

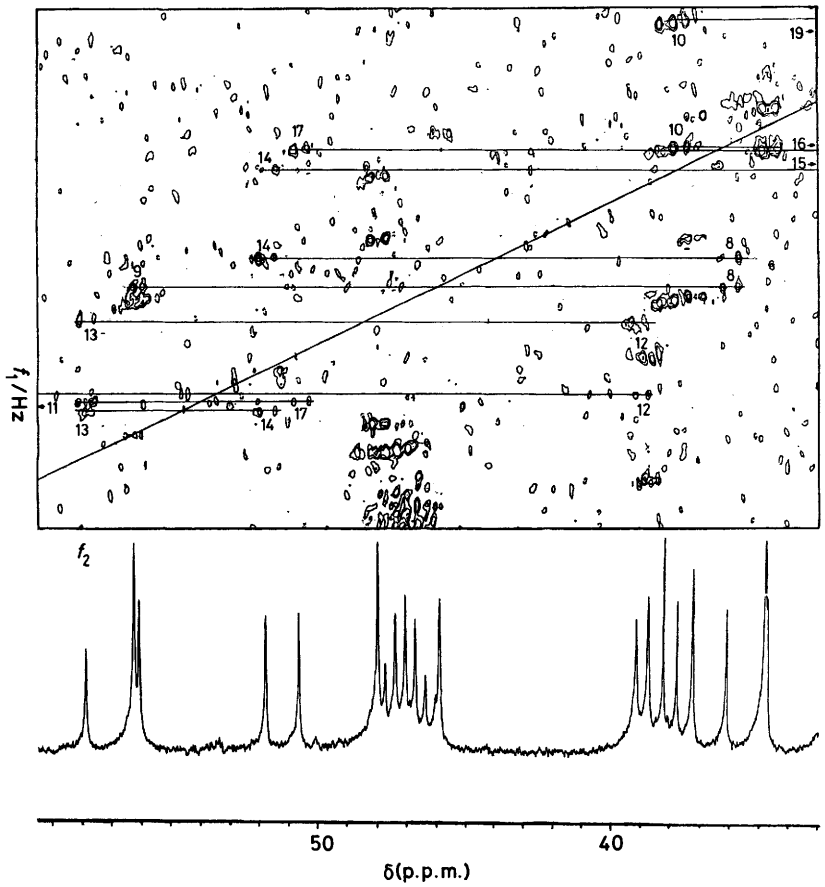


Figure 4. Part of the two-dimensional 62.5 MHz INADEQUATE ^{13}C n.m.r. spectrum of aldosterone for the chemical-shift range δ 10—126. A higher vertical scale was used on the plot than for Figure 3. Only the correlations for the carbon atoms in the 20-oxo form (**2**) are shown

Table 1. ^{13}C Chemical shifts for aldosterone (solvent CD_3OD; δ in p.p.m. relative to Me_4Si as internal standard)

Carbon no.	18,20 Hemiacetal (1)	20-Oxo (2)	Carbon no.	18,20 Hemiacetal (1)	20-Oxo (2)
1	34.92	34.85	12	38.88	39.27
2	31.85	32.46	13	63.52	58.01
3	199.80	199.82	14	48.08	51.89
4	122.55	122.63	15	28.77	24.10
5	171.80	172.00	16	24.90	22.46
6	32.46	32.06	17	46.05	50.69
7	31.22	30.44	18	105.84	95.30
8	37.39	36.23	19	17.14	17.06
9	56.34	56.16	20	206.34	209.01
10	38.40	37.81	21	66.42	67.50
11	80.77	73.77			

INADEQUATE experiments) were used for the interpretation of experiments carried out on the C_5D_5N solution.

The heteronuclear correlation experiment was repeated on the C_5D_5N solution, thereby giving the shifts of all the protons in both hemiacetal and oxo forms. A 2D *J*-resolved spectrum was acquired on the sample, and the proton assignments obtained from the heteronuclear correlation were used as an aid to its interpretation. This showed that many of the resonances that had been overlapped in the first 2D *J*-resolved spectrum were sufficiently resolved to allow analysis of their multiplet structures. This made it possible to obtain all the geminal and vicinal ^1H–^1H coupling constants for rings *A*, *B*, and *C*.

Table 2. ^1H Chemical shifts for aldosterone (δ in p.p.m. relative to Me_4Si as internal standard)

	Solvent: CD_3OD		Solvent: C_5D_5N	
Proton	18,20-Hemiacetal (1)	20-Oxo (2)	18,20-Hemiacetal (1)	20-Oxo (2)
1α	1.64	1.62	1.43	1.45
1β	2.12	2.10	1.95	1.92
2α	2.26	2.26	2.25	2.22
2β	2.45	2.45	2.37	2.39
4	5.61	5.61	5.75	5.76
6α	2.26	2.26	2.10	2.10
6β	2.45	2.45	2.27	2.29
7α	1.16	1.08	1.05	0.97
7β	1.96	1.88	1.77	1.68
8β	1.72	1.65	1.68	1.56
9α	1.05	0.97	0.90	0.84
11α	4.71	4.46	4.72	4.52
12α	1.39	1.50	1.40	1.51
12β	2.26	2.57	2.34	2.61
14	1.44	1.46	1.38	1.38
15α	1.35	1.39	1.54	1.31
15β	1.81	1.86	1.82	1.69
16α	1.51	1.77	1.62	1.84
16β	2.06	2.22	2.49	2.45
17α	2.49	2.83	2.91	2.98
18	5.30	4.90	5.72	5.26
19	1.23	1.19	1.19	1.17
21-H_2	3.30, 3.37dd	4.14, 4.32dd	3.84, 3.95dd	4.56, 4.82dd

Table 3. ^1H–^1H Coupling constants for aldosterone

	^2J	$^3J_{ax-ax}$	$^3J_{ax-eq}$	$^3J_{eq-eq}$
1α,1β	13.0			
1α,2α			5.0	
1α,2β		14.0		
1β,2α				3.8
1β,2β			5.4	
2α,2β	16.9			
6α,6β	15.0			
6α,7α			4.5	
6α,7β				3.4
6β,7α		13.2		
6β,7β			5.4	
7α,7β	13.2			
7α,8		11.9		
7β,8			3.4	
8,9		11.9		
8,14		8.4		
9,11α			~0	
11α,12α			~0	
11α,12β				6.2
12α,12β	11.2			

Chemical shifts for the protons of the two main forms of aldosterone are listed in Table 2, and those couplings that were measured are given in Table 3.

As a confirmation of the proton assignments a COSY spectrum was run on the CD_3OD solution. Owing to the large number of protons present in the spectrum it was decided to use the phase-sensitive method, with the pulse sequence devised by Wuthrich and his co-workers[23] for use on peptides, where it was also necessary to separate correlations for very large numbers of protons. In order to maximise the resolution this experiment was carried out on the restricted range δ 0.8—2.9, where most of the signals occur. The values used for the transmitter offset frequency and sweep width were chosen to minimise the effects of folding artefacts. A digital resolution of 1 Hz per point was obtained for f_1 and f_2. This was sufficient to enable most of the ^1H–^1H couplings to be observed. The majority of the signals downfield from δ 2.9 did not have any correlations to peaks in the region chosen, so very little information was lost.

As stated earlier, the coupling constants for ring *D* for (**1**) and (**2**) could not be derived from the 2D *J* spectrum. This meant that the conformation of ring *D* could not be inferred from the coupling constants. Instead the conformation was obtained with the aid of the COSY spectrum. First it was necessary to identify which of the protons at positions 15 and 16 in (**1**) and (**2**) were in the β-configuration. This was achieved by carrying out an NOEDS experiment on the CD_3OD solution, irradiating at frequencies corresponding to H-18 at δ 4.90 and 5.30 for (**1**) and (**2**), respectively. When the frequency of H-18 in (**1**) was irradiated a positive nuclear Overhauser enhancement (nOe) was observed for the protons resonating at δ 1.81 and 2.06, amongst others. These protons were identified from the heteronuclear correlation as being attached to C-15 and C-16, respectively, and were therefore the 15β- and 16β-protons. No nOe was observed for the α-protons attached to positions 15 and 16. When the frequency of H-18 in (**2**) was irradiated a positive nOe was observed for the proton signals at δ 1.86 and 2.22, similarly identified as being due to the 15β- and 16β-protons in the oxo form. For both isomers, the resonances of the β-protons at positions C-15 and C-16 were further downfield than for the corresponding α-protons. This was to be expected as the β-face of the molecule around ring *D* is exposed to the deshielding effects of several oxygen atoms. From the COSY spectrum it was noted that there was little if any coupling (probably < 1 Hz) between 16β- and 15β-protons in the hemiacetal form (**1**), or between 16β- and 15α-protons in the 20-oxo form (**2**), implying torsion angles close to 90° in slightly different conformations of ring *D* for the two tautomers in solution.

The final problem to be resolved by the NOEDS method concerned the respective assignments for protons at C-2 and C-6, signals for which clustered and/or overlapped in the region δ 2.25—2.5. The 2β- and 6β-proton signals were recognised by their appearance in a NOEDS experiment involving *irradiation* at the frequency of the C-19 protons, which are close in space to 2β-H and 6β-H. The 6β-proton signal was further distinguished by its allylic coupling[4] to 4-H. Similarly, NOEDS with irradiation at the frequency of 4-H revealed the signal from the neighbouring 6α-H, and the 2α-H signal was recognised from its small long-range W coupling to 4-H.[4] The conclusions reached from these experiments are fully compatible with indications of interproton couplings from the COSY experiment, which on its own could not be used for unambiguous assignments of signals from the various protons at C-2 and C-6.

In conclusion, we draw attention to certain features of our ^1H assignments which are of particular value as starting points for the study of spectra of other compounds in the aldosterone series, and especially for the stepwise analysis of COSY spectra. The composition of the mixture of the predominant hemiacetal and 20-oxo forms is readily ascertained by inspection and integration of the 18-H singlets, the 11-H_2 doublets, and the 21-H_2 double doublets, with characteristic relative chemical shifts as shown clearly in Figure 2. The 17α-H signal, especially the triplet (or double-doublet) at δ *ca.* 2.8 for the 20-oxo form (δ values quoted for CD_3OD solvent), is well separated from all other signals, and its correlation peaks with 16α-H and 16β-H in the COSY spectra make location of the latter signals straightforward. The 9α-H signals are prominent doublets at exceptionally high field (δ *ca.* 1.0), showing couplings only to 8β-H, the signal of which is also easily located from the COSY spectrum. The 9α-H,C(9),C(11),11α-H torsion angle is close to 90°, causing *ca.* zero 9α-H,11α-H coupling; the protons of ring *C* (11α, 12α, 12β) therefore constitute an effectively isolated three-spin system which is easily traced out from the COSY spectrum. As a conspicuous part of this system, the signal due to 12β-H in the 20-oxo form is clearly seen as a double doublet at δ *ca.* 2.6, whereas that due to 12α-H in each form appears as a clear doublet (coupled only to 12β-H) in the region δ *ca.* 1.4—1.5.

Experimental

Carbon–Carbon Correlation (*at* 62.5 MHz).—For the ^{13}C 2D INADEQUATE experiment the following parameters were used. The pulse sequence was that of Mareci and Freeman;[20] an extended Ernst-type phase cycle was employed to suppress single quantum peaks. A value of 6 ms was used for τ [$\tau = (2n + 1)/4J_{CC}$, $n = 0$]; 512 and 4 096 data points for a sweep width of 7 352 Hz for both f_1 and f_2 gave 14.4 and 1.8 Hz per data point, respectively. For each of the 256 values of t_1 128 scans were accumulated. A sine-squared bell shifted by π/2 was used as the window function in both domains. Data accumulation took 43 h and the transformation took 3 min using an Aspect 3000 computer fitted with an array processor.

Carbon–Proton Correlation [*at* 100 MHz *for* ^{13}C *and* 400 MHz *for* ^1H).—The pulse sequence of Bax and Morris[25] was used. A value of 144 Hz was used for $J_{C,H}$ to calculate the values for the delays in the pulse sequence; 256 and 2 048 data points for sweep widths of 1 072 for the f_1 and 10 638 Hz for the f_2 dimension gave 4.2 and 5.2 Hz per data point, respectively. For each of the 128 values of t_1 64 scans were accumulated. A sine-squared bell shifted by π/2 was used as the window function in

both domains. Data accumulation took 18 h and transformation 50 min.

2D-J *Resolved* (^{1}H-*Homonuclear*) *Spectra* (*at* 400 MHz).—For each of the 32 values of t_1 8 scans were accumulated; 64 and 1 024 data points were used for sweep widths of 71 Hz for the f_1 and 2 272 Hz for the f_2 dimension, which gave 1.1 and 2.2 Hz per data point, respectively. An unshifted sine-squared bell was used as the window function in both domains. Data accumulation took 20 min and transformation 15 min.

Phase-sensitive Double Quantum Filtered COSY Spectra (*at* 500 MHz).—The pulse sequence of Marion and Wuthrich[23] was used with data acquisition for phase-sensitive mode with DQF with pure absorption/dispersion lineshapes without phase twist. Quadrature detection was used in f_1, with time-proportional phase increments. For each of the 1 024 values of t_1 16 scans were accumulated; 2 048 data points were used for a sweep width of 1 050 Hz for f_1 and f_2, which gave 1.03 Hz per data point for each domain. A sine-squared bell, shifted by $\pi/8$ in both domains, was used as the window function. Data accumulation took 10 h and transformation 1.5 h.

Acknowledgements

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