CARBON–13 NUCLEAR MAGNETIC RESONANCE SPECTRAL ANALYSIS OF NATURALLY OCCURRING WITHANOLIDES AND THEIR DERIVATIVES

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ABSTRACT.—The ^{13}C NMR spectra of the naturally occurring withanolides: withaferin A, 2,3-dihydrowithaferin A, and seventeen related derivatives have been recorded at 15.03 MHz. The signal due to each carbon atom in these withanolides has been assigned with the help of standard techniques and the spectra have been analyzed in terms of substituent effects. Particular attention is directed towards features of the spectra which are most useful for structure determination and identification of the basic withanolide skeleton in these steroidal lactones.

The withanolides consist of a group of structurally complex and unusual steroidal lactones which occur in the *Solanaceae* family (1). To date, about seventy withanolides have been isolated from nature (2, 3). Some of these compounds are known to have various biological activities and one of them, withaferin A (**1**), is highly cytotoxic and active in several tumor systems (3, 4). Withaferin A also possesses antibacterial activity against acid-fast bacilli and gram-positive microorganisms (3). The structure elucidation of these complex withanolides required extensive chemical work and spectral interpretation. Proton nmr spectroscopy and mass spectrometry have played important roles in determining the structures of withanolides in the last fifteen years. In spite of extensive proton nmr and mass spectral data available for this class of compounds (2, 3), it is not always possible to establish the various substitution patterns on the withanolide skeleton unambiguously, e.g., the complete structures of several withanolides still remain unknown (5, 6). Although the ^{13}C nmr data of several hundred steroids have been published (7), the application of ^{13}C nmr spectroscopy to the structure elucidation of this class of compounds has been largely ignored.

In this paper, we report for the first time the ^{13}C nmr spectral assignments of various withanolides and discuss the way in which this information can be used for structure elucidation of these highly oxygenated steroidal lactones. The general procedure for ^{13}C nmr data acquisition and assignment of the carbon resonances for the compounds reported here involved determination of the noise-decoupled and the single-frequency off-resonance decoupled (SFORD) spectra. The chemical shifts were assigned with the aid of complete and partial decoupled spectra, direct analysis of non-protonated carbon centers, acetylation of hydroxyl functions, application of known chemical shift rules (8), steric effects, and from comparisons of spectra from compound to compound.

During our search for tumor-inhibitory natural products from plants, we isolated the known antineoplastic agent, withaferin A (**1**), from *Physalis viscosa*. The structure of withaferin A has been elucidated by chemical studies and x-ray crystallography by two different research groups (9–11). ^{13}C chemical shifts assignments of withaferin A and its derivatives are reported in table 1. The ^{13}C nmr spectrum of withaferin A shows twenty-eight signals for twenty-eight carbons in the molecule. The single frequency off-resonance decoupled (SFORD) spec-
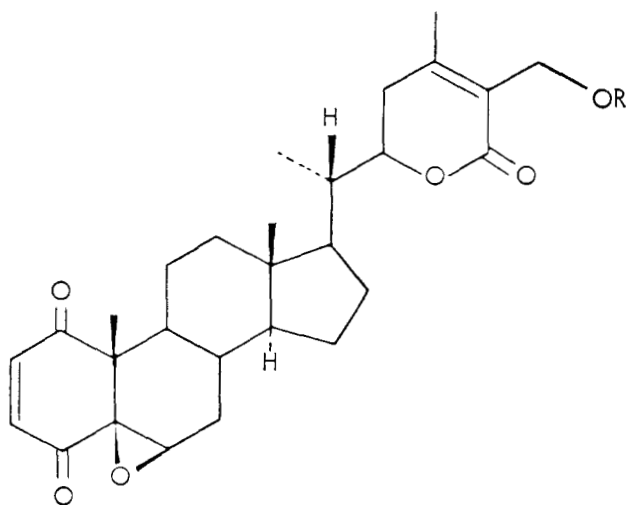
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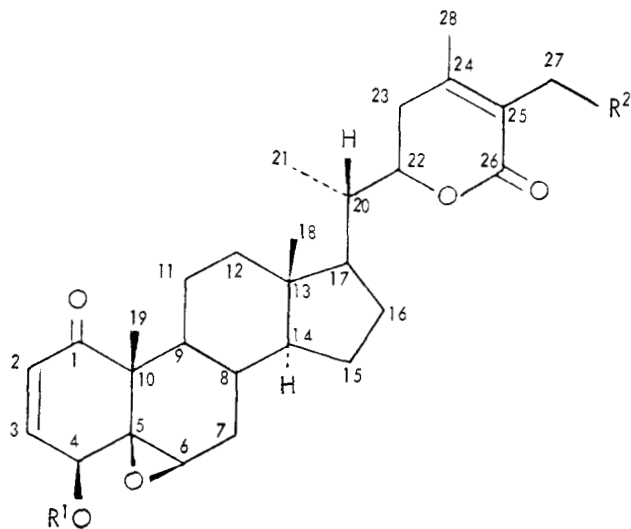
1   R¹=H; R²=OH Withaferin-A
2   R¹=Ac; R²=OAc
3   R¹=Ac; R²=H

4   R=H 4-Dehydrowithaferin-A
5   R=Ac

trum of **1** shows 7 singlets, 10 doublets, 7 triplets and 4 quartets.   The downfield singlets at 202.3 and 167.0 ppm can be assigned unambiguously to the carbonyl groups of the α,β unsaturated ketone and lactone at C–1 and C–26, respectively. The two doublets at 132.3 and 142.5 ppm in compound **1** are assigned to the double bond between C–2 and C–3, respectively.   The chemical shifts at 153.5 and 125.6 ppm in **1** are singlets and can be accommodated only by the double bond of the α,β-unsaturated lactone.   The singlet at 63.9 ppm and doublet at

61.7 ppm are assigned to C-5 and C-6 of the epoxide ring. The chemical shift of C-4 (hydroxy group) is observed upfield at 69.8 ppm in withaferin A as compared with the corresponding carbon in the diacetate derivative 2 because of the substitution of hydroxy by acetoxy functionality. The low field doublet at 78.7 ppm in compound 1 is assigned to C-22 of the lactone ring because this signal remains constant in all α,β unsaturated lactone-containing withanolide derivatives. The triplet at 57.0 ppm can be assigned definitely to C-27 (only primary hydroxy group) in 1. Of the two singlets at 47.8 and 42.5 ppm, the former is assigned to C-10 and the latter is assigned to C-13 by comparison with the corresponding shifts in 2,3-dihydrowithaferin A (7) (table 2). The four methyl group quartets at 11.6, 13.3, 17.0 and 20.0 are assigned to C-18, C-21, C-19 and C-28 in 1, respectively, because the C-19 and C-28 methyl group signals are shifted in various derivatives while the chemical shifts of C-18 and C-21 remain constant. The remaining chemical shifts of withaferin A are assigned by comparison with the corresponding shifts of various known steroids (7).

The spectrum of the diacetate 2 shows extra signals for the two acetate groups and some minor changes in the chemical shifts of the double bonds, the carbonyl groups and the C-4 carbon when compared with that of withaferin A. The

TABLE 1. Chemical shifts and assignments of withaferin A derivatives.[a]

|	1	2	3	4	5	6
C-1	202.3(s)	201.1	201.6	202.0	201.1	209.3
C-2	132.3(d)	133.8	134.2	139.2	139.2	37.5
C-3	142.5(d)	139.8	140.1	141.6	141.6	126.2
C-4	69.8(d)	72.1	72.4	193.8	193.8	130.7
C-5	63.9(s)	61.0	61.2	63.9	63.9	65.3
C-6	61.7(d)	60.3	60.4	63.5	63.5	61.7
C-7	29.8(t)	30.1	29.7	29.7	29.6	29.6
C-8	31.1(d)	31.1	31.2	30.5	30.5	31.1
C-9	44.0(d)	44.2	44.3	43.6	43.6	42.1
C-10	47.8(s)	48.1	48.3	49.8	49.8	50.2
C-11	21.8(t)	21.3	21.3	23.4	23.4	23.0
C-12	27.2(t)	27.3	27.3	27.2	27.2	27.2
C-13	42.5(s)	42.6	42.6	42.6	42.6	42.7
C-14	56.0(d)	56.1	56.3	55.5	55.6	55.5
C-15	24.2(t)	24.2	24.3	24.2	24.2	24.2
C-16	39.2(t)	39.2	39.3	39.4	39.4	39.3
C-17	51.8(d)	51.9	52.1	52.0	52.0	52.1
C-18	11.6(q)	11.6	11.6	11.8	11.8	11.6
C-19	17.0(q)	15.7	15.7	19.1	19.1	17.3
C-20	38.7(d)	38.8	38.9	38.7	38.7	38.8
C-21	13.3(q)	13.3	13.5	13.3	13.3	13.3
C-22	78.7(d)	78.2	78.4	78.6	78.2	78.5
C-23	29.8(t)	29.6	29.7	29.6	30.1	28.8
C-24	153.5(s)	157.0	149.2	153.1	157.0	149.0
C-25	125.6(s)	121.9	122.3	125.7	121.8	122.0
C-26	167.0(s)	165.2	167.3	166.9	165.2	167.0
C-27	57.0(t)	58.0	12.5	57.2	58.0	12.4
C-28	20.0(q)	20.5	20.6	20.0	20.6	20.5
C-4-C=O	---	170.0	170.5	—	—	—
CH₃	—	20.8	21.0	—	—	—
C-27-C=O	—	170.8	---	—	170.8	—
CH₃	—	20.8	—	—	20.9	—

[a]Chemical shifts are recorded as ppm downfield from Me₄Si. The samples were dissolved in CDCl₃.

spectrum of **2** also confirms the chemical shift assignments of the hydroxy group-containing carbons C-4 and C-27.   The C-4 β-acetoxy group in **2** showed some shielding effect on the C-19 methyl group when the spectrum of **2** was compared with that of **1**.   This shielding effect can be explained by the 1,3 diaxial interaction between the C-4 acetoxy and the C-19 methyl groups.   Comparison of the spectrum of 27-deoxy-4-acetylwithaferin A (**3**) with that of compound **2** revealed the absence in **3** of the chemical shifts due to the C-27 acetoxy group, a major change in the chemical shift of C-27, and some minor changes in the chemical shifts of the lactone ring carbons.   These observations lead to the conclusion that the methyl group is present at the C-27 position (12.5 ppm) in compound **3**. Comparison of the ¹³C nmr spectrum of 4-dehydro-withaferin A (**4**) with that of withaferin A revealed the presence of an additional singlet at 193.8 ppm and the absence of a secondary hydroxy group-containing carbon doublet, results which indicate the presence of the carbonyl group at C-4 in compound **4**.   The carbonyl group at C-4 in **4** releases the steric compression on the methyl group (C-19) and results in the downfield shift of the C-19 methyl group when compared with similar shifts in compounds **1** to **3**.   The assignment of the triplet at 57.0 ppm in **4** to C-27 was further confirmed by acetylation of compound **4** to 4-dehydrowithaferin A acetate (**5**).
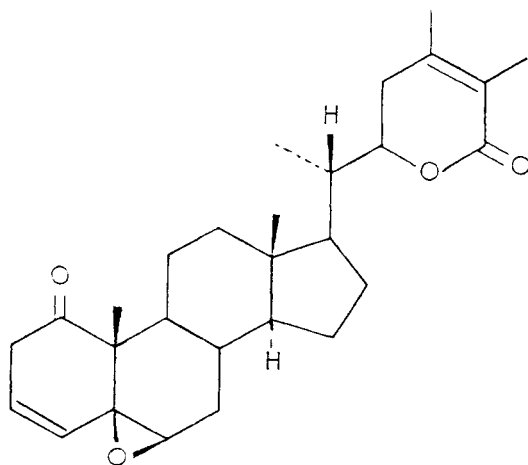
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During the transformation of withaferin A to various naturally occurring withanolides, we have prepared (12) compound **6**.   The pattern of ¹³C chemical shifts in **6** is similar to that of compounds **1** to **5**.   However, comparison of the spectrum of **6** with those of withaferin A and compound **3** revealed the absence of the chemical shifts due to the hydroxy group-containing carbons C-4 and C-27 and changes in chemical shifts of the ring-A carbons in **6**.   The chemical shift of the C-1 carbonyl group at 209.3 ppm indicated that the double bond in ring-A is not in conjugation with the C-1 ketone.   Therefore, two doublets at 126.2 and 130.7 ppm can be assigned to the double bond between C-3 and C-4 in compound **6**.   The downfield shift of C-5 further confirmed the presence of the double bond in ring A in **6**.   The chemical shifts of compound **6** are in agreement with its assigned structure.   This example demonstrates the usefulness of ¹³C nmr spec-
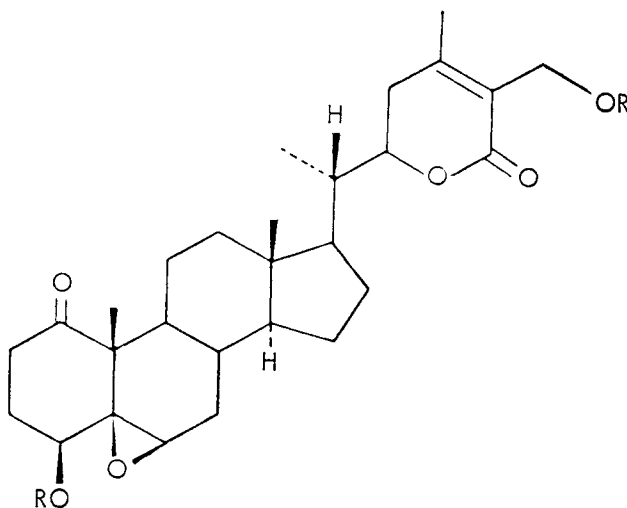
7   R=H 2,3-Dihydrowithaferin-A
8   R=Ac

TABLE 2.   Chemical shifts and assignments of 2,3-dihydrowithaferin-A derivatives.[a]

|	7	8	9	10	11	12
C-1	211.5	210.3	211.5	210.6	211.4	210.3
C-2	31.8	31.4	31.6	31.8	31.6	31.4
C-3	26.5	30.9	26.2	31.8	26.2	31.4
C-4	72.8	75.6	72.8	75.6	72.8	75.6
C-5	66.6	63.5	66.7	63.5	66.7	63.5
C-6	59.1	57.7	58.5	57.7	58.5	57.7
C-7	29.9	30.0	29.6	29.6	29.4	29.2
C-8	31.4	31.4	31.4	31.3	31.4	31.4
C-9	43.1	43.3	43.1	43.3	43.0	43.4
C-10	50.5	51.0	50.4	51.1	50.4	51.1
C-11	21.5	21.0	21.3	21.1	21.2	21.1
C-12	27.3	27.3	27.2	27.3	27.5	27.5
C-13	42.7	42.6	42.6	42.6	42.6	42.6
C-14	56.3	56.1	56.2	56.2	56.1	56.2
C-15	24.3	24.2	24.2	24.4	24.2	24.4
C-16	39.2	39.0	39.0	38.8	38.9	38.9
C-17	52.0	51.8	51.9	51.9	51.9	52.0
C-18	11.6	11.5	11.5	11.5	11.5	11.5
C-19	15.5	14.1	15.2	14.2	15.2	14.2
C-20	38.8	38.7	38.8	38.8	38.2	38.2
C-21	13.4	13.3	13.4	13.4	12.7	12.7
C-22	78.7	78.2	78.3	78.2	80.0	80.0
C-23	29.4	29.2	29.4	29.2	29.2	29.1
C-24	152.8	157.0	149.1	148.9	27.2	27.2
C-25	125.8	121.8	121.9	122.0	38.9	38.9
C-26	167.0	165.2	167.0	166.9	176.2	176.1
C-27	57.5	58.0	12.4	12.4	12.0	12.1
C-28	20.0	20.5	20.5	20.5	18.1	18.1
C-4-C=O	—	169.7	—	169.8	—	169.8
CH₃	—	20.8	—	21.1	—	21.1
C-27-C=O	—	170.7	—	—	—	—
CH₃	—	21.0	—	—	—	—

[a]Chemical shifts are recorded as ppm downfield from Me₄Si.   The samples were dissolved in CDCl₃.

troscopy in determining new structures of withanolides without tedious chemical work.

Table 2 presents the ^{13}C chemical shifts and assignments for 2,3-dihydro-withaferin A (7) and its derivatives. The spectrum of 7 reveals the absence of a double bond between C-2 and C-3 and major changes in the chemical shifts of ring A carbons when compared with that of withaferin A. The chemical shifts of C-1, bearing a carbonyl group, and C-4, bearing a hydroxy group, shifted downfield as expected. Two new triplets appeared as expected at 26.5 and 31.8 ppm in compound 7, which have been assigned to C-3 and C-2, respectively. Hydrogenation of the double bond in ring A also caused a shielding effect on the chemical shift of the C-19 methyl group and deshielding effects on the chemical
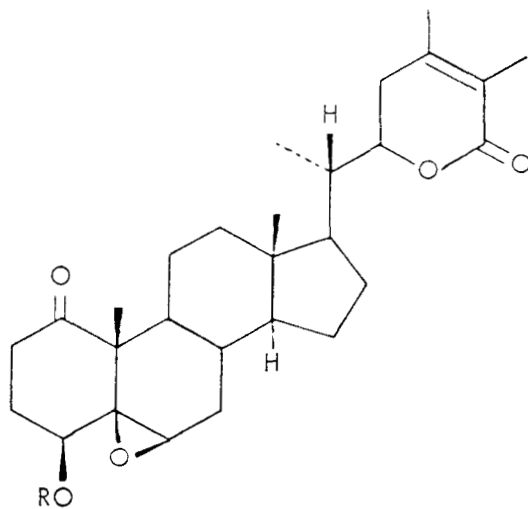
9   R = H 2,3-Dihydro-27-deoxywithaferin-A
10   R = Ac
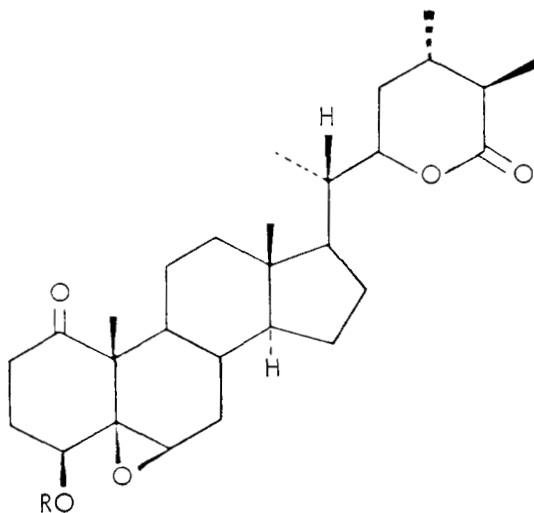
11   R = H
12   R = Ac

shifts of two quaternary carbons C–5 and C–10 of ring A. Ring A in withaferin A (1) exists in a boat form in solution as well as in the solid state (9, 10). The major changes in the chemical shifts of ring-A carbons in 2,3-dihydrowithaferin A can be explained only by a conformational change in ring-A. Reduction of the double bond between C–2 and C–3 must have released the strain on the carbons of ring A in compound 7.

Examination of the ^{13}C nmr spectrum of the diacetate derivative 8 of 2,3-dihydrowithaferin A revealed the presence of new peaks at 20.8, 21.0, 169.7, and 170.7 ppm for the two acetoxy groups at the C–4 and C–27 positions. The changes in chemical shifts of carbons 3, 4, 5, 24, 26, and 27 of ring A and the lactone ring in 8 as compared with compound 7 were as expected. Similar changes were observed also between the chemical shifts of withaferin A and its acetylation product.

The absence of a hydroxy group at C–27 was confirmed by observing a new quartet at 12.4 ppm in derivative 9 as compared with compound 7. Acetylation of compound 9 afforded only a C–4 monoacetate derivative which further supported

TABLE 3. Chemical shifts and assignments of some withaferin-A derivatives.[a]

|	13	14	15	16	17	18	19
C-1	210.8	209.8	208.5	210.0	209.2	210.6	209.2
C-2	42.6	39.6	40.5	40.3	41.0	40.5	41.1
C-3	68.5	77.5[b]	76.3[b]	75.4	77.1	75.3	77.1
C-4	76.9	75.9[b]	75.4[b]	75.4	73.5	75.4	73.5
C-5	64.6	64.9	61.4	64.9	61.6	65.1	61.6
C-6	60.1	60.1	58.5	59.9	58.8	59.8	58.8
C-7	29.7	29.8	30.1	29.8	29.4	29.6	29.7
C-8	31.0	31.2	31.1	31.2	31.3	31.3	31.3
C-9	42.9	42.8	43.1	42.8	43.2	42.9	43.2
C-10	50.4	50.4	50.9	50.4	51.0	50.5	51.1
C-11	21.4	21.5	21.0	21.4	21.1	21.4	21.1
C-12	27.3	27.3	27.3	27.3	27.3	27.3	27.3
C-13	42.7	42.7	42.6	42.6	42.8	42.7	42.7
C-14	56.2	56.0	56.0	56.1	56.2	56.2	56.2
C-15	24.3	24.2	24.2	24.3	24.3	24.3	24.3
C-16	39.2	39.1	38.9	39.1	39.1	39.2	39.1
C-17	52.1	52.0	51.8	51.9	52.0	52.1	52.1
C-18	11.6	11.5	11.5	11.5	11.5	11.6	11.5
C-19	15.7	15.5	14.0	15.4	14.1	15.2	14.1
C-20	38.9	38.8	38.7	38.7	38.9	38.9	39.0
C-21	13.4	13.3	13.3	13.3	13.3	13.4	13.4
C-22	78.5	78.7	78.2	78.7	78.3	78.5	78.4
C-23	29.2	29.4	29.2	29.4	30.1	29.5	29.4
C-24	149.2	153.0	156.9	153.0	157.3	149.5	149.2
C-25	122.2	125.7	121.8	125.7	122.1	122.2	122.3
C-26	167.6	167.0	165.2	167.0	165.6	167.5	167.2
C-27	12.4	57.4	58.0	57.3	58.1	12.5	12.5
C-28	20.6	20.0	20.5	20.0	20.6	20.6	20.5
C-3-OCH$_{2(3)}$	—	56.8	57.1	64.4	65.0	64.6	65.0
CH$_3$ C-4-C=O	—	—	—	15.4	15.4	15.5	15.4
	—	—	169.5	—	171.1	—	169.8
CH$_3$ C-27-C=O	—	—	20.8	—	21.0	—	21.1
	—	—	170.8	—	169.8	—	—
CH$_3$	—	—	20.9	—	21.0	—	—

[a]Chemical shifts are recorded as ppm downfield from Me$_4$Si. The samples were dissolved in CDCl$_3$.
[b]Values in any vertical column may be interchanged.

the absence of the C–27 hydroxy group.   Two new resonances at 21.1 and 169.8 ppm in compound **10** appear as a quartet and singlet, respectively, in the SFORD spectrum and must be assigned to the C–4 acetate group.   The ¹³C nmr spectrum of compound **11** shows no chemical shifts assignable to double bonds.   Some new ¹³C chemical shifts were observed in **11** as compared with compound **9**.   The up-field methine carbons resonances at 27.2 and 38.9 ppm are assigned to C–24 and C–25, respectively, in 27-deoxy-2,3,24,25-tetrahydrowithaferin A (**11**).   Two quartets at 12.4 and 20.5 ppm in compound **11** can be assigned only to C–27 and C–28, respectively.   The chemical shifts of the carbonyl group and C–22 of the lactone ring also moved downfield owing to the absence of the conjugated double bond in the lactone ring of compound **11**.   The introduction of an acetoxy group at C–4 in **12** showed similar changes that were observed in compounds **7** and **8**.

The ¹³C chemical shifts and assignments for 3-hetero substituted-2,3-dihydro-withaferin A derivatives (**13–19**) are presented in table 3.   All of the assignments in compound **13** except the carbons of ring A were made by comparison with com-pound **9**.   The presence of a β-hydroxy group at C–3 produced expected changes in the chemical shifts of the carbons of ring A in **13**.   Two doublets at 68.5 and 76.9 ppm are assigned to C–3 and C–4, respectively, in the spectrum of compound **13**.   Acetylation of **13** with acetic anhydride and pyridine afforded compound **3** which indicates the β-configuration for the C–3 hydroxy group.   The substitution of a methoxy group for a hydroxy group on C–3 produced an expected downfield shift in compound **14**.   The neighboring carbons C–2 and C–4 in **14** are shifted upfield 3.0 and 1.0 ppm (α effect), respectively, relative to **13**.   Acetylation of the secondary hydroxy group at position C–4 in **14** has almost no effect on the chemical shift of that carbon and the assignments for **15** were made by comparison with compound **13**.
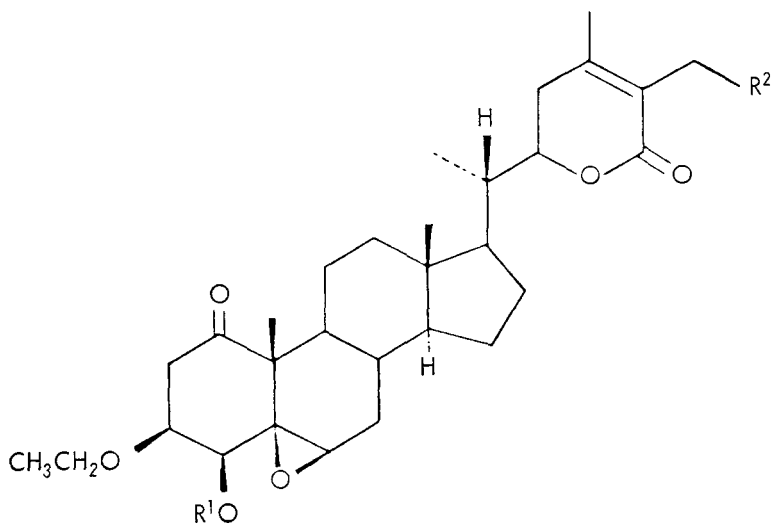
During the isolation of withaferin A from *Physalis viscosa*, we have isolated and identified a new compound as 2,3-dihydro-3-ethoxywithaferin A (**16**).   We believe that compound **16** is an artifact.   The use of ethanol as a solvent during the
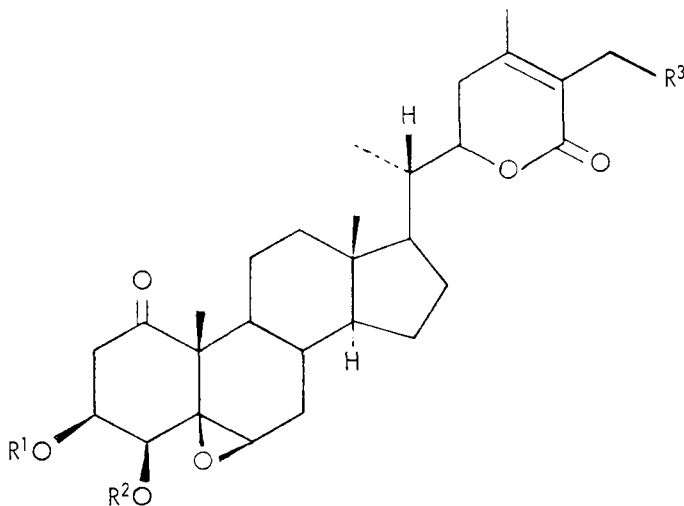
13   R¹=R²=R³=H
14   R¹=CH₃; R²=H; R³=OH
15   R¹=CH₃; R²=Ac; R³=OAc

16    $R^1 = H; R^2 = OH$
17    $R^1 = Ac; R^2 = OAc$
18    $R^1 = R^2 = H$
19    $R^1 = Ac; R^2 = H$

extraction of the plant material probably led to a Michael addition of ethanol to withaferin A. A methanol adduct (14) of withaferin A has been isolated as an artifact during isolation work (9, 10). The ^{13}C nmr spectrum of 16 was complicated in comparison with compound 14. The signals due to the ethoxy group were merged with other signals, which prompted us to prepare several derivatives of compound 16 for unambiguous assignments in this compound. The presence of the ethoxy group at C–3 was confirmed by observing a quartet at 15.4 and triplet at 64.4 ppm in the spectrum of compound 16. All the carbons in compounds 16, 17, 18 and 19 are assigned as shown in table 3, on the basis of available comparison data for the closely related compounds mentioned earlier.

We note that the presence of a heterosubstituted group at the C–3 position in 2,3-dihydrowithaferin A derivatives has a marked effect on the chemical shifts of the ring-A carbon atoms. It is particularly noteworthy that the variation in the substitution pattern of ring-A and lactone-ring carbons does not effect the chemical shifts of the remaining carbons of the molecule. These results indicate that the data reported here will be useful for identifying the presence of various groups in rings B, C and D of these withanolides. A particularly important feature in these withanolides is the constant pattern of shifts exhibited by the quaternary carbons except where major structural or substitution changes occur. The ability to assign all the quaternary carbons without ambiguity is of great value in detecting substitution occurring in the vicinity of these carbons. This catalogue of ^{13}C chemical shifts of an important group of steroidal lactones should assist in the structure elucidation of new withanolides without laborious and time-consuming chemical work.

EXPERIMENTAL

The ^{13}C spectra were determined at 15.03 MHz in the Fourier mode using a JEOL FX–60 spectrometer in conjunction with a JEC–980 computer. The spectra were determined at 30°C

in deuterochloroform solution (which provided the lock signal) with Me$_4$Si as an internal reference. Chemical shifts are recorded as ppm downfield from internal Me$_4$Si. Samples were contained in precision ground 5 and 10-mm o.d. tubes. The spectrometer was used in the crosscoil configuration. On the average, a 45° tilt angle was employed. For the average spectral width of 4000 Hz, the delay between pulses was 2 to 3 seconds. Acquisition times averaged 1-3 h over 8K data points for concentrations of the order of 0.4-1.0 M. For off-resonance spectra the time was 6-15 hours.

The known withanolides and their derivatives used in this study were isolated and/or prepared by procedures given in the literature cited. Certain new compounds used for the ^{13}C nmr analysis were prepared (12) during work on the transformation of withaferin A to naturally occurring withanolides.

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