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THE WITHANOLIDES AS A MODEL IN PLANT GENETICS: CHEMISTRY, BIOSYNTHESIS AND BIOLOGICAL ACTIVITY *

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ABSTRACT: The withanolides are oxygenated steroidal lactones occurring in a number of species of the Solanaceae plant family. In *Withania somnifera* (L.) Dun., which has a fairly wide geographical distribution, several chemotypes have been identified. They differ primarily in their respective composition in withanolides displaying a variety of substitution patterns.

Through hybridization performed between different types originating from various parts of the world, followed by chemical analysis of the constituents present in the offsprings, genetic characters were identified at the chemical level. Several hybrids were chemically analysed and thereby a study of the inheritance characteristics of a number of chemical groups was made possible. Biosynthetic pathway are suggested based on the occurrence of appropriate intermediates. A summary of the biological activity is provided.

KEY-WORDS: Withanolides: chemistry, biosynthesis, biological activity.

INTRODUCTION

Withania somnifera (L.) Dun. is a shrub of the Solanaceae plant family, with a fairly wide geographical distribution. In Israel it grows at least as three distinct chemotypes differing in their leaf content of the various withanolides, C-28 oxygenated steroidal lactones with various substitution patterns.[1] Chemotypes are considered as groups of plants which morphologically are similar in all respects, however, differ by their chemical constituents; in the present case through the composition of the withanolides in the leaves, and the respective quantities.

In this paper we will discus the constituents of a number of chemotypes, describe some highlights of their structures, analyse and discuss

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the composition and substitution pattern of the compounds formed in the hybrids obtained by the cross pollination between different chemotypes.

This approach enabled an understanding of the inheritance at the chemical level, and led to the disclosure of biosynthetic pathways. It could provide a certain control of the formation of specific compounds in the plant.

It is interesting to note that other genera of this family (Solanaceae) as Physalis, Acnistus, Jaborosa, and Nicandra, also produced withanolides and related structures which showed certain skeletal alterations. [2]

A number of withanolides were found to have antineoplastic activity and immunosuppressive properties. [3]

RESULTS AND DISCUSSION

The compounds found in chemotype I have been described in an earlier publication. [4] Withaferin A (*1*) is always the major product which is formed, whereas the others, *2-5*, are obtained in small quantities. Chart 1 shows some of the compounds present in this type. It could be seen that OH groups are present in various positions of the carbon skeleton, however, no such group could be found at position C-20 of the side chain. In rings A/B the 4β-OH, 5β, 6β-epoxy system is observed in most compounds and is present in the product formed in the largest quantity, withaferin A (1). This system is a predominant feature of the compounds of this type. [5]

The compounds identified in chemotype II (Chart 2) have all an OH group at position C-20, a feature inherent to the compounds of this group. [4, 5] Here again the 4β-OH,5β,6β-epoxy system is present in most cases. The compound which accumulates and is present in the largest quantity is withanolide D (*6*). It forms 88% of the total withanolide content of the leaves, whereas the others are formed by far in smaller quantities.

In chemotype III several compounds have been isolated, some of which are presented in Chart 3. [6, 7] Two groups of compounds have been identified, those having an isolated double bond in ring B, in addition to the usual 2-en-1-one system of all the whithanolides; these compounds in most cases have a 14α-OH group, recently identified in all of them [7] together with the usual β-oriented lactone side chain. The second group is represented by withanolide E (*13*) in which the side chain is α-oriented. [8, 9] This inverted stereochemistry is a unique feature in a naturally occurring steroid, and was found so far only in this group of compounds. In all the products existing in chemotype III, no 4β-OH has been detected. Only in withanolide E (*13*) is an epoxide present at position 5,6 of ring B.

The above observations led to the idea that the formation of the different compounds, with their inherent characteristics concerning the substitution patterns, is produced by specific enzymatic reactions genetically controlled. Therefore, it was interesting to see how genetic mani-

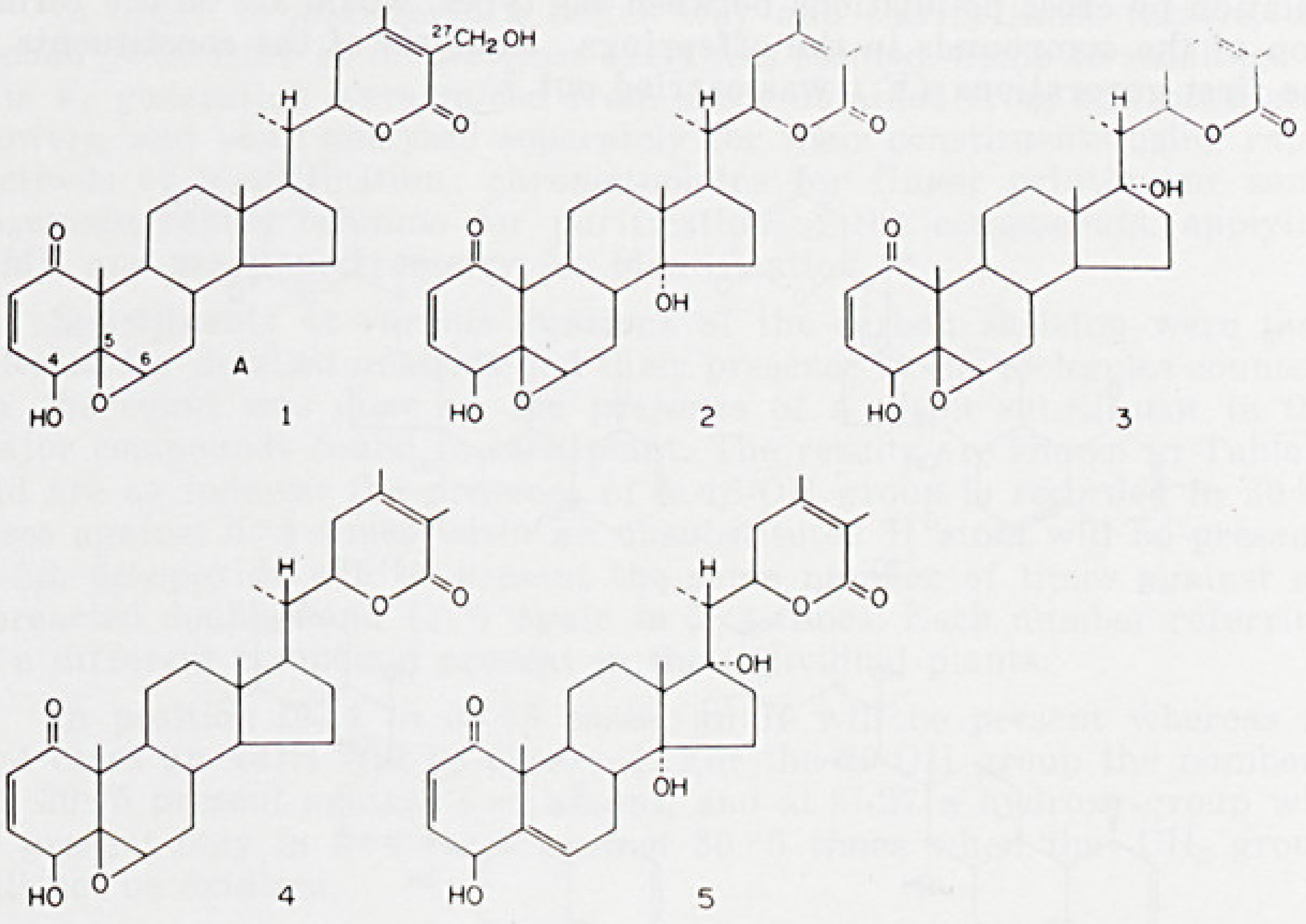


Chart 1 Chemotype I

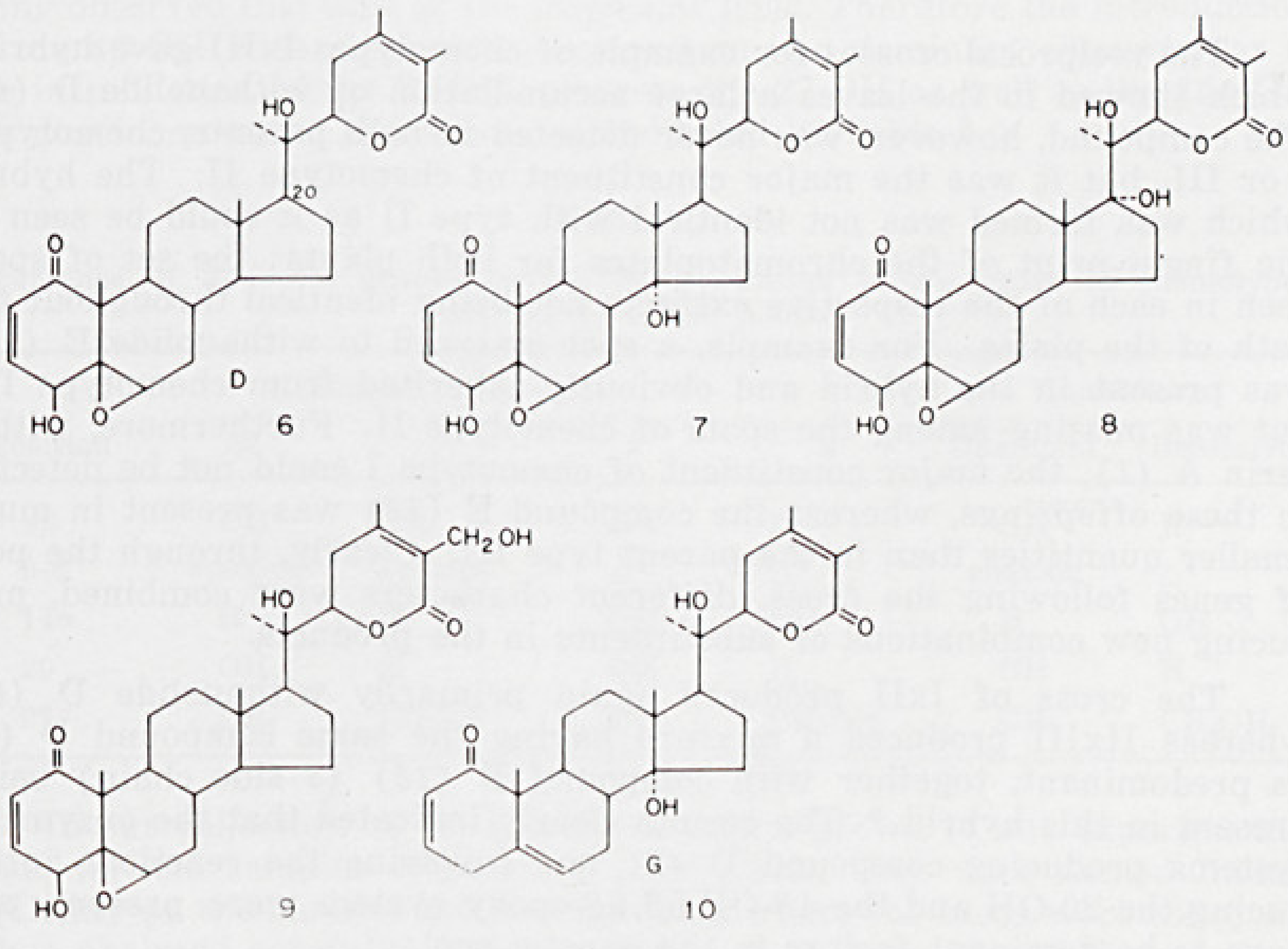
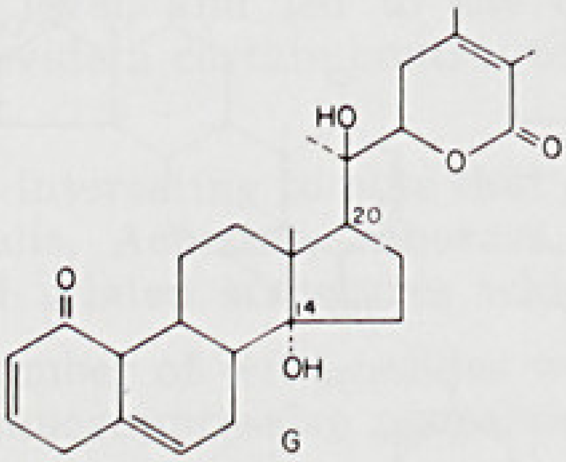


Chart 2 Chemotype II

pulation by cross pollinations between the types, would act on the formation of the compounds in the offsprings. Analysis of the constituents of the first generations (F_1) was carried out. [5]



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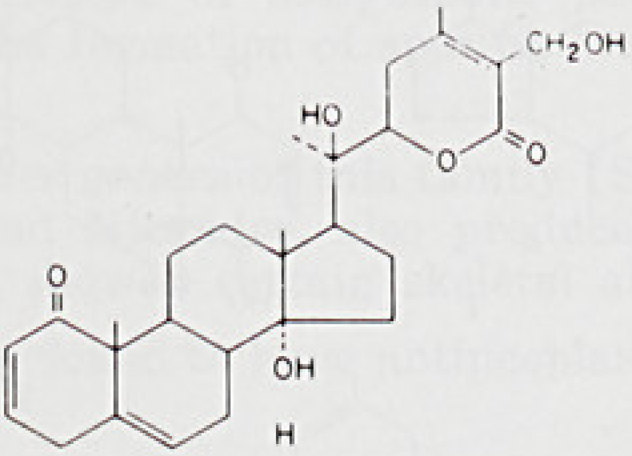

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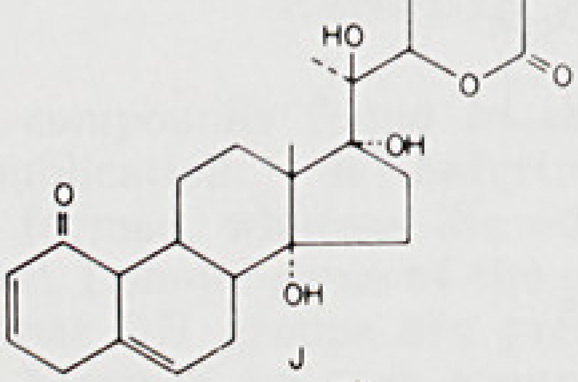

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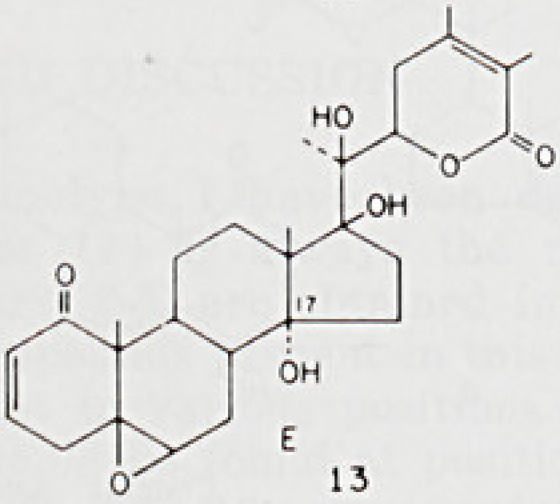

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Chart 3 Chemotype III

The reciprocal crosses for example of chemotypes IxIII gave hybrids which showed in the leaves a large accumulation of withanolide D (*6*). The compound, however, was never detected in both parents, chemotypes I or III, but it was the major constituent of chemotype II. The hybrid which was formed was not identical with type II as it could be seen in the finger-print of the chromatoplates for both plants; the set of spots seen in each of the respective extracts not being identical throughout the path of the plates. For example, a spot assigned to withanolide E (*13*) was present in the hybrid and obviously inherited from chemotype III, but was missing among the spots of chemotype II. Furthermore, withaferin A (*1*), the major constituent of chemotype I could not be detected in these offsprings, whereas the compound E (*13*) was present in much smaller quantities than in the parent type III. Clearly, through the pool of genes following the cross, different characters were combined, producing new combinations of substituents in the products.

The cross of IxII produced again primarily withanolide D (*6*), whereas IIxIII produced a mixture having the same compound D (*6*) as predominant, together with compound E (*13*) (α side chain) being present in this hybrid. [5] The results clearly indicated that the enzymatic systems producing compound D (*6*) and involving the reactions introducing the 20-OH and the 4β-OH,5β,6β-epoxy system were present, and have to be dominant factors in the genetic pool.

In order to analyse in a better way and clarify these factors, the second generation F_2 of the cross IxIII was studied. Some 45 seedlings of this F_2 generation were raised from seeds obtained from self pollinated flowers, and were analysed separately for their constituents using rapid methods of identification: chromatoplates for finger printing or small chromatographic columns for purification of the components, applying NMR and mass spectrometry for identification.

Substituents at various locations of the carbon skeleton were then selected for detailed analysis and their presence in the molecules counted, i.e. the count was done on the presence of a given substituent in the major compounds found in each plant. The results are shown in Table 1 and are as follows: the presence of a 4β-OH group is recorded in 30+6 cases against 5+4 times when an unsubstituted H atom will be present. A 5β, 6β-epoxide will be present the same number of times against an unreacted double bond ($\triangle^5$) again in 5+4 times. Each number referring to a different compound present in the individual plants.

In position C-14 in 30+6 cases, an H will be present whereas in 5+4 cases an α-OH will be observed. For the 20-OH group the numbers are 30+5 present against 6+4 absent, and at C-27 a hydroxy-group will be present only in 6+4 cases against 30+5 times when the $-CH_3$ group will not be oxidized.

The relation between occurrence and absence of groupings in the selected positions is 1:3.5 to 1:4, a relation fitting very well into the genetic inheritance rules, and since we observe the products of enzymatic reactions taking place in the cells which are controlled by genetic characters, one can refer to these characters as *dominant* or *recessive* while being observed this time at the *molecular level.* Therefore the introduction of the 4β-OH with a 5β, 6β-epoxy system is a dominant character as is the introduction of the 20-OH, whereas the 27-OH as well as the 14α-OH are due to recessive characters as indicated in Table 1.

TABLE 1

Occurrence of different groups in the F_2 generation of the cross of chemotypes IxIII in 45 single plants

	Number of cases				Characters	
Position	30	6	5	4	Dominant	Recessive
4β	OH	OH	H	H	OH	H
5-6	epoxide	epoxide	$\triangle^5$	$\triangle^5$	epoxide	$\triangle^5$
14α	H	H	OH	OH	H	OH
20	OH	H	OH	H	OH	H
27	CH_3	CH_2OH	CH_3	CH_2OH	CH_3	CH_2OH

In our collection of *W. somnifera* plants grown in our experimental plots, we had samples originating from India, they were named Indian I (Delhi) on the basis of the location from where the seeds were collected. When analysed carefully, a large number of withanolides of the most varied nature were isolated and identified [10]. Chart 4 shows some representative structures indicating that the outstanding feature in this chemotype is the appearance of a different and characteristic substitution pattern in rings A and B, namely the 5α-OH, 6α, 7α-epoxy system as observed in withanone * (14). This compound has also a 17α-OH. Withaferin (1) was also found to be present in this type in sizable quantities so that the plant has the outstanding ability of producing concurrently the two systems in rings A/B, the 5α-OH, 6α, 7α-epoxy, and the 4β-OH, 5β, 6β-epoxide. Advantage was taken of this characteristic in our subsequent genetic studies and cross-breedings. The most interesting feature inherent to this plant was, however, the fact that at position C-20 in *all* compounds no OH group was ever found to be present [10].

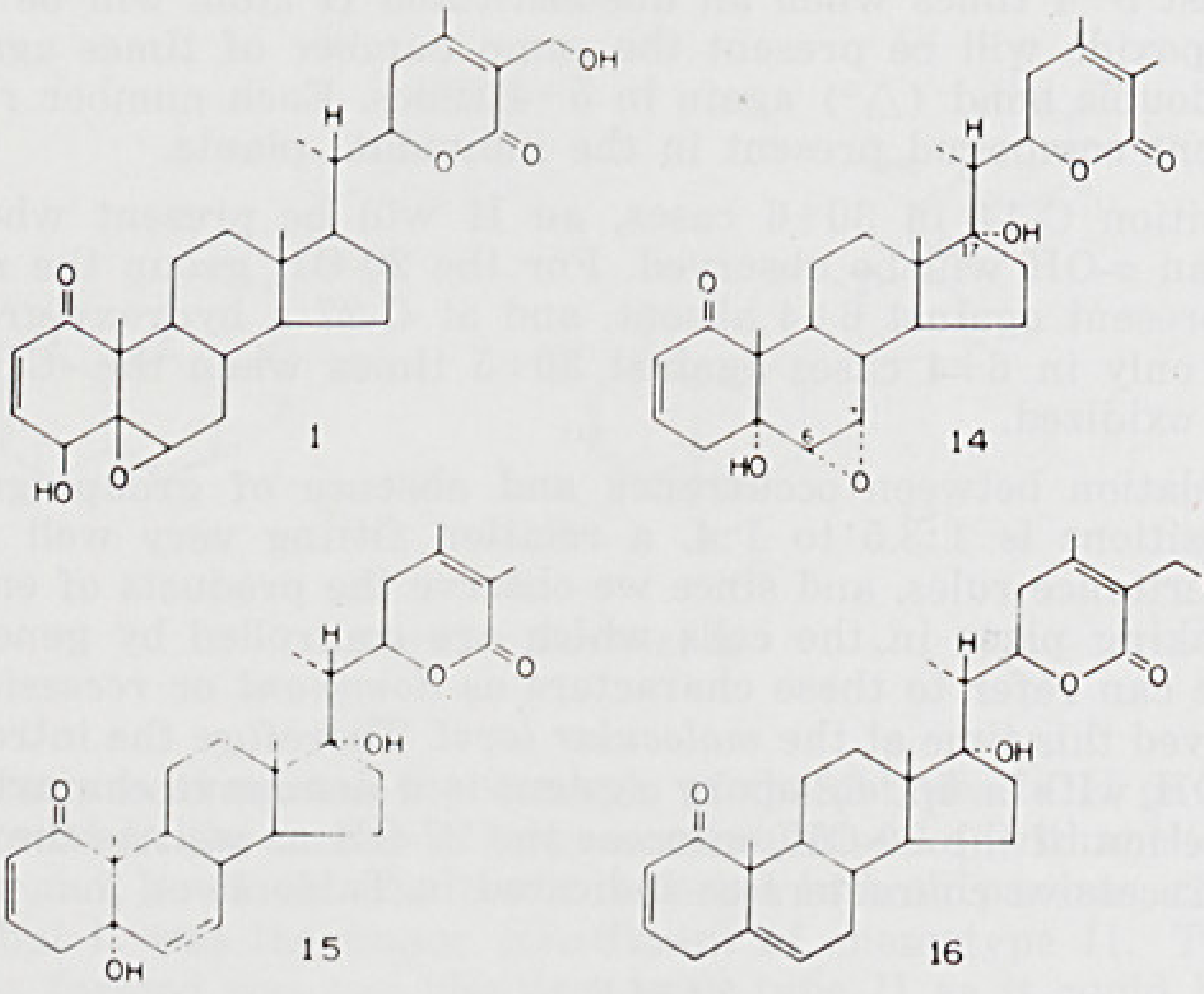

Chart 4 Chemotype Indian I

Following the hybridization of Indian I by chemotype III offsprings were obtained in which withanolide D (6) was formed replacing withaferin A (1) which disappeared from the mixture, i.e., a compound with a 20-OH, dominant character, instead of the recessive 27-OH. Furthermore, a new "non natural" withanolide was produced, withanolide T (17), which is a 20-OH withanone, a compound that the plant Indian I was unable to prepare. Such a compound was the product of the new enzymatic changes originating from the genetic pool of the hybrid [11].

Recently the analysis of the cross of chemotypes III by Indian I was repeated [12]. Also a study of the hybrid of chemotypes II by Indian I was carried out [12]. Table 2 provides the compounds isolated from these two F_1 hybrids given in percent of the respective total withanolide con-

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* Withanone is the name given to a compound isolated from plants collected in India, and first identified as compound IV in Ref. 10. This name is now adopted.

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TABLE 2

Withanolides given as percent relative to their total occurrence

Compound	Chemotypes			Hybrids	
	Ind.I *[10]	II *[4]	III *[6]	II x Ind. I	III x Ind. I
Withanolide G (10)			3.9	1.0	8.0
Withanolide I			0.4		2.4
Withanolide J (12)			2.6		0.4
Withanolide U					2.4
Withanolide E (13)			91.9		
Withaferin A (1)	33.4				
Withanolide D (6)		88.0		62.0	49.4
14-Hydroxy-20-desoxywithanolide D	0.9				
27-Hydroxy-withanolide D (9)		10.7		2.5	
Withanone (14)	58.0			18.6	16.7
20-Hydroxy-17-desoxywithanone (18)				3.6	6.3
Withanolide T (17)				8.3	9.6
14α-Hydroxy withanone					0.3
14β-Hydroxy withanone				4.1	2.1
6,7-Desoxy withanone (15)	0.9				

* The values are computed on the basis of the reported data.

tent. The data of the relevant parents are also incorporated. Table 3 provides the combined quantitative occurrence of each substituent in relation as percent of the total withanolide content, each being treated as a separate item.

TABLE 3

Calculated occurrence in percent of each substitution in relation to the total withanolide content

Substituents	III (Isr.) x Ind. I	III (Isr.)	Indian I	II (Isr.)	II (Isr.) x Ind. I
4β-OH	51	—	35	99	64
5β,6β-Epoxide	49	92	35	99	64
20-OH	78	100	—	99	77
14α-OH	13	99	1	1	1
17α-OH	27	3	64	0.5	31

From the cross breedings described above, we know that three substituents are introduced due to dominant factors, those are the $20\alpha_F$-OH, the 4β-OH and the 5β, 6β-epoxide. One can see that if theoretically each of these substituents should occur as 75% of the mixture, due to the dominant character, for the first (in II by Indian I), we have recorded 78%, for the second 51% and for the third 49%. The lower values for the last two positions are due to the fact that there is in this case a competition with other enzymes, those producing the 5α-OH and 6α, 7α-epoxide, characteristic of the withanone system (14) present in the Indian I chemotype. The 14α-OH and 17α-OH are both due to recessive characters and instead of the expected 25% the observed quantities are 13% for the former and 27% for the latter. The lower value of 13% may be attributed to some interference by the 17α-OH. Also some 14α-OH may have been converted to 14β-OH derivatives through formation of a Δ^{14} followed by reoxidation as described earlier. [12, 13]

Concerning the hybrid II by Indian I, [12] similar conclusions can be drawn. The 20-OH appeared in 77%, the 4β-OH and 5β, 6β-epoxide in 64% each, again in lower values due to competition with the withanome (14) rings A/B system. The 14α-OH is missing, or occurring in trace quantities in the two parents and so in their hybrid (1%), whereas the 17α-OH is present in a normal level 31%.

Analysis of a hybrid derived from the cross of chemotype II (Chart 2) with a type originating from South Africa, [14] Chart 6, grown in our plots, indicated that the dominant characters present in both parent, namely 4β-OH and 5β, 6β-epoxide prevailed (100%). Concerning the 20-OH group present only to the extent of 10% in the African parent, [15] it must be due to a different enzyme than the one encountered in all other cases, and known to be connected with a dominant character. Indeed, following the cross with chemotype II, 95% of this group was produced in the compounds.

The double bond reducing capacity (specific to this type) which was present in the South African parent [15] to a small extent, 5.8% for the 24,25-dihydro compounds (20, 21) should be considered as a recessive character. Following cross with chemotype II it reached the expected level of 27.5%. Interestingly, this character expressed itself also by reducing the 2,3-double bond only in the hybrids and to the extent of 22.4% (Table 4).

Table 4 provides the occurrence in percent of various substitutions in chemotypes II (Israel) and the South African, and in the product of their cross; it sums up some of the remarks provided above.

TABLE 4

Calculated occurrence in percent of substituents in *W. somnifera* chemotypes South African, II (Isr.) and their cross

Substituents	South African (%)	II (Israel) (%)	Cross (%)
4β-OH	100	99.6	100
Δ^5	—	0.3	—
5β, 6β-Epoxide	100	99.6	100
$20\alpha_F$-OH	10.5	99.0	95.0
24,25-Dihydro	5.8	—	27.5
2,3-Dihydro	—	—	22.4
2-Ene-1,4-dione	—	—	2.8

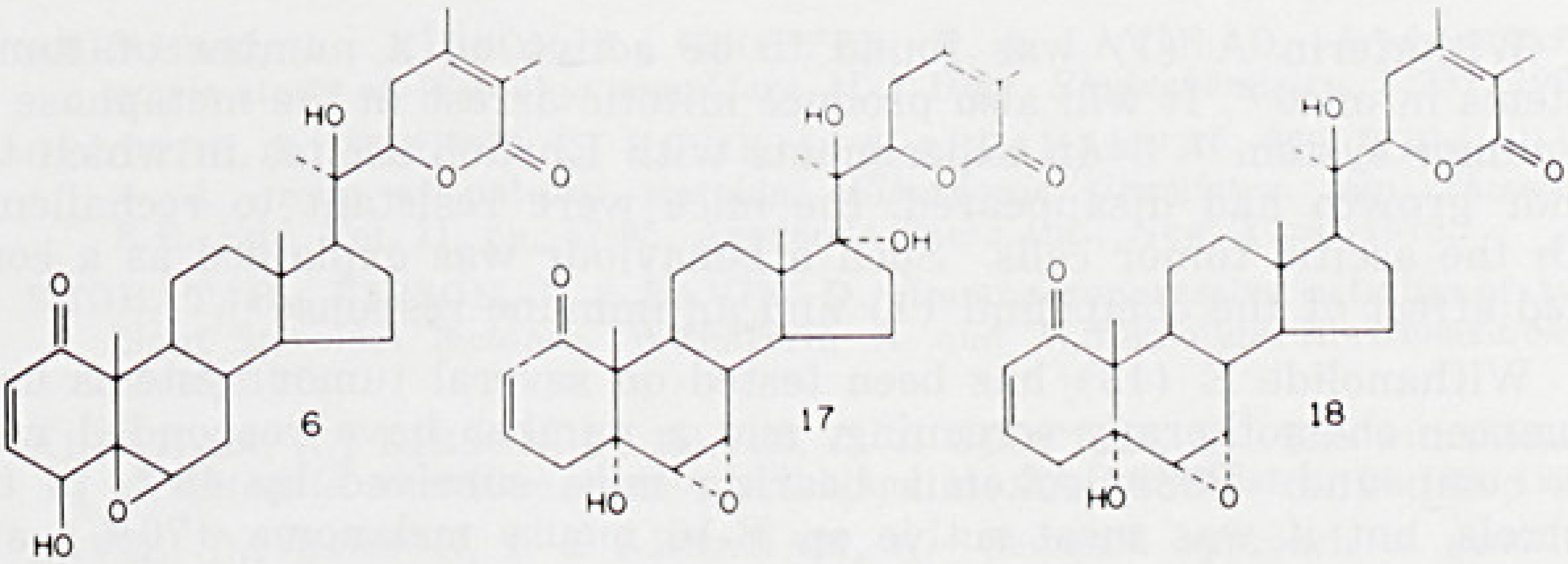

Chart 5 Hybrid of Indian I by Chemotype II

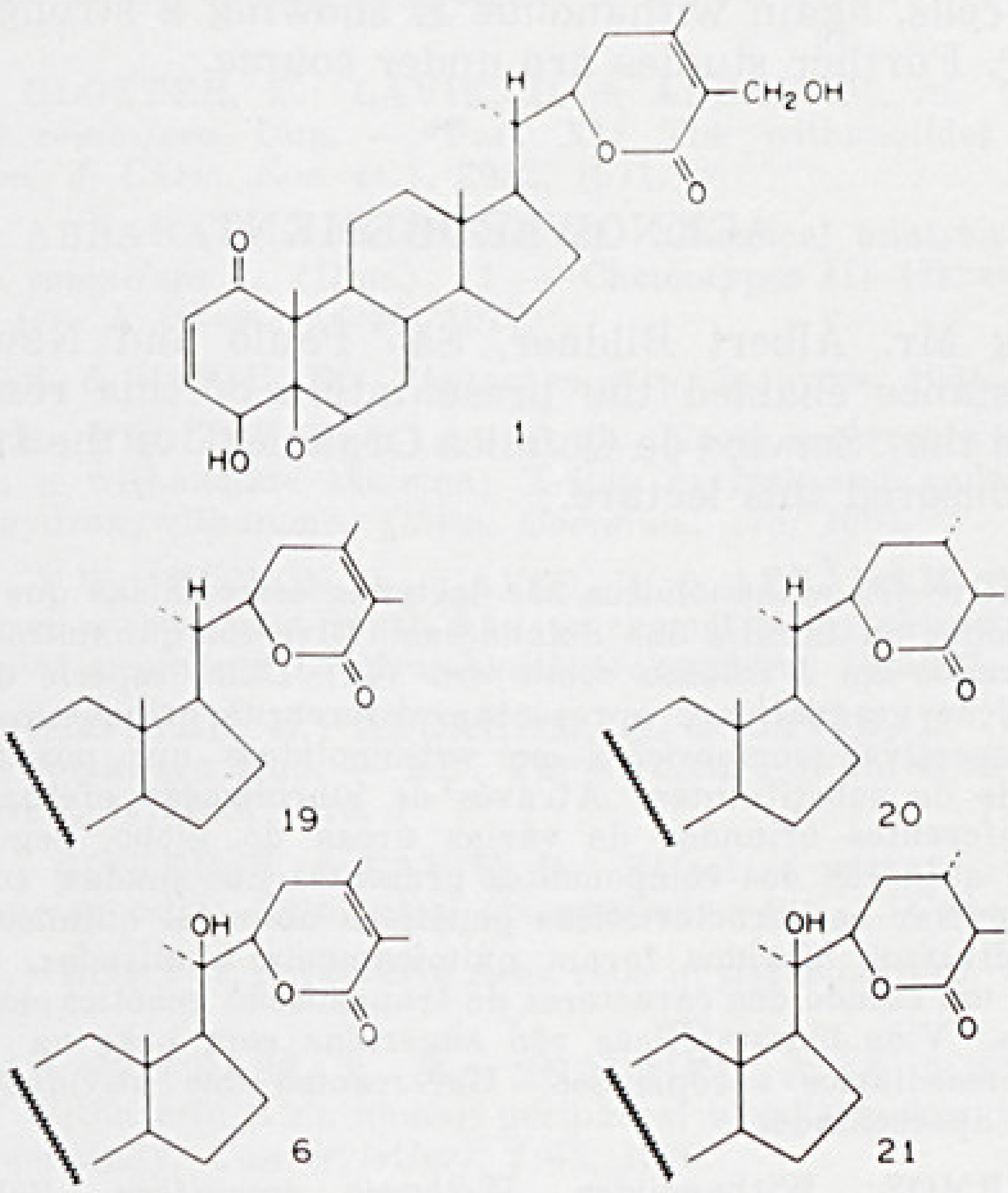

Chart 6 South African Chemotype

BIOLOGICAL ACTIVITY

Withaferin A (1) was found to be active on a number of tumor systems in mice[16]. It will also produce mitotic arrest in the metaphase of the tumor system[17,18]. In experiments with Ehrlich ascites in which the tumor growth had disappeared, the mice were resistant to rechallenge with the ascitic tumor cells. Such a behaviour was explained as a combined effect of the compound (1) and an immune response[17].

Withanolide E (13) has been tested on several tumor systems used in cancer chemotherapy screening, and a number have responded with this compound. P388 leukemia bearing mice survived by 48% to the controls, but it was most active on B-16 mouse melanoma (70%) and Lewis lung carcinoma (unpublished results).

The immunosuppressive effects of the two compounds (1) and (13) were tested on a number of systems[3]. Four tests were performed with human T and B lymphocytes since the main interest was the mode of action of these compounds on the human immune system. Both compounds showed an effect on E and C_3 human lymphocytes receptors, withanolide E (13) affecting more the human lymphocyte E receptor (T cells), while withaferin A (1) had a stronger action on C_3 receptors (B cells).

The inhibitory effects of both compounds on the local graft versus host reaction demonstrate that they had also an effect on the functional activity of T cells, again withanolide E showing a stronger action than withaferin A[3]. Further studies are under course.

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RESUMO: Os withanolídeos são lactonas esteroídicas que ocorrem em espécime da família das Solanaceas. Diversos quimiotipos foram identificados em *Withania somnifera* (L.) Dun., espécie de ampla distribuição geográfica e apresentam diferenças principalmente nas suas respectivas composições em withanolídeos que possuem uma variedade de substituintes. Através de hibridização efetuada entre tipos diferentes oriundos de várias áreas do globo, seguida por análises químicas dos componentes presentes nas mudas, conseguiu-se evidenciar as características genéticas ao nível químico. Assim sendo, diversos híbridos foram quimicamente analisados, tornando possível um estudo dos caracteres de transmissão genética dos grupos químicos. Vias biossintéticas são sugeridas com base na presença dos intermediários apropriados. Um resumo das atividades biológicas é apresentado.

UNITERMOS: Withanolides, *Withania somnifera*, Solanaceae, lactonas esteroídicas, fitogenética.

UNITERMS: Withanolides, *Withania somnifera*, Solanaceae, steroidal lactones, plant genetics.

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