Ryanoid Insecticides: Structural Examination by Fully Coupled Two-dimensional ^1H–^{13}C Shift Correlation Nuclear Magnetic Resonance Spectroscopy

Andrew L. Waterhouse,* Ian Holden,* and John E. Casida
Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, California 94720, U.S.A.

The two-dimensional pulse sequence, (π/2, ^1H)–Δt_1–(π/2, ^1H; π/2, ^{13}C)–acquire, for 'fully coupled' ^1H–^{13}C shift correlation n.m.r. has been applied to structural analysis of ryanodine (**1**) and 9,21-didehydroryanodine (**2**) (a new botanical insecticide from *Ryania speciosa*). This procedure allowed total assignment of the ^{13}C n.m.r. spectra using one-, two-, and three-bond ^1H–^{13}C couplings, stereochemical assignments of protons, and some conformational data *via* relative $^3J_{CH}$ couplings. The structure (**2**) defined by n.m.r. was confirmed by reductive conversion into ryanodine (**1**) and 9-*epi*-ryanodine (**3**).

The ground stemwood of the plant *Ryania speciosa* (marketed as ryania powder) has been used for more than 30 years to control agricultural and garden pests.[1] An active constituent was isolated and identified as the alkaloid ryanodine (**1**).[1] The structure (**1**) was defined by Wiesner's group[2] in 1966 using classical methods and later confirmed by *X*-ray crystallography;[3] the hydrolysis product, ryanodol, has also been the subject of a total synthesis.[4] The high toxicity of (**1**)[1] is due to an action on the transverse tubular system in muscles.[5,6] Ryanodine is a potential key to unravelling the molecular mechanism of this poorly understood organelle involved in transduction of information from the nerve termini to the muscle cell sarcoplasmic reticula. Unfortunately, the currently available purified ryanodine contains a major contaminant,[7] structurally related to (**1**).[8] Subsequent investigation of fresh ryania powder revealed that the contaminant (**2**) was three times as abundant as (**1**).[8]

In order to determine the structure of the new 'ryanoid' by n.m.r., assignments for (**1**) were sought, but only partial low-field ^1H data were available.[9] Our first attempts to assign the ^1H and ^{13}C n.m.r. spectra of (**1**) and (**2**) using standard one- and two-dimensional methods were hampered by overlapping multiplets and the multitude of quaternary carbons. We therefore re-examined the neglected 'fully coupled' ^1H–^{13}C correlation method and found it to be a superior technique in unambiguously identifying (**2**) as 9,21-didehydroryanodine.

Several two-dimensional (2D) n.m.r. methods (Figure 1) were utilized for spectral assignment of (**1**). A homonuclear COSY spectrum[10] revealed the ^1H–^1H interactions, and a conventional ^1H–^{13}C correlation spectrum[11,12] established the identity of carbon atoms directly attached to known protons. These methods, however, left the eight quaternary carbons unassigned and some ambiguities in the proton spectrum. The connectivity of the quaternary carbons could be determined in principle by observing either the ^{13}C–^{13}C couplings or long-range ^1H–^{13}C couplings. In the former case the modified one-dimensional DANTE[13] sequence is tedious, and the 2D INADEQUATE[14,15] method was impractical because of the prohibitively large sample required. The recently developed relay heteronuclear correlation experiment[16–20] provides one method for observing long-range ^1H–^{13}C coupling, but unfortunately it is inapplicable to quaternary carbon atoms. Although an adapted conventional correlation method determines, in an additional experiment, the long-range ^1H–^{13}C interactions,[21] the fully coupled ^1H–^{13}C correlation method[22–24] described here simultaneously reveals this long-range coupling, plus one-bond coupling with coupling constant and carbon multiplicity.

The fully coupled ^1H–^{13}C 2D correlation method using the original three-pulse sequence, (π/2, ^1H)–Δt_1–(π/2, ^1H; π/2, ^{13}C)–acquire, with recent modification for quadrature detection,[25,26] is uniquely powerful in directly detecting one-, two-, and three-bond ^1H–^{13}C coupling. It reveals not only carbon–carbon connectivity, but some relative stereochemical information as well. The method is now much more useful than when originally described, for two reasons. First, quadrature detection eliminates the unmodulated high-intensity ^{13}C spectrum at the ^1H transmitter frequency,[12] so the full dynamic range of the instrument can be utilized in detecting the sometimes weak cross-peaks, greatly increasing sensitivity. Secondly, the field strength of current high-field n.m.r. magnets reduces the relative size of the complex primary coupling arrays (up to 350 Hz wide) to a reasonable portion of the spectrum. The fully coupled correlation method simultaneously detects all the ^1H–^{13}C

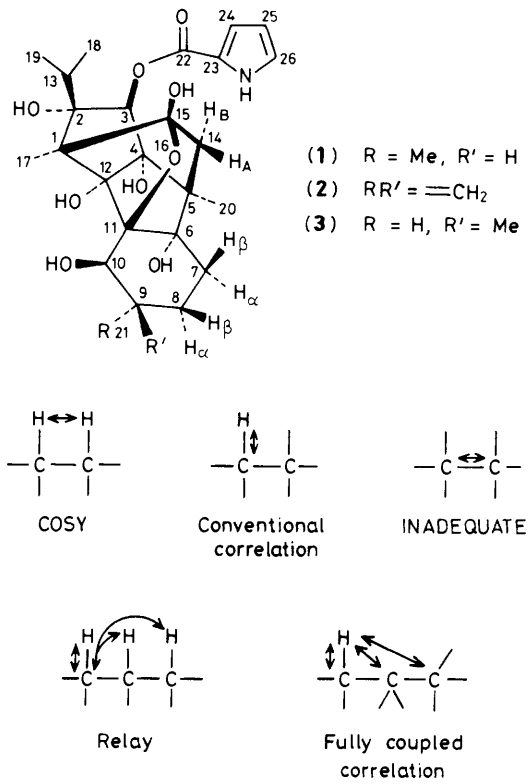

Figure 1. ^1H–^1H and ^1H–^{13}C interactions important in 2D shift correlation spectroscopy

Table 1. Complete ^1H and ^{13}C n.m.r. assignments (δ; *J*/Hz) with two-dimensional data (COSY and fully coupled) for ryanodine (**1**) and 9,21-didehydroryanodine (**2**)

	Ryanodine (**1**)				Didehydroryanodine (**2**)			
	^1H shift and COSY ^1H cross-peaks		^{13}C shift with associated fully coupled ^1H cross-peaks		^1H shift and COSY ^1H cross-peaks		^{13}C shift with associated fully coupled ^1H cross-peaks	
Position	δ(^1H)	Cross-peaks	δ(^{13}C)	Cross-peaks[a]	δ(^1H)	Cross-peaks	δ(^{13}C)	Cross-peaks[a]
1			65.7	2.51, 2.19			65.6	6.71, 2.43, 2.17
2			83.6	6.69, 2.19, 1.55, 1.15			83.4	6.71, 2.17, 1.55, 1.16
3	6.69(s)		90.9	6.69(dd)	6.71(s)		90.7	6.71(dd)
4			92.1	6.69, 2.51, 1.38			91.9	6.71, 2.43, 1.40
5			49.5	6.69, 3.25, 2.51, 1.38			48.8	6.71, 3.24, 2.43, 1.40
6			85.5	3.25, 1.62, 1.38			85.3	3.24, 2.45, 1.54, 1.40
7α	1.47(m)	2.32, 1.90	26.9	2.32, 1.47(td)	1.54(m)	2.98, 2.45, 2.37	27.5	2.37, 1.54(td)
7β	2.32(m)	1.90, 1.62, 1.47			2.37(dd) (*J* 5, 12)	2.98, 2.45, 1.54		
8α	1.90(m)	2.40, 2.32, 1.62, 1.47	29.1	1.90, 1.62(td); 1.29(ts)	2.98(m)	5.73, 5.23, 2.45, 2.37, 1.54	29.6	2.98, 2.45(td); 5.73, 5.23(ts)
8β	1.62(m)	2.32, 1.90			2.45(m)	2.98, 2.37, 1.54		
9	2.40(m)	4.55, 1.90, 1.29	35.2	2.40(dd); 1.47, 1.29(ds)			149.5	5.73, 5.60, 2.98, 2.45, 1.54
10	4.55(d) (*J* 10)	2.40	72.5	4.55(dd); 1.62, 1.29(ds)	5.60(s)	5.73, 5.23	69.6	5.60(dd); 5.73, 5.23, 2.45(ds)
11			87.5	1.47			88.5	5.60, 1.54
12			97.0	2.19			96.7	2.17
13	2.84(sept) (*J* 6.5)	1.55, 1.15	30.5	2.84(dd); 1.55, 1.15(ds)	2.84(m)	1.55, 1.16	30.3	2.84(dd); 1.55, 1.16(ds)
14A	2.51(d) (*J* 14)	3.25	42.4	3.25, 2.51(td); 1.38(ts)	2.43(d) (*J* 14)	3.24	42.3	3.24, 2.43(td); 1.40(ts)
14B	3.25(d) (*J* 14)	2.51			3.24(d) (*J* 14)	2.43		
15			102.7	2.51, 2.19			102.5	3.24, 2.43, 2.17
17	2.19(s)		10.9	2.19(qd)	2.17(s)		10.7	2.17(qd)
18	1.15(d) (*J* 6.5)	2.84	19.3	1.15(qd), 1.55(qs)	1.16(d) (*J* 6.5)	2.84	18.9	1.16(qd), 1.55(qs)
19	1.55(d)[b] (*J* 6.5)	2.84	19.8[b]	1.55(qd), 1.15(qs)	1.55(d)[b] (*J* 6.5)	2.84	19.6[b]	1.55(qd), 1.16(qs)
20	1.38(s)		13.1	1.38(qd)	1.40(s)		13.0	1.40(qd)
21	1.29(d) (*J* 6)	2.40	19.1[b]	1.29(qd)	5.23(d) (*J* 2)	5.73, 5.60, 2.98	107.5	5.73, 5.23(td); 2.45(ts); 5.60
					5.73(d) (*J* 2)	5.23, 5.60, 2.98		
22			161.1	6.69			160.9	7.39, 6.71
23			123.0	7.38[c]			123.0	7.39[c]
24	7.29(br s)	6.50, 7.38	116.1	7.29(dd), 7.38(ds)	7.30(dd) (*J* 2, 4)	6.51, 7.39	116.1	7.30(dd); 7.39, 6.51(ds)
25	6.50(br s)	7.29, 7.38	110.5	6.50(dd), 7.38(ds)	6.51(dd) (*J* 3, 4)	7.30, 7.39	110.4	6.51(dd); 7.39, 7.30(ds)
26	7.38(br s)	6.50, 7.29	124.9	7.38(dd); 7.29, 6.50(ds)	7.39(dd) (*J* 2, 3)	7.30, 6.51	124.8	7.39(dd); 7.30, 6.51(ds)

[a] Multiplicities are given when $J > 100$ Hz, designating ^{13}C first and ^1H second, but not when a singlet in both dimensions. $^1J_{CH}$ values are the same for (**1**) and (**2**) as follows: 127 Hz for 7α, 7β, 8α, 8β, 14A, 14B, 17—20; 150 Hz for 3, 10; 173 Hz for 24, 25; 187 Hz for 26. In (**1**) only, 125 Hz for 9, 21. In (**2**) only, 150 Hz for 21. [b] Not distinguishable relative to 18. [c] Additional peaks may be obscured.

couplings because none is eliminated by refocusing pulses or broad-band decoupling. In addition, no delays are required to observe couplings of a particular magnitude, thereby allowing all couplings to be seen (from 180 Hz to an estimated 4 Hz) and eliminating the possibility of instrumental timing imperfections encountered in highly complex pulse sequences.

The fully coupled 2D method is used to advantage in the analysis of structures (**1**) and (**2**), allowing the total assignments of the ^{13}C spectra of both compounds and the unambiguous structural determination of (**2**).

Results and Discussion

Analysis of Ryanodine (**1**).—*Conventional n.m.r. methods.* The 300 MHz n.m.r. spectrum is suitable to assign the pyrrole protons (H-24, H-25, and H-26) to the 12-line AMX system at low field, H-3 to the singlet at δ 6.69, H-10 to the doublet at δ 4.55, and the methylene protons at C-14 to the doublets at δ 2.51 and 3.25. The methyl singlets for positions 17 and 20 are not differentiated, nor are the methyl doublets for 18, 19, and 21. The remaining signals are not directly assignable. Using a 2D ^1H homonuclear COSY experiment[10] (Figure 1), the methine protons at positions 9 and 13 are readily assigned by their

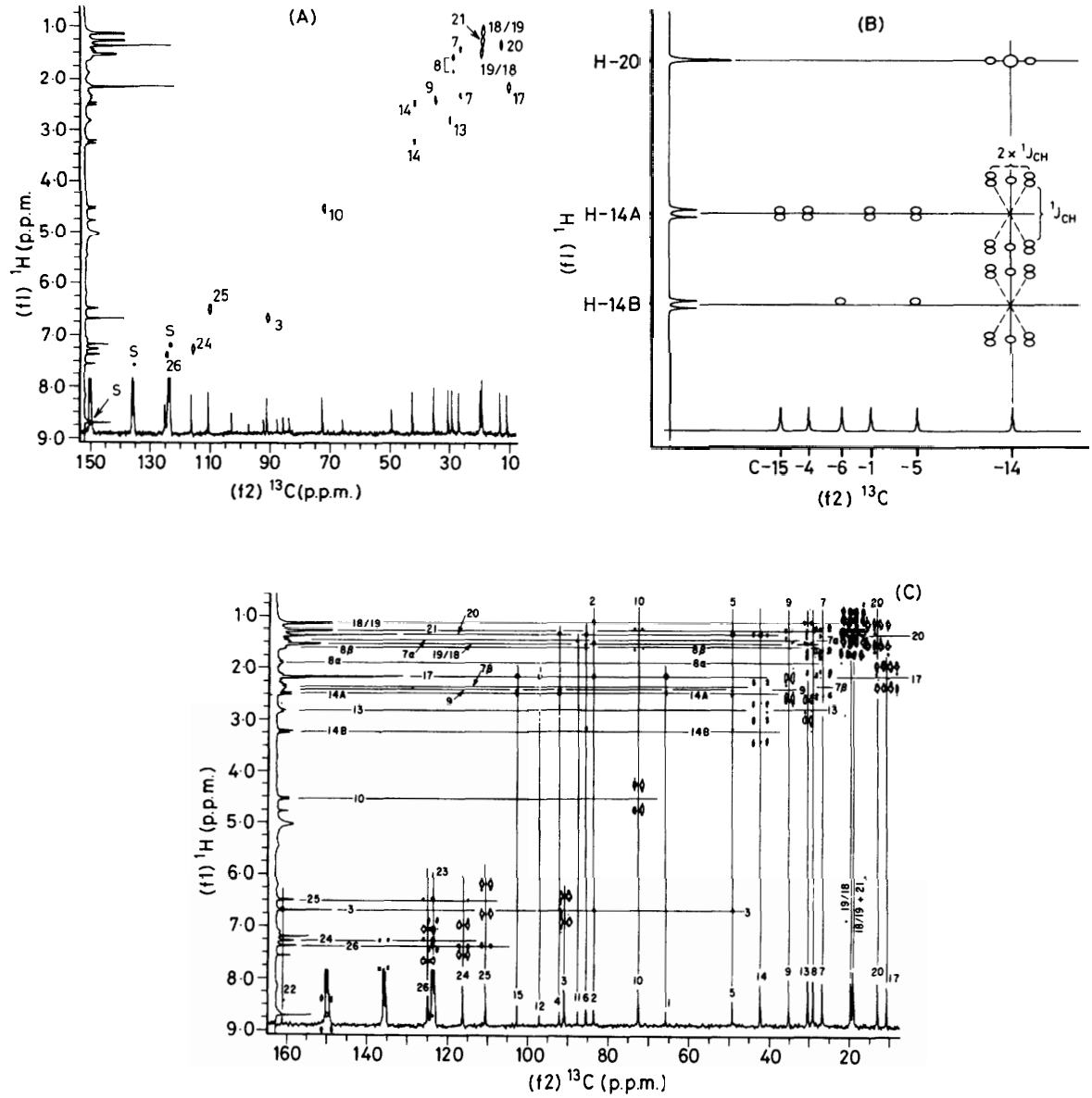

Figure 2. 2D shift correlation spectra for ryanodine (**1**): (A) conventional correlation spectrum, (B) a partial schematic of the fully coupled spectrum, (C) the fully coupled spectrum

coupling to the now easily distinguishable single methyl (H-21) and the pair for H-18 and -19. Also, its coupling with H-10 confirms the assignment of H-9 and its interaction with another proton indicates this to be one of the C-8 protons. Further coupling of this H-8 to the other three signals indicates these are the remaining H-8 and H-7 methylene protons.

The ^{13}C spectrum exhibits 25 lines (one obscured by solvent) and a DEPT[27] experiment ($\theta = 3\pi/4$) reveals, as expected, five methyl groups (at high field), four aliphatic and three aromatic methines (all 12 with positive peaks), and three methylenes (with negative peaks), leaving nine quaternary carbon atoms and one carbonyl nulled.

A conventional 2D ^{1}H–^{13}C correlation experiment[11,12] (Figures 1 and 2A) readily assigns the carbon absorbances for C-3, -7, -8, -9, -10, -13, -14, -24, -25, and -26 by their correlation with assigned protons. The highest field ^{13}C signals are assigned to the pair of methyl groups at C-17 and -20 but neither this pair nor the C-18/19 pair are differentiated. In addition, the ^{13}C resonance for C-21 was simply too close to one of the C-18/19 pair to be distinguished. The carbonyl signal for C-22 and the other aromatic signal for C-23 are easily assigned on the basis of their chemical shifts, leaving the remaining eight quaternary carbon signals unassignable, and the C-17 and -20 methyl signals indistinguishable.

At this point the fully coupled technique was applied to ryanodine (**1**) and this yielded a self-consistent set of assignments the interpretation of which was initially aided by the conventional ^{1}H–^{13}C correlation spectrum.

Fully coupled 2D ^{1}H–^{13}C *shift correlation n.m.r. spectrum.* The directly observed long-range ^{1}H–^{13}C coupling is the key to spectral assignment because, although it does not directly establish carbon–carbon connectivity, it maps out groups of connected carbon atoms, and the common couplings to proximal protons can make structural assignment unequivocal. An early communication used this method to detect geminal methyl groups[24] and thereby indirectly noted its value for detecting long-range couplings. When the original method was modified,[11] the long-range coupling information was unfor-

tunately lost and this method has not been exploited significantly since then.[26]

Interpretation of the complicated 2D contour plot (Figure 2C) is facilitated by first considering an idealized representation of the partial spectrum for C-14, H-14A, H-14B and interacting nuclei (Figure 2B). Each of two rectangular arrays due to one-bond C–H coupling is defined by six cross-peaks, centred at the same frequency along the f2 axis as the C-14 signal. These arrays are 230 Hz wide triplets in f2 and 110 Hz wide doublets in f1, and along the f1 axis they line up with the two ^{1}H doublets assigned to the 14-methylene. The carbon signal is split into a triplet by the two hydrogen atoms; thus a 115 Hz coupling is observed in the f2 dimension. The protons are coupled to one ^{13}C, and the signals are split into doublets of 110 Hz, as close as can be expected to the f2 coupling with the relatively low precision in our 512 W (f2) × 512 W (f1) data set. The C-14/H-20 cross-peak shows the ^{13}C atoms at C-14 to be coupled to protons on C-20. Since C-14 bears two protons, this signal still shows a large $^1J_{CH}$ of 110 Hz in the f2 dimension due to the C-14 protons, but since 99% of protons at C-20 are bonded to ^{12}C, there is no evident large splitting in the proton (f1) dimension. The long-range $^3J_{CH}$ is too small to be observed with the available resolution. The other carbon atoms that couple with the 14-protons do not bear any protons of their own and thus exhibit singlets in f2. In f1, however, the strong 15 Hz ^{1}H–^{1}H couplings are still evident. The presence of these long-range interactions indicates significant geminal or vicinal ^{1}H–^{13}C coupling, but there are frequently protons and carbons two and three bonds distant which have couplings too small to be observed. The intensity of the geminal coupling is as difficult to predict here as it is for ^{1}H–^{1}H coupling, whereas vicinal coupling is useful, as described later.

Comparison of one-bond f1 and f2 coupling aids discrimination amongst methyl, methylene, and methine groups. This is particularly helpful with methylenes where the centre peak of the triplet in the carbon dimension has very low intensity and often does not appear, *e.g.*, C-7 and C-8 (Figure 2C). Thus, a methylene is clearly present at both C-7 and C-8 since their arrays show coupling in f2 of twice that in f1. On the other hand, a methine shows a clear four-peak doublet of doublets with the sides having equal sizes in Hz, while methyl groups show a distinctive rectangular quartet of doublets (carbon/proton multiplicity) with f2 coupling three times that in f1.

Analysis of the full 2D spectrum (Figure 2C) started with distinguishing the C-17 and C-20 methyl groups. Both the methyls exhibit the large eight-peak quartet of doublet arrays from direct $^1J_{CH}$ coupling. Each has four additional ^{13}C cross-peaks, all but one on unidentified carbon atoms. The known carbon is C-14, and since it is three bonds from H-20 and four from H-17, it was clear that C-14 couples only with the C-20 methyl group, because four-bond coupling is small and rarely observed in non-conjugated systems.[28] This enabled assignment of H-20 to the upfield methyl singlet, and H-17 to the downfield one. The other carbon atoms coupled to H-20 through two or three bonds were thus C-4, -5, and -6. The farthest upfield signal (δ 49) is most likely due to the quaternary C-5 with no deshielding heteroatoms; this is also coupled to H-3 and weakly to both 14-protons. One of the other two carbon atoms is coupled to one H-8 (as well as to the downfield H-14), clearly indicating that this (δ 85) peak is due to C-6, leaving the remaining carbon atom as C-4, which in addition to H-20 is also coupled to H-3 and the upfield H-14.

Four unidentified carbon atoms were coupled to H-17, and within three bonds these were C-1, -2, -12, and -15. One of these is coupled to H-18 and -19, and in view of the limit of three-bond coupling this must be C-2. Again, the farthest upfield signal (δ 66) is assigned to C-1 as it is not bonded to a heteroatom. The C-15 atom but not C-12 can interact with H-14, and coupling with the δ 102 signal thereby enables assignment of C-15 to leave the C-12 signal as the δ 97 peak. The above assignments left only one undetermined carbon atom, by elimination C-11.

Table 2. Comparison of calculated torsion angles and observation of vicinal coupling for ryanodine chair conformation

	^{13}C position and torsion angle	
^{1}H Position	Coupling observed	Coupling not observed
3	5, 150°	1, 124°; 12, 99°; 13, 100°
7α	9, 171°; 11, 178°	5, 67°
7β		5, 51°; 9, 73°; 11, 64°
8α		6, 71°; 10, 73°; 21, 51°
8β	6, 174°; 10, 171°	21, 63°
9		7, 70°; 11, 66°
10		6, 62°; 8, 69°; 12, 60°; 21, 55°
14A	1, 175°; 4, 174°	6, 64°; 20, 59°
14B	6, 179°	1, 70°; 4, 70°; 20, 58°

The fully coupled 2D method apparently distinguishes larger from smaller vicinal couplings, which are dependent on torsion angles.[28–30] A model shows that the 7α-proton makes a torsion angle near 180° with C-11, which favours coupling, whereas the 7β-proton makes a torsion angle near 90°, with coupling close to zero. Thus the selective coupling of C-11 with one 7-proton allows its assignment as H-7α. This stereochemical analysis based on vicinal couplings is also possible elsewhere in the molecule and particularly for the 14-protons with C-4 and -6. Each of these carbon atoms makes a favourable torsion angle with the opposite 14-proton and an unfavourable angle with the other, allowing unequivocal assignment of H-14A and H-14B and confirmation of the C-4 assignment.

The conformation of the potentially flexible cyclohexane ring is evident from the observed torsion angle-dependent vicinal couplings. Analysis of the ^{1}H–^{1}H coupling data is ambiguous in distinguishing between chair and boat conformation. Calculations using Allinger's MM2 method[31] strongly favoured the chair by 7 kcal, and by using the torsion angles determined by these calculations a close correlation was established between favourable torsion angles and observed interactions (Table 2). These findings concur with the *X*-ray data of a ryanodine derivative which indicate a chair conformation.[3]

Identification of New Ryanoid as 9,21-*Didehydroryanodine* (**2**).—^{1}H N.m.r. comparison of (**2**) with (**1**) showed the lack of one methyl doublet and the H-10 methine at δ 4.55 (Table 1). Instead, there were three new peaks in the δ 5.2—5.7 region, two of which exhibit 2 Hz coupling. The rest of the spectrum was largely unchanged. These new peaks are attributed to a terminal methylene and a vinylic alcohol at positions 9 and 10. This assignment is supported by a ^{1}H–^{1}H COSY 2D experiment[10] which showed that the 2-isopropyl group is intact. Also, the three new signals at δ 5.2—5.7 are coupled to each other as well as having the olefinic protons coupled to one ring methylene proton, presumably at C-8. These assignments were corroborated by a conventional ^{1}H–^{13}C correlation experiment,[10,11] which showed both the vinyl signals coupled to one low-field carbon atom that has a negative sense in the DEPT experiment ($\theta = 3\pi/4$).[27] All this evidence points to a terminal methylene group at position 21.

The fully coupled 2D spectrum of (**2**) is similar to that of (**1**), with some notable exceptions. Two of the ^{13}C signals (C-9 and -23) are masked by solvent peaks in the ^{13}C spectrum of (**2**) but they are evident in the fully coupled experiments by their long-range ^{1}H couplings. Since they bear no protons they were not

observed in other experiments. It is clear that bonding hybridization can affect long-range ^1H–^{13}C coupling,[28] and the sp^2 carbon at C-9 exhibits geminal coupling different from the sp^3 carbon at the same position. It shows weak geminal coupling across the double bond to only one exocyclic hydrogen in (**2**), whereas coupling was very strong to the methyl group in (**1**). On the other hand, C-9 in (**2**) has strong geminal coupling across single bonds to both 8-protons and H-10, which is not present in (**1**). Despite these differences, vicinal coupling through single bonds is expected to be torsion angle-dependent, and C-9 shows $^3J_{CH}$ interaction only with H-7α. This supports the pseudo-chair conformation of the cyclohexane ring in (**2**). In the pseudo-chair, C-9/H-7α coupling is favoured, whereas in the pseudo-boat C-9/H-7β coupling is favoured. In either case, H-7α can be assigned on the basis of its predicted and observed interaction with C-11. The pseudo-chair assignment is also supported by the absence of both C-12/H-10 and C-8/H-10 couplings.

As a chemical proof of structure, (**2**) was catalytically reduced to ryanodine (**1**) and 9-*epi*-ryanodine (**3**). It is interesting that (**2**) was apparently lost in early isolations from ryania,[32] probably at the step of recrystallization. Thus, when we recrystallized a 1:1 mixture of (**1**) and (**2**) twice from diethyl ether, only negligible amounts of (**2**) remained.

Conclusions

The neglected fully coupled ^1H–^{13}C correlation method is clearly a valuable tool for determining the structure of organic molecules. Using a very simple pulse sequence, accessible on any n.m.r. instrument with 2D capability, the method yields not only carbon–carbon connectively but also conformational and stereochemical information. By comparison, it is more sensitive than the INADEQUATE though less sensitive than the standard correlation experiment and gives more information than either of these techniques. Here the structural determination of 9,21-didehydroryanodine was made a straightforward procedure by using this powerful 2D method.

Experimental

N.m.r. Details.—All spectra were taken with a Bruker WM-300 wb (^1H 300 MHz; ^{13}C 75 MHz) spectrometer equipped with an ASPECT 3000 data system. COSY and conventional correlation spectra were obtained by using standard software. The fully coupled ^1H–^{13}C correlation spectra were recorded using the pulse sequence, $(\pi/2,^1H)$–Δt_1–$(\pi/2,^1H;\pi/2,^{13}C)$–acquisition, with quadrature detection. The pulses were phase-shifted according to Bleich *et al.*[25] The spectral width in f2 (^{13}C) was 11 904 Hz and in f1 (^1H) 2 941 Hz. The probe was tuned and the π/2 pulses were carefully determined for each sample. The f2 dimension was exponentially weighted using a line-broadening factor of 2.0 Hz; the f1 dimension was modified with a shifted (π/4) sine bell squared function before transformation. The f2 dimension was collected and transformed over 1 K data points and the f1 dimension was generated over 256 experiments and transformed over 512 words as a power spectrum. The fully coupled spectra were determined with 0.5 mmol ryanoid and 12 h acquisition time in $[^2H_5]$pyridine.

Analysis of Ryania Powder and Isolation of Ryanodine (**1**) and 9,21-*Didehydroryanodine* (**2**).—The powdered stemwood of *Ryania speciosa* grown in Trinidad (a gift of Progressive Agri-Systems, Stockertown, Pennsylvania, U.S.A.) was extracted using the wet chloroform procedure.[32] The extract was partitioned with water three times and the aqueous fraction analysed directly by h.p.l.c. [4.6 × 250 mm Altex Ultrasphere-ODS, 1 ml min^{-1}, methanol–water 1:1; 268 nm detection; t_R/min (**2**) 7.6, (**1**) 9.6]. Peak-area measurement corrected for absorptivity showed that (**1**) and (**2**) were present in 1:3 ratio. Methanolic extracts (cold stirring or Soxhlet) were directly analysed by the same h.p.l.c. method, and gave identical ratios of (**1**) to (**2**). The identity of the two components was confirmed by their isolation and ^1H n.m.r. spectra. Larger quantities of (**1**) and (**2**) were obtained by purifying commercial ryanodine (Penick Co., Lyndhurst, New Jersey, U.S.A.) with an identical preparative (10 mm × 250 mm) column, and a 4 ml min^{-1} flow rate.

9,21-*Didehydroryanodine* (**2**) had m.p. 175—178 °C (from diethyl ether); $[\alpha]_D^{20}$ +17° (*c* 1.13 in methanol) (Found: C, 59.3; H, 6.9; N, 2.6. $C_{25}H_{33}NO_9 \cdot H_2O$ requires C, 58.9; H, 6.9; N, 2.7%); $\lambda_{max.}$(methanol) 268.5 (ε 17 500 dm^3 mol^{-1} cm^{-1}) and 204 nm (6 470); $\nu_{max.}$ 1 678, 1 545 cm^{-1}; *m/z* 514 (M^+ + 23) (fast atom bombardments). Ryanodine (**1**) had $[\alpha]_D^{20}$ +15° (*c* 0.8 and 0.5 in methanol) (lit.,[32] $[\alpha]_D^{25}$ +26°).

Reduction of 9,21-*Didehydroryanodine* (**2**) *to Ryanodine* (**1**) *and* 9-epi-*Ryanodine* (**3**).—Compound (**2**) (45.6 mg, 93 μmol) was dissolved in water (5 ml) and 10% Pd/C (10 mg) was added. The solution was vacuum-degassed four times (venting with hydrogen) and then stirred under hydrogen (1 atm). After 60 min the h.p.l.c. [as before, t_R/min of (**3**) 11.4] profile remained constant, and at 3 h the solution was filtered and purified by preparative h.p.l.c. (as before) to yield ryanodine (**1**) (1.18 mg, 2.4 μmol, 3%) and 9-epi-*ryanodine* (**3**) (34.6 mg, 70 μmol, 75%), m.p. 232—233 °C (decomp.) (from diethyl ether); $[\alpha]_D^{20}$ +2.7° (*c* 1.17 in methanol) (Found: C, 59.0; H, 7.1; N, 3.0. $C_{25}H_{35}NO_9 \cdot H_2O$ requires C, 58.7; H, 7.3; N, 2.7%); $\lambda_{max.}$ 267.5 nm (ε 12 500 dm^3 mol^{-1} cm^{-1}); $\nu_{max.}$ 1 665 cm^{-1}; δ_H (300 MHz, $[^2H_5]$pyridine; standard $[^2H_4]$pyridine, δ 8.71) 1.14 (3 H, d, *J* 6 Hz, H-19/18), 1.40 (3 H, s, H-20), 1.45 (1 H, m, H-7β), 1.54 (1 H, m, H-8β), 1.55 (6 H, d, *J* 7 Hz, H-18/19 + -21), 2.18 (3 H, s, H-17), 2.48 (1 H, m, H-8α), 2.53 (2 H, m, H-7α + -9), 2.53 (1 H, d, *J* 13 Hz, H-14A), 2.83 (1 H, sept, *J* 6 Hz, H-13), 3.27 (1 H, d, *J* 13 Hz, H-14B), 5.06 (1 H, d, *J* 6 Hz, H-10), 6.49 (1 H, dd, *J* 2 and 4 Hz, H-25), 6.67 (1 H, s, H-3), 7.29 (1 H, dd, *J* 1 and 4 Hz, H-24), and 7.38 (1 H, dd, *J* 1 and 2 Hz, H-26); δ_C (75 MHz, $[^2H_5]$pyridine, δ 135.5) 11.0 (C-17), 13.2 (C-20), 15.9 (C-21), 19.1 (C-19/18), 19.7 (C-18/19), 23.5 (C-7), 27.0 (C-8), 30.4 (C-13), 34.8 (C-9), 42.1 (C-14), 49.4 (C-5), 65.5 (C-1), 67.4 (C-10), 83.4 (C-2), 85.3 (C-6), 88.2 (C-11), 90.9 (C-3), 91.9 (C-4), 97.4 (C-12), 102.5 (C-15), 110.5 (C-25), 116.2 (C-24), 124.8 (C-26), and 161.0 (C-22) (C-23 obscured by solvent); *m/z* 494 (M^+ + 1) (fast atom bombardment).

Acknowledgements

This study was supported in part by a grant from the United States National Institutes of Health. We thank Dr. David Moreland for carrying out the MM2 calculations, and Molecular Design, Ltd., Hayward, California, U.S.A., for providing the computing facilities.

Note added in proof: Recently, another ^1H–^{13}C long-range shift correlation method has appeared (H. Kessler, C. Griesinger, J. Zarbok, and H. Loosli, *J. Magn. Reson.*, 1984, **57**, 331). An application of the fully coupled method described herein has also been published (R. L. Halterman, N. H. Nguyen, and K. P. C. Vollhardt, *J. Am. Chem. Soc.*, 1985, **107**, 1379).

References

1 D. G. Crosby, in 'Naturally Occurring Insecticides,' eds. M. Jacobson and D. G. Crosby, Dekker, New York, 1971, p. 198.
2 K. Wiesner, *Adv. Org. Chem.*, 1972, **8**, 295.
3 S. N. Srivastava and M. Przybylska, *Can. J. Chem.*, 1968, **46**, 795.

4 A. Bélanger, D. T. F. Berney, H.-T. Borschberg, R. Brousseau, A. Doutheau, R. Durand, H. Katayama, R. Lapalme, D. Leturc, C.-C. Liao, F. N. MacLachlan, J.-P. Maffrand, F. Marazza, R. Martino, C. Moreau, L. Saint-Laurent, R. Saintonge, P. Soucy, L. Ruest, and P. Deslongchamps, *Can. J. Chem.*, 1979, **57**, 3348.
5 L. Procita, *J. Pharmacol. Exp. Ther.*, 1956, **117**, 363.
6 Z. J. Penefsky, *Pflügers Arch.*, 1974, **347**, 173.
7 J. L. Sutko and J. T. Willerson, *Circ. Res.*, 1980, **46**, 332.
8 A. L. Waterhouse, I. Holden, and J. E. Casida, *J. Chem. Soc., Chem. Commun.*, 1984, 1265.
9 D. R. Babin, J. A. Findlay, T. P. Forrest, F. Fried, M. Götz, Z. Valenta, and K. Wiesner, *Tetrahedron Lett.*, 1960, 31.
10 A. Bax and R. Freeman, *J. Magn. Reson.*, 1981, **44**, 542.
11 R. Freeman and G. A. Morris, *J. Chem. Soc., Chem. Commun.*, 1978, 684.
12 A. Bax and G. A. Morris, *J. Magn. Reson.*, 1981, **42**, 501.
13 J. Brondeau, C. Millot, and D. Canet, *J. Magn. Reson.*, 1984, **57**, 319.
14 A. Bax, R. Freeman, and S. P. Kempsell, *J. Am. Chem. Soc.*, 1980, **102**, 4849.
15 T. H. Mareci and R. Freeman, *J. Magn. Reson.*, 1982, **48**, 158.
16 P. H. Bolton and G. Bodenhausen, *Chem. Phys. Lett.*, 1982, **89**, 139.
17 P. H. Bolton, *J. Magn. Reson.*, 1982, **48**, 336.
18 O. W. Sørensen and R. R. Ernst, *J. Magn. Reson.*, 1983, **55**, 338.
19 H. Kessler, M. Bernd, H. Kogler, J. Zarbock, O. W. Sørensen, G. Bodenhausen, and R. R. Ernst, *J. Am. Chem. Soc.*, 1983, **105**, 6944.
20 A. Bax, *J. Magn. Reson.*, 1984, **57**, 314.
21 A. Bax, 'Two-dimensional Nuclear Magnetic Resonance in Liquids,' Delft University Press, London, 1982.
22 A. A. Maudsley and R. R. Ernst, *Chem. Phys. Lett.*, 1977, **50**, 368.
23 G. Bodenhausen and R. Freeman, *J. Magn. Reson.*, 1977, **28**, 471.
24 G. Bodenhausen and R. Freeman, *J. Am. Chem. Soc.*, 1978, **100**, 320.
25 H. Bleich, S. Gould, P. Pitner, and J. Wilde, *J. Magn. Reson.*, 1984, **56**, 515.
26 H. Bleich (IBM Instruments, Inc., Danbury CT, U.S.A.), personal communication.
27 D. M. Doddrell, D. T. Pegg, and M. R. Bendall, *J. Magn. Reson.*, 1982, **48**, 323.
28 P. E. Hansen, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1981, **14**, 175.
29 M. Karplus, *J. Am. Chem. Soc.*, 1963, **85**, 2870.
30 R. Wasylishen and T. Schaefer, *Can. J. Chem.*, 1972, **50**, 2710.
31 N. L. Allinger, S. H.-M. Chang, D. H. Glaser, and H. Hönig, *Isr. J. Chem.*, 1980, **20**, 51.
32 E. F. Rogers, R. R. Koniuszy, J. Shavel, Jr., and K. Folkers, *J. Am. Chem. Soc.*, 1948, **70**, 3086.

Received 6*th August* 1984; *Paper* 4/1379