El profesor David Lavie, del Instituto Weizmann de Rehovot, Israel, presentó el siguiente tema en una serie de conferencias que, como profesor visitante, impartió durante el mes de agosto de 1970 en el Instituto de Química.

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STEROIDAL LACTONES OF THE WITHANOLIDE TYPE

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Popular medicine, especially in India and South Africa, has attributed many physiological properties to crude extracts of roots and leaves of *Withania somnifera* (L.) Dun. Solanaceae. Sedative, hypnotic and antiseptic activity have been ascribed to these extracts.

Within the framework of a survey of the Israeli flora, the leaves of this species were thoroughly investigated for their content of the non-alkaloidal constituents. In the early stage of this investigation the structure of the steroidal lactone, withaferin A 1 was elucidated.[1] Since this compound could not be isolated from plant material collected in other regions of this country, a thorough study of this species was initiated in order to clarify the possibility of ontogenetic changes or the chemical variability of the species.

Our interest in withaferin A and in related compounds is also due to their interesting physiological action. In addition to the remarkable bacteriostatic activity this compound has also been found to have anti tumor properties.[2]

From the plants obtained from various sources, several such steroidal lactones have been isolated. The name "withanolide" has

been proposed[3] for this new type of compounds characterized by a C-28 basic skeleton with a 9 carbon atoms side chain having a six membered ring lactone 2. From the biogenetic point of view, the withanolides can be considered as possessing a highly oxygenated cholestane type side chain bearing an extra methyl group at C-24.

Leaves from samples of 24 populations of *Withania somnifera* from various parts of Israel were examined for their content in steroidal lactones, and three well-defined chemotypes of this species were disclosed.[3] Chemotype I contains predominantly withaferin A **1**[1] (about 0.2% of oven dry leaves) accompanied by small amounts of an isomer 27-desoxy-14α-hydroxy withaferin **3**.[4] Chemotype II contains as a main constituent 27-desoxy-20α (R)-hydroxy withaferin A also called withanolide D **4**. This compound is isomeric as well with withaferin A, its empirical formula being $C_{28}H_{38}O_6$.

The close structural similarity of this compound with the isomeric lactones **1** and **3** was disclosed by most of the signals in their nmr spectra, and facilitated thereby the identification of the functional group and its characterization.[5]

The presence of only one secondary OH group (at C-4) was confirmed by preparing a monoacetate **4b** which was different from the acetate of **3**.

Whereas during catalytic hydrogenation of the diacetate of withaferin A **1b** two moles of hydrogen were absorbed, the first for the reduction of the Δ^2 and the second for the hydrogenolysis of the 27-allylic acetoxy group, the monoacetate **4b** absorbed only one mole of hydrogen to give the corresponding 2,3-dihydoderivative. In all acetylated compounds **1**, **3** and **4** the nmr spectra showed the signal of the 4-H as a narrow triplet at δ 4.62, 4.58 and 4.54 indicating an axial orientation of the geminal 4-acetoxyl group.

The ultraviolet absorption spectra were instructive as well. The band at 217 nm (ε 19,500) is due to the overlapping of two chromophores, the unsaturated ketone in ring A and the unsaturated lactone of the side chain. Reduction of the double bond in ring A is accompanied by a slight shift and a significant lowering of the intensity of the U. V. absorption band λ_{max} 225 nm (ε 9,600) now being due to the effect of the unsaturated lactone.

In compound **4** since no proton is present at C-20 the signal of the three protons at 21 (methyl group) are unsplit and appear as a singlet, however shifted downfield by 0.3 ppm due to the deshielding effect of the 20-OH group. The absence of a 20-H proton, as in **1** and **3**, is also confirmed by the pattern of the 22-H signal, appearing in this case as a double doublet due to interaction only with the two vicinal protons at C-23. In the former two compounds this signal is a double triplet, produced by the additional coupling with the C-20 proton present in them.

Chemical proof was given by the elimination of the tertiary 20-OH group as the elements of water using thionyl chloride in pyridine solution. Two compounds were obtained **5** and **6**, the latter being a minor constituent. The structure of **5** which was the required product, bearing a tetrasubstituted double bond $\Delta^{20(22)}$, was obtained through the nmr spectrum of the compound, in which one could see that the 22-H signal present in **4** has disappeared while the strong signal of the 21-methyl group, now attached to a double bond, is at δ 1.58 (in **4** this signal is at 1.25).

Compound **5** could easily be isomerised over alumina to the α-pyrone **7** being a mixture of the 20R and 20-S isomers. The ultraviolet of this compound show a band at 300 nm ($\varepsilon \sim 5000$), and infrared band at 1570 and 1540 cm^{-1}. In the nmr the 23-vinylic proton appears as two one half proton signal each for the R and S stereoisomers.

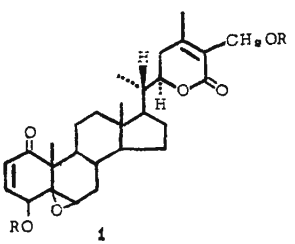
Compound **5** was cleaved by ozonolysis to the corresponding pregnane derivative **8** the characterization of which was done by spectroscopic means as well as by its mass spectra data. Obtention of the methyl ketone as a side chain was a good corrobration for the presence of a hydroxyl group at C-20.

The stereochemistry at C-20 was found to be 20α(R)* using nmr data. Advantage was taken of the known chemical shift of the 18, 19 and 21 methyl groups in cholesterol, 20α-hydroxy and 20β-hydroxy-cholesterol.[7] In the last two compounds, the signal of the 18-methyl group is shifted by very close values (0.13 and 0.12

* The nomenclature at C-20 is according to Fieser's designation; in a 20α configuration the substituent (OH group) is oriented upwards and in a 20β this group is downwards.[8]

ppm, respectively) in comparison to cholestane, however, the chemical shift of the 21-methyl is differently deshielded by the adjacent OH group by 0.30 ppm when 20β and by 0.13 ppm when 20α.[3, 5] Similar shifts have also been observed in the ecdysterol group.

Since in withaferin A 1 the 20-H is 20α as in cholesterol, (earlier proven by degradation to bisnor-5α-cholanic acid)[1c] the chemical shift of the 21-methyl group was compared with the corresponding shift in 4 and the results are in good agreement for a 20α stereo-



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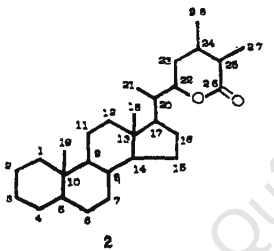

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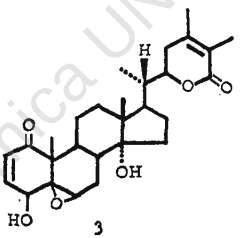

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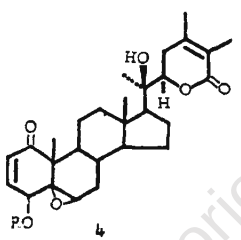

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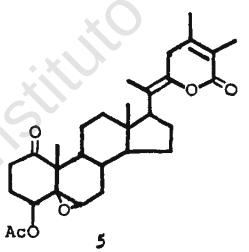

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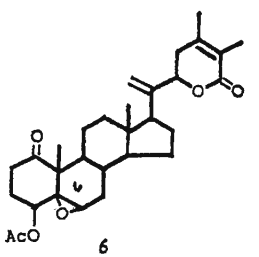

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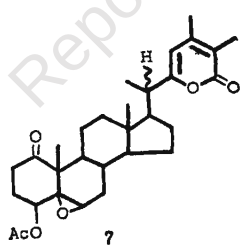

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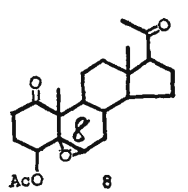

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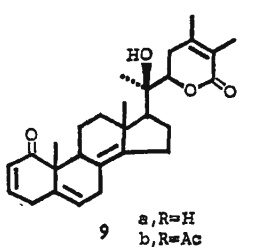

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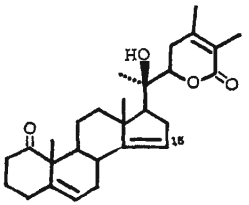
b, R=Ac



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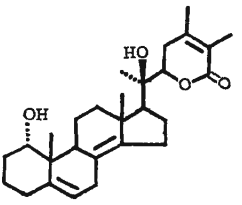

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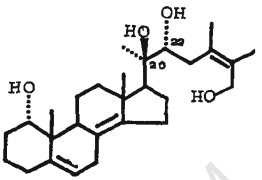

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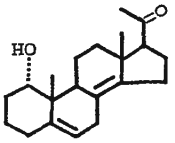

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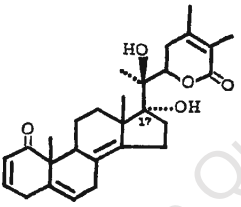

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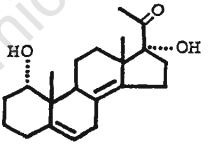


14c

chemistry. These data were obtained and compared in several derivatives.

While in chemotypes I and II a limited number of compounds have been isolated from each, chemotype III contains at least seven related withanolides,[2] we will deal presently with two most important compounds. In the two previous types described above a 4β-hydroxy group and 5,6 epoxide, also β oriented were consistently present, however, in type III in most of the compounds no substitution was observed at C_4. Only one compound in this group possesses a 5,6 oxirane ring, and since its structure has not yet been definitively elucidated it will not be discussed in this presentation.

One of the major constituents was shown to have structure **9** The ultraviolet spectrum had a λ_{max} 223 nm (ε 19,400) for the two unsaturated carbonyl chromophores. The nmr spectrum was however most informative, it disclosed and identified the functional groups of the molecule: the two vinylic methyl groups of the unsaturated lactone ring, the double doublet signal of C_{22}-H indicating only two vicinal protons at C_{23} which implied the presence of a

substituent at C_{20}, and then the set of vinylic protons signal related to C_2-H, C_3-H and C_6-H. The presence of a tertiary OH group at C_{20} was indicated by the position of the C_{21} methyl signal at lower field δ 1.30 ppm, as well as by using the recently described reagent, trichloro-acetyl isocyanate,[8] which reacts with all OH groups in *situ* in the nmr tube to form trichloroacetyl carbamates. This derivative can readily be detected by a signal at low field due to the newly formed N-*H* proton placed between two carbonyl groups inducing the strong deshielding effect.

The double bond Δ^2 was easily hydrogenated over $Pd/CaCO_3$ to produce the reduced ring A derivative, while the action of mineral acid on this reduced compound induced the migration of the tetrasubstituted ring C double bond $\Delta^{8(14)}$ to ring D assuming a 14-15 position **10**, thereby the double bond could be observed through a new signal in the nmr at δ 5.23 connected with the vinylic 15-H. This migration enables to detect the double bond at 8-14 in compound **9**.

In order to obtain chemical proof for the structure of the side chain the following degradation sequence was carried out, $NaBH_4$ reduction of the 2,3-dihydro-derivative of compound **9**, produced predominantly the 1α-hydroxy isomer **11** which could easily be separated from some 1β-hydroxy isomer accompanying the reaction product: the two isomers 1α-OH and 1β-OH could be distinguished by their respective nmr spectra taken in pyridine. It should be reminded that pyridine forms with hydroxyl groups collision complexes which influence in a clear way the location of certain neighbouring signals in the nmr.[9] For example, the 1α-OH derivative in pyridine has a small influence in the location of the C-10 methyl signal, while the 1β-OH isomer shows in this solvent a strong downfield shift of about 0.32 ppm, since the two groups are oriented on the same side of the plane of the molecule.

Reduction with lithium aluminum hydride of **11** produced **12** which was characterized by the corresponding triacetate. Sodium periodate oxidation of **12** cleaved the 20,22 glycol system producing the methyl ketone **13**. This compound was compared with pregnenolone acetate in order to determine the stereochemistry at C_{17},

using nmr as well as circular dichroism data. It has been found that indeed the side chain is β-oriented.

The second compound to be described in this presentation is **14**. This compound is very close in its structure to **9** differing in a second hydroxy-group which is located at C_{17}. The spectoscopic data in the two compounds were very close, while with the reagent trichloroacetyl isocyanate,[8] two carbamate derivatives were obtained as indicated by two low field singlets for the two -NH groups produced by the reagent. Following a similar degradation sequence as for **9** the 17-hydroxy-methyl ketone was obtained **14c** which was used in order to place unequivocally the OH group at C_{17}. The α orientation of this group was attained using again solvent shifts effect with pyridine.[9]

Having described briefly the major constituents of each of the chemotypes, one should add that it was found that each of them has a definite geographical distribution area. In order to examine the possible influence of the habitat and the surroundings (edaphic influence) on the formation of withanolides in this plant, samples of each of the chemotypes taken from four different soil and moisture conditions were examined, but no significant differences could be detected. The constancy of the types was also shown when plants of all the types raised from seeds in a nursery showed the same content of withanolides as those taken from their natural habitat. A comparison of the morphological characteristics of the chemotypes was carried out on several specimens of living plants of the same age and no difference could be found between them. The only variation is therefore of chemical nature on the content of the steroidal lactones of the withanolide type.[2]

Based on the above considerations it has been deduced that the differences existing in the formation of the various compounds have to be of a genetic character. A confirmation was obtained through cross-breedings by pollination of the different types.

For example, the cross of chemotypes I x III gave an offspring F_1 in the leaves of which compound 4 was produced predominantly. This compound which was never detected in chemotypes I or III is a major constituent of the withanolides present in chemotype II. However, this offspring F_1 (I x III) was not identical

with type II as seen on a chromatoplate, the spots distribution not being identical. As described, chemotype I contains as a major constituent compound 1 while III has several compounds, among which 9 and 14 are predominating. It was clear therefore that a combination of genes had taken place inducing changes in the oxygenated substitution pattern of the withanolide skeleton (cf 2).

The crossing of II x III and I x II resulted in plants producing again the same compound 4. These results implied that the enzymatic systems involved in the oxidation reactions producing compound 4 were a dominant factor. For example the OH group at C_{20} present in 9 of type III, however, not occuring in 1 of type I, had to be considered as dominant since it occurred in 4. Similarly hydroxylation at C_4 seemed also to be dominant since it was not present in 9 but occurred in 1, and after the cross is present in 4.

These observations could be far better analysed when the second generation F_2 of I x III, was studied. Some 50 single plants were analysed of this F_2 generation in which the various genetic factors became split and each plant appeared with some of the characters of the parent plants. By observing the presence of the various substituents in the compounds identified in the F_2 generation offsprings, a relationship between dominant substituents and recessive ones could be obtained, disclosing the characters of the corresponding enzymatic reactions responsible for these various reactions in the plant.

The analysis of these results is still preliminary although convincing. It will have to be corroborated through additional experiments on a large number of offsprings as well as on various combinations of crossings.

Let us deal now with the constituents of a different type of *W. somnifera*. This study was done on the leaves of plants raised on experimental plots from seeds obtained from South Africa. They should therefore be considered as a South African type which was different from all the other types investigated in our laboratory.[3]

Chromatography of the crude methanol extract resulted in the separation of 5 withanolides,[3] two of which were readily identified

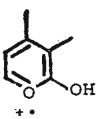
as **1a** and **4a**. The third was found to be **15** which is a 27-desoxy-derivative of **1**.

The characterization of compound **15** is based on the analysis of the spectral data as well as by degradation to the pregnane derivative **8** which has been independently obtained by a corresponding sequence performed on **4**.[5]

Comparison of the nmr signals of **16** and **4** shows a close similarity as far as the characteristic protons of the carbocyclic system are involved. The high field region of the spectrum of the former was clearly different. Instead of the signals of the two vinylic Me groups in **4a**, two doublets [at δ 1.21 (d, $J = 6$ Hz) and δ 1.16 (d, $J = 6$ Hz)] were present implying two secondary Me groups. Since the UV absorption band of **16a** (λ_{max} 214 nm, ε 9500) pointed towards the presence of only one α,β-unsaturated carbonyl system in this molecule, it can be concluded that the 6-membered ring lactone is saturated. Supporting evidence was found in the IR spectrum which showed in the carbonyl region two bands at 1681 and 1730 cm^{-1} (unsaturated 6-membered ring ketone and saturated 6-membered ring lactone, respectively).

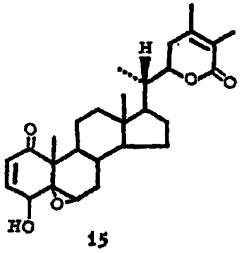
Upon acetylation the monocetate **16b** was obtained, characterized by the new signal of the acetoxy Me group. The catalytic hydrogenation proceeded with the absorption of only one mole of hydrogen, yielding the dihydroderivative **17** devoid of any absorption in the UV region. The reduction of the double bond (Δ^2) could be also followed in the nmr spectrum of **17** by the disappearence of the signals of the vinylic C_2-H and C_3-H and the change in the pattern of the C_4-H (from a doublet at δ 4.60 to a narrow triplet at δ 4.58).

These structural assignments are confirmed through the analysis of the mass spectra of compounds **16a** and **17** (molecular ions M^+ 472 and 516, respectively). These spectra do not have the m/e 125 peak whicr is present in all the withanolides with a double bond (Δ^{24}) in the lactone ring. This peak is due to cleavage of the C_{20}-C_{22} bond, resulting in the ion:



In these two compounds the 100% peak (m/e 345 and 389, respectively) is due to the fission of the same bond and loss of 127 m. u. (the saturated lactone). The presence of the C_{20}-OH facilitates the cleavage of the C_{17}-C_{20} bond yielding a fragment m/e 171 (the whole side chain); the same cleavage in 4a leads to the formation of the ion m/e 169.

Elimination of the tertiary C_{20}-OH in 17 was smoothly performed with thionyl chloride in excess pyridine to give the corresponding crystalline enol-lactone 18. In the previous elimination perform-



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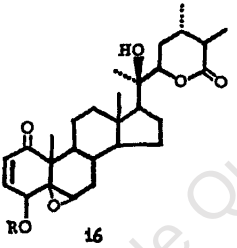

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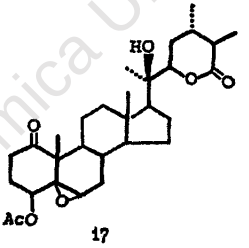

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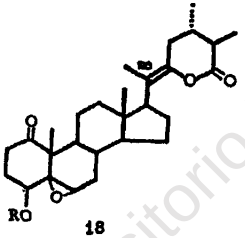

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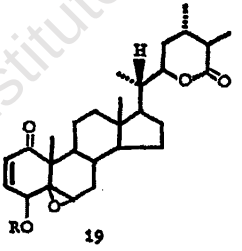

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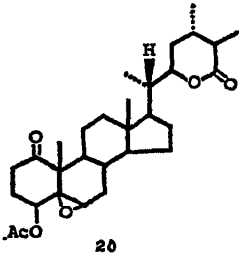

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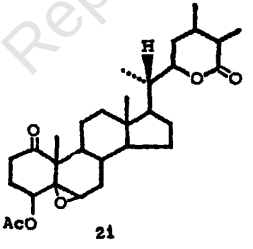

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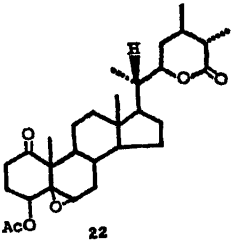

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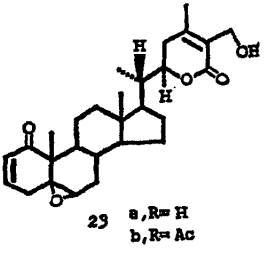
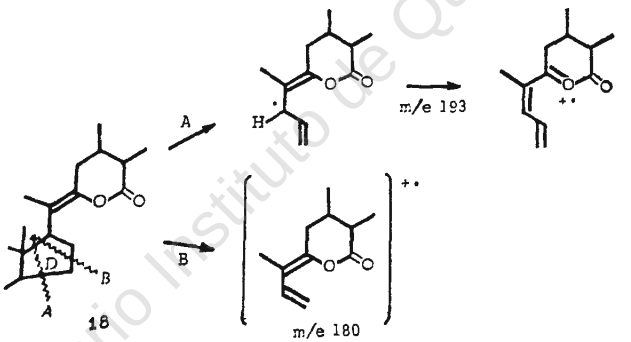


23 a, R= H
b, R= Ac

ed on a derivative of 4 the product (enol-lactone) could not be purified, since already during chromatography on silica gel the exocyclic Δ^{20} entered into conjugation with the endocyclic Δ^{24} to give a mixture of two stereoisomeric pyrone derivatives 7.

The structure of the enol-lactone 18 was determined by inspection of its nmr spectrum and comparison with that of 17, one could observe the disappearence of the signal of C_{22} as well as the expected changes in the pattern of the C_{20}-Me (downfield shift from δ 1.26 in 17 to δ 1.51 in 18 in agreement with its olefinic character.

The enol-lactone was further characterized by three important peaks in its mass spectrum: 498 (M^+; 100% peak), 193 and 180. The m/e 193 signal arises presumably through the fragmentation path A while the fragment m/e 180 is due to cleavage of ring D according to path B.



To ascertain these fragmentations, the $SOCl_2$ induced elimination of the C_{20}-OH was repeated with a deuteriated compound in which the two C_2 hydrogens and the C_{25}-H are exchanged by deuterium (M^+ 501). The mass spectrum of deuteriated 18 shows peaks at m/e 194 and 181 instead of the peaks at m/e 193 and 180 in the non deuteriated compound, i. e. with one m. u. more, respectively. The asumption that these ions contain the lactone ring is thereby confirmed.

Ozonolysis of 18 produced the methyl ketone 8 identical with that obtained from the degradation of 4. The stereochemistry at

C_{20} in **16** is 20α(R) using the same criteria which served for this stereochemical assignment in compound **4**. The data presented herewith firmly establish the structure of **16** as 4β,20α(R)-dihydroxy-1-oxo-5β,6β-epoxywith-2-enolide; the only points which remain for further study are the asymmetric centers of the lactone ring (C_{22}, C_{24} and C_{25}).

The last compound which was isolated from the crude extract only in minute amounts was **19a**; it is related to **15** in the same way as compound **16a** is related to **4a**, i. e. it is the 24-dihydroderivative of **15**. The relevant signals of the protons on the carbocyclic skeleton of **19** are at the same positions and have the same multiplicities, however **19** features two doublets at δ 1.21 and 1.11 indicating two secondary Me groups on saturated C atoms. These data, in conjunction with the ultraviolet spectrum (λ_{max} 214 nm, ε 9550) similar to that of **16** and infrared absorption bands at 1730 and 1681 cm^{-1}, point unambigously to the presence of a saturated lactone ring in **19** as well.

Further insight into the structure of this compound was obtained by acetylation and catalytic hydrogenation to a dihydroderivative **20** devoid of major UV absorption.

At this stage it is worthwhile to refer to the catalytic hydrogenation of withaferin A diacetate **1b**.[1a] The hydrogenation of **1b** could be performed stepwise: reduction of Δ^2, hydrogenolysis of the allylic C_{27}-OH, and saturation of the tetrasubstituted double bond in the lactone ring. The product tetrahydrodesoxywithaferin A acetate **21** was however different from compound **20**.

The close similarity of these two isomeric compounds was revealed by a quasi-identical fragmentation pattern under electron impact. Comparison of their nmr spectra suggested however, that the difference should be in the stereochemistry of the lactone ring. Since compound **21** was obtained by catalytic reduction, a *cis* orientation was assigned to the C-24 and C-25 Me groups; when exposed to sodium methoxide in methanol the product underwent epimerization to **22**, the Me groups becoming *trans*.

Under similar alkaline conditions followed by reacetylation of the C_4-OH **19b** was left unchanged; it was however, different from compond **22**.

It can be concluded therefore that there are subtle differences between the lactone rings in the natural occurring withanolides **16** and **19** and the saturated compounds **21** and **22** prepared in the laboratory through hydrogenation and epimerization procedures.

The conformation of the lactone ring as well as the configuration of the substituents at this ring (C-22 H, C-24 Me, C-25 Me) were elucidated by analysis of the solvent shifts of the corresponding nmr signals together with circular dichroism measurements.[10]

The following analysis which was done for the three stereoisomeric componds **21**, **22** and **20** is based on the assumption that they possess the same configuration at C_{22}; indeed, CD measurements *(vide infra)* confirm the 22R configuration of **21** and **22**, the same as in withaferin A **1a**. The NMR signals of the C_{22}-H show the same multiplicity (double triplet) and appear at about the same position (δ 4.22, 4.37 and 4.33 respectively). Furthermore, one can safely assume that the C_{20} atom, carrying the whole steroid skeleton is equatorially attached to the C_{22} of the lactone ring.

Conformational studies on 6-membered lactones performed by X-ray analysis have shown that the carbonyl, the ethereal oxygen and the two adjacent C atoms lie generally in the same plane.[11] The lactone ring can therefore assume either a half chair or a half boat conformation. Both conformations have actually been found in the crystalline state, the energy difference between them not being therefore too large; if flagpole interactions are however involved the half boat conformation cannot be adopted.

Circular dichroism measurements. Some time ago the configuration at C_{22} of another withanolide, jaborosalactone A **23** was determined, by comparison of its CD with that of parasorbic acid.[12] Since dihydro-**1** as well as several other derivatives also shows a positive Cotton effect in the range of the R band of the conjugated lactone, at about 250 nm they should possess the same stereochemistry at C_{22} (i. e. 22R). The same conclusion was reached independently through the X-ray analysis of a bromobenzoate derivative of withaferin A.[13] Withanolide D acetate **4b** has also similar CD band and it follows that it possesses the same 22 R configuration.

The sign of the $n \rightarrow \pi^*$ band of nonplanar lactones in the

region around 215 nm is determined by the torsion angle along the O = C—C—C system of the ring.[14] Considering the 22 R configuration and the equatorial orientation of the linkage between the lactone and the whole steroid skeleton, a half chair conformation of the lactone should lead to a positive and a half boat conformation to a negative CD band in the aforementioned region.

Actually it was found that the n → π* band in compounds 21 and 20 is negative whereas in compound 22 is positive.

NMR solvent shift measurements. The stereochemical implications of the solvent shifts ($\Delta_{C_6H_6}^{CDCl_3}$) of protons lying in the proximity of a ketone group are well documented.[15] It is known for instance that a proton (or a Me group) on a carbon adjacent to and a weak (usually downfield) shift if equatorial oriented. For the ketone will exhibit a strong upfield solvent shift if axial, lactones the available information is very limited. In the case of 5-membered ring lactones (the study was performed on a whole series of sesquiterpene lactones) a pseudo-equatorial Me group next to the lactone CO shows an upfield solvent shift ($\Delta_{C_6H_6}^{CDCl_3}$) of ~ 14 Hz, whereas a pseudo-axial methyl has a shift of ~ 28 Hz. Both shifts are upfield, but the trend is the same as in ketones: axial substituents have stronger upfield shifts than equatorial counterparts.

The solvent shifts ($\Delta_{C_6H_6}^{CDCl_3}$) measured for the C-25 Me in compounds 20, 21 and 22 (see Table) are +4.5, +5 and +4 Hz, respectively. The conclusion is that in all these compounds the C-25 Me groups have to be similarly oriented with respect to the lactonic CO; to account for such a weak solvent effect, this orientation should be equatorial.

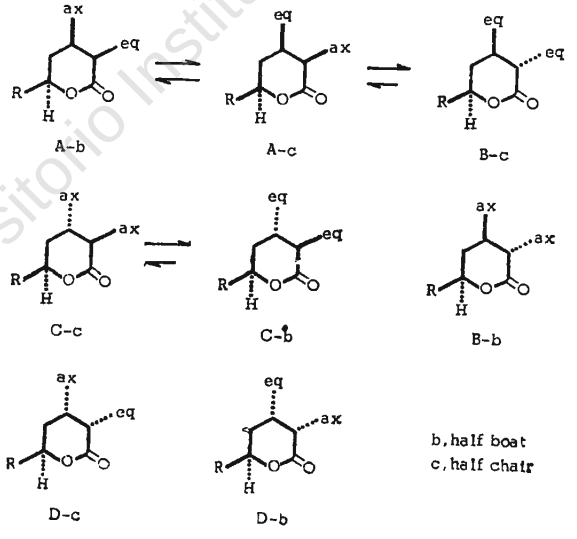
For the purpose of the present discussion the four theoretically possible δ-lactones are designated as A, B, C and D. In each case the half chair and half boat conformations are marked by the symbols *c* and *b*, respectively. The numbering of the C atoms is that used in steroids, and the substituents are referred to as α and β oriented; the skeleton attached equatorially at C_{22} is always β oriented.

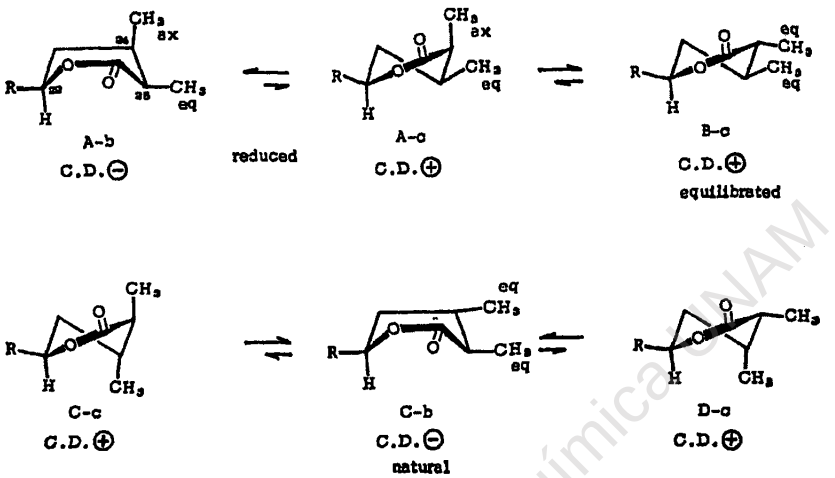
In the lactone ring of compound 21 which is obtained by catalytic hydrogenation of 1b the two Me groups at C-24 and C-25

should be in a *cis* relationship.[1b] In principle four possibilities are available for this lactone: with the two Me groups β oriented (structures A-c or A-b) or α oriented (structures D-c and D-b); since the Cotton effect in the 215 nm region is negative, the two chair possibilities (A-c and D-c) can be disregarded.

Conformation D-b is very improbable due to flagpole interactions; furthermore, the axial C-25 Me should be able to undergo epimerization in alkaline conditions to yield a compound in which this Me is equatorial; the product of such an epimerization would have the lactone in conformation C-b which is, however, incompatible with the positive sign of the Cotton effect (half chair conformation) measured for compound **22**.

The last possibility left for the lactone in compound **21** is therefore conformation A-b, suggested both by the negative CD curve and by the equatorial orientation of C-25 Me, as deduced from the nmr solvent shifts data. Thus, the process of epimerization of compound **21** into **22** can best be imagined through a sequence involving first the flipping of structure A-b to A-c, in which the C-25 Me becomes axially oriented, being therefore readily epimerized. The lactone ring in **22** should be consequently assigned





structure B-c with the two Me groups in a trans diequatorial relationship. This conclusion is supported by the positive Cotton effect of the latter in the n → π* band region; since 22 has been obtained in equilibrating conditions, the equatorial orientation of the C-25 Me is obvious, being thermodinamically more stable, Furthermore, according to the solvent shifts data this Me group should indeed be very close to the plane of the lactonic CO. A boat conformation (B-b) for this lactone is highly improbable due to flagpole interactions.

NMR Solvent Shifts Data for the C-24 and C-25 Me Groups in Certain Withanolides (in Hz at 60 MHz)

	C-25 CH_3			C-24 CH_3		
Compound	$CDCl_3$	C_6H_6	$\Delta \frac{CDCl_3}{C_6H_6}$	$CDCl_3$	C_6H_6	$\Delta \frac{CDCl_3}{C_6H_6}$
21	68	63.5	+4.5	55.5	39.5	+16
22	79.5	74.5	+5	65	41.5	+23.5
20	73	69	+4	67	42	+25
17	72.5	65	+7.5	69.5	38.5	+31

The same analysis can now be done for compound **20** which, in contrast to **21** and **22** possesses a saturated 6-membered ring lactone of natural occurrence. The negative sign of the Cotton effect associated with the lactone ring in **20** excludes the possibility of a chair conformation, as represented by structures C-c and D-c; since the compound remains unchanged upon exposure to alkaline conditions, i. e. it does not undergo epimerization at C-25, structure D-b can be discarded as well, the last possibility being that described by C-b. Supporting evidence for this structure is found again in the weak solvent shift measured for the equatorial C-25 Me.

It is noteworthy that in all the structures proposed for the lactone (A-b, B-c and C-b, respectively) 1,3 diaxial repulsive interactions are reduced to minimum.

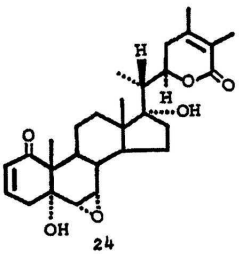
The lactone ring in compound **17** should also possess structure C-b, by the same arguments used for compound **20**.

Supporting evidence for the above conclusions can be found in a work due to Wolf,[14] showing that the CD maximum of a half chair conformation should lie at longer wavelengths than that of the corresponding half boat conformation. Indeed, λ_{max} (EtOH solution) for **22** (structure B-c) is longer by 11 and 7 nm, respectively, than for compounds **21** and **20** (structures A-b and C-b).

In connection with the nmr solvent shifts of these compounds there is still another observation which deserves further comment. The $\Delta_{C_6H_6}^{CDCl_3}$ values for the C-24 Me in **21**, **22**, **20** and **17** are +16, +23.5, +25 and +31 Hz, respectively. According to the above analysis this Me group is axial in **21** and equatorial in the three other compounds. It follows therefore that a Me group on a C atom β to the lactonic CO experiences an upfield solvent shift; however, the shift of an equatorial group is larger than that of the axial counterpart. These observations made for shifts induced by benzene in 6-membered ring lactones are not without precedent in the cyclic ketones,[16] they can be rationalized in terms of the geometry of the aromatic solvent solute collision complex which is formed. In such a complex, the benzene molecule orients itself on the positive side of the CO dipole, at about right angles to it. Such a model would explain also the larger upfield solvent shift experienced by the equatorial C-24 Me, in the lactones **20**, **21** and

17 as compared to the shift of the corresponding axial Me in the lactone 21. According to the specific geometry of the aromatic solvent-solute complex, an equatorial methyl β to the CO is closer to the aromatic molecule than an axial Me.

Extending the analysis of the types to populations growing in various parts of the world we would like to discuss shortly our studies on a population naturally occurring in North Western In-



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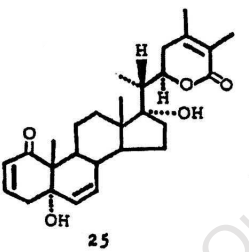

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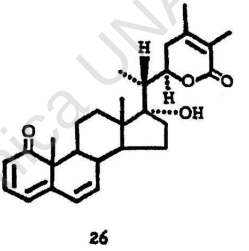

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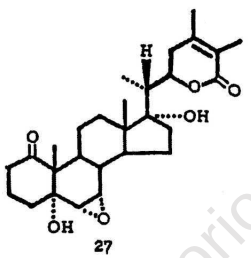

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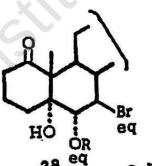
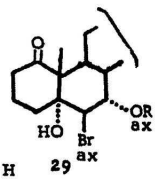


28 a, R=H b, R=Ac



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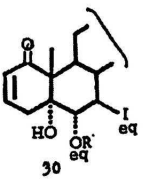

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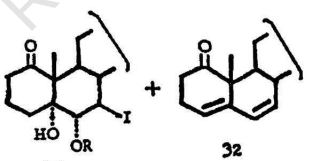

31 a, R=H b, R=Ac

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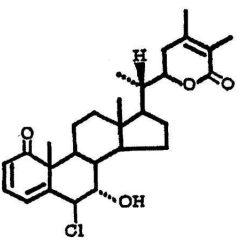

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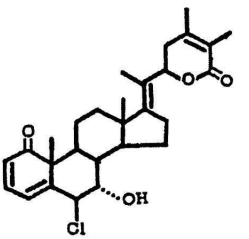

34

dia. This population was found to be different from those previously investigated by us and constitutes a chemotype which we call "Indian I".

Chromatographic separation of the crude extract of the leaves afforded twelve withanolides, and the structures of the most characteristic two compounds **24** and **25** are hereby discussed. Without entering into the detailed structural elucidations of these compounds let us describe some of the interesting reactions connected with both of them.

The allylic position of the 5-OH in (25) is demonstrated by its smooth acid catalysed elimination under mild conditions yielding the trienone **26** in which the second OH group (at C_{17}) remained unchanged. This compound has also been obtained by the hydroiodic acid reduction of **24** as later described. The presence of two OH groups in **25** is supported as well by the nmr of its bis-trichloroacetylcarbamate derivative in which two characteristic N-*H* signals[8] are observed at δ 8.60 and 8.66. The respective orientations of the 5-OH and of the oxirane ring had to be assigned. Steroidal 1-ones (5α and 5β) can be readily distinguished according to the solvent shift ($\Delta_{C_6H_6}^{CDCl_3}$) of the 19-methyl protons: in 5α-1-one, this shift is +15 Hz,[17] whereas in a 5β-1-one it is -7 Hz.[18] The corresponding shift of the 19-H in **27**, (the 2,3-dihydro-derivative of **24**) is +15.6 Hz in perfect agreement with a *trans* junction between rings A and B (OH α-oriented). Additional evidence was given by the pyridine induced shift ($\Delta_{C_6H_6}^{CDCl_3}$)[9] which accounts for the relative orientation of the 5-OH to the 19-methyl group. The corresponding values are: for 5α-hydroxy-1-oxocholest-2-ene, −0.05 ppm, and for 5β-hydroxy-1-oxocholest-2-ene, −0.35 ppm, while compound **24** showed a value of −0.07 ppm in accordance with the former stereochemistry.

The steric course of the epoxidation with peracid of allylic alcohols is well known.[19, 20] In a steroid, the rear approach of the epoxidising agent is favoured because of steric hindrance on the top side of the molecule; provided that the allylic 5-OH is α oriented, the directing effect of the latter will contribute to the exclusive formation of the α *cis* epoxy alcohol, **2** → **24**, producing a 5α-hydroxy-6α,7α-epoxy derivative. In the event of a 5β-OH the

above factors should act in opposite directions to give a mixture of 6α, 7α and 6β, 7β epoxides. The epoxidation of **25** proceeded stereoselectively and therefore, the first possibility (6α, 7α epoxide) is the most probable orientation. With these considerations we can now analyse some opening reactions of the epoxide ring, which substantiate the α orientation of both substituents of ring B.

In a rigid system, opening of an oxirane ring leads usually to the formation of a *trans* diaxial derivative (cf. the Fürst-Plattner rule[21]). The steric course of the reaction can be, however, changed under the influence of a neighbouring hydroxyl or acetate group.

In the present case, treatment of **27** with HBr led to a mixture of two bromohydrins, the *trans* diequatorial **28a** and the *trans* diaxial **29a** in a ratio of 7:3 in **28a** the nmr signal of 6-H is a doublet at δ 3.76 (J = 10 Hz) whereas the 7-H features a broad triplet at δ 4.06. Following acetylation, the signal of the 6-H in the bromoacetate **28b** is shifted downfield by a 1.5 ppm, whereas the signal of the 7-H remains practically unchanged (δ 4.11). The relationship between the 6-H and 7-H could be ascertained by double resonance. The large coupling constants between the 7-H and the adjacent 6-H and 8-H determine the *trans* diaxial relationship between 6-H and 7-H and consequently the *trans* diequatorial orientation of the acetoxyl and bromo substituents.

The second bromohydrin **29a** exhibits a doublet at δ 3.39 (J = 5 Hz) and a double doublet at δ 3.90 (J = 5 and 4.5 Hz); in the corresponding bromoacetate **29b** only the double doublet is shifted downfield (δ 4.97) pointing therefore towards a 6-bromo-7-hydroxy arrangement. Irradiation of the 6-H doublet (the experiment was performed on **29a** as well as on **29b**), induced the collapse of the double doublet to a doublet (J = 4.5 Hz), while irradiation at the frequency of the 7-H led to the appearance of a sharp singlet for 6-H. The magnitude of the coupling constants between 6-H and 7-H (5 Hz) and between 7-H and 8β-H (4.5 Hz) is indicative for a *trans* diequatorial and equatorial-axial relationship, respectively. The hydroxyl (acetoxyl) and bromo-substituents have to be therefore *trans* diaxial oriented. Supporting evidence for the β-axial orientation of the 6-bromo substituent was also found in the large deshielding (1,3-diaxial interaction) experienced by the 19-

methyl signal [from δ 1.21 in **27** to δ 1.42 in **29a**; in **28a** this signal is at δ 1.30]. The preponderant formation of the *trans* diequatorial bromohydrin **28** illustrates clearly the neighbouring group effect on the steric course of the opening of the epoxide ring.

The relationship between the different substituents in **24** was further confirmed by treatment with hydroiodic acid leading again to the opening of the epoxide ring. The major product of this reaction (60%) was the *trans* diequatorial iodohydrin **30a**, also characterized as the corresponding iodoacetate **30b.** The nmr signals of the 6-H and 7-H followed closely the corresponding signals in the bromohydrin **28a** and its acetate **28b** respectively, their relationship being again confirmed by decoupling experiments. The second product of this reaction (40%) was **26** (with a trienone system in rings A/B) characterized by the u. v. absorption, λ_{max} 357 nm (ε 3600) and 223 nm (ε 9000), the IR bands at 1695, 1653 and 1626 cm^{-1}, and the nmr signals for 5 vinylic protons. The 2-H and 4-H are split by the 3-H, appearing therefore as doublets at δ 5.89 (J = 9.5 Hz) and 5.92 (J = 5.5 Hz), whereas the 3-H gives a double doublet at δ 6.95 (J = 9.5 and 5.5 Hz). The 6-H and 7-H are reciprocally coupled by 10 Hz; the 7-H couples also with 8β-H and appears therefore as a double doublet at δ 6.13 (J = 10 and 2.5 Hz), while the doublet of the 6-H is only slightly broadened by long range coupling. The trienone **26** owes its formation to the intermediate *trans* diaxial iodohydrin (6β-iodo-7α-hydroxy) which eliminates the elements of HOI with the excess HI present in the reaction mixture leading to the subsequent formation of free iodine; water is then eliminated from the intermediate allylic alcohol leading ultimately to the trienone. The elimination of HOI is possible only from the *trans* diaxial iodohydrin in which the substituents are favourably disposed (antiparallel) for such a reaction. Indeed, no reaction whatsoever occurred when the diequatorial iodohydrin **30** was treated with an excess of HI.

The same reaction (with HI) performed on compound **27** proceeded in a similar way leading to a mixture of the *trans* diequatorial iodohydrin **31** and the heteroannular 4,6-dien-1-one **32**, in the same ratio of 6:4. Compound **32** was characterized by its u. v.

spectrum, λ_{max} 234 nm (ε 31,500) and shoulder at 249 nm (ε 20,000). It should be kept in mind that this absorption results from overlapping with the unsaturated lactone chromophore: 226 nm (ε 7900). This structure is supported by the low field nmr signals of the three vinylic protons.

An additional step in the elucidation of compound **24** was made by treatment with thionyl chloride taking place with elimination of the two hydroxyl groups to yield a mixture of compounds **33** and **34** which could be separated by chromatography. This reaction confirmed the previous assignment of one tertiary OH at C-5 and allowed the unequivocal allocation of the other hydroxyl at C_{17}.

In order to determine the orientation of the 17-OH, advantage was taken of the shifts induced by pyridine on the protons situated in the proximity of the hydroxyl group.[9] These figures for **24** are in good agreement with an α orientation of the 17-OH.

	18-H	21-H	22-H
$\Delta_{C_5H_5N}^{CDCl_3}$	−0.01	−0.16	−0.22 ppm

Indeed in saturated cyclic systems protons in a 1,3-diaxial relationship with a hydroxyl group undergo a downfield shift ($\Delta_{C_5H_5N}^{CDCl_3}$) of 0.2-0.4 ppm. Truly, the 17α-OH and the 22α-H are not involved in such a system, however, the crowing around the C_{17}-C_{20} bond (the lactone and the whole steroid skeleton) makes highly probable a preferred staggered conformation in which the groups (17-OH and 22-H) taking part in the interaction are in front of each other in a way similar to a 1,3-diaxial disposition, producing thereby a solvent shift for the 22-H, within the above range. Conversely, the 18-methyl protons should experience a very small downfield shift since the dihedral angle substended by the C_{13}-C_{18} and the C_{17}-O bond is ~ 160°; indeed, in 17α-hydroxy-5α,14α-androstane for example,[9] the corresponding shift is −0.05 ppm. As for the 21-methyl group, the downfield shift of −0.16 ppm is acceptable for a *gauche* relationship within the 17α-OH.

Acknowledgement

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