Phytochemistry, Vol. 26, No. 6, pp. 1797–1800, 1987.
Printed in Great Britain.

0031-9422/87 \$3.00 + 0.00
Pergamon Journals Ltd.

WITHANOLIDE Y, A WITHANOLIDE FROM A HYBRID OF *WITHANIA SOMNIFERA*

ROBERTO BESSALLE, DAVID LAVIE and FELIX FROLOW

Department of Organic Chemistry and Chemical Services, Weizmann Institute of Science, Rehovot 76100, Israel

(*Revised received* 20 *September* 1986)

Key Word Index—*Withania somnifera*; Solanaceae; withanolides; steroidal lactones; X-ray structure; trichloroacetyl isocyanate derivatives; biogenesis.

Abstract—The structure of a withanolide isolated from the hybrid of *Withania somnifera* chemotype III (Israel) by Indian I (Delhi) has been elucidated as (20*R*,22*R*)-5α,6α-epoxy-7α-17α,20-trihydroxy-1-oxo-witha-2,24-dienolide by X-ray single crystal analysis. This compound is the first example of a 5α,6α-epoxy-7α-hydroxy system among the withanolides, and thus provides an additional step in the biosynthetic pathway. Its reaction with trichloroacetyl isocyanate has been followed by ^{1}H NMR and compared with withanolide T.

INTRODUCTION

The withanolides are a group of naturally occurring oxygenated ergostane-type steroids generally having a δ lactone in the side chain and a 2-en-1-one system in ring A. They were first isolated from *Withania somnifera* L. Dun. from which they derive their name. Subsequently they were found in other genera of the plant family Solanaceae [1, 2].

During recent years, several studies concerning the chemistry [3, 4], biological properties and genetics of withanolides [1, 5] have been carried out. Genuine interest arose in the whole group of withanolides when it was found that they have anti-tumour activity in a number of experimental tumours in animals. In addition, immunosuppressive [6] and antiinflamatory properties were found [7].

In Israel *Withania somnifera* grows as a number of distinct chemotypes [8, 9] differing in their total leaf content of withanolides with various substitution patterns. These substitutions are characteristic for each chemotype and seem to be genetic in character [1]. Through cross pollination, hybrids were obtained between different chemotypes providing new and different combinations of substituents of the withanolides which themselves appear in different quantities [1, 5, 10].

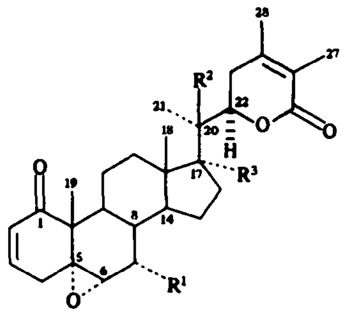
During earlier studies on the hybrids of chemotypes III (Israel) by Indian I (Delhi) grown in our experimental farm, leaves were collected, dried and extracted with methanol as described [11]. The chloroform fraction was fractionated by column chromatography and the fractions purified affording 15 withanolides, seven of which possessed new structures [12]. At that time, the mother liquors of the crystallization process were combined, evaporated and kept dried for further analysis. During the isolation process of several withanolides (carried out from the above mother liquors) a new one was found in a very low concentration and identified as (20*R*,22*R*)-5α-6α-epoxy-7α,17α,20-trihydroxy-1-oxo-witha-2,24-dienolide. This compound was named withanolide Y (**1a**).

RESULTS AND DISCUSSION

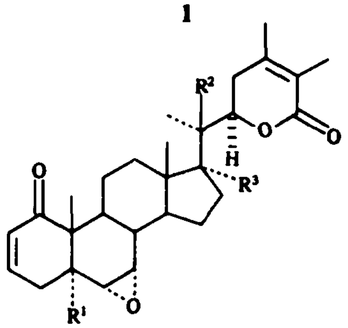
Given the small sample available (9 mg), an X-ray analysis was performed so as to provide the most comprehensive structural data and stereochemistry of **1a** (Fig. 1). Several difficulties were encountered in the crystallization process due to the tiny amount of material and because no adequate crystals could be obtained. From the several solvent systems tried, only CH_2Cl_2–EtOAc (2:1) with hexane and toluene (1:1) as a vapour phase in a closed system afforded pure crystals. They were as long as 8 mm and were formed during a period of 3 days.

The ^{1}H NMR spectrum of **1a** was characteristic for the steroidal structure of the withanolide group. The 2-en-1-one system of ring A presented the usual chemical shifts (δ) and coupling constant (*J*) for H-2, H-3 and both geminal H-4. The ^{1}H NMR signals are collected in Table 1 and the relation between the dihedral angles and the coupling constant of the relevant protons of **1a** are shown in Table 2.

An important feature was the rather high value of *J* (11.0 Hz) for the H-7 formed by the vicinal proton of the hydroxyl group. This was resolved by decoupling the vicinal H-6 and H-8 resulting in very close values of *J* = 11.0; 5.0 for both protons respectively. Furthermore when D_2O was added the signal of the 7-OH disappeared



1



1a, 2a $R^1 = R^2 = R^3 = OH$
1b, 2b $R^1 = R^2 =$ OTAC, $R^3 = OH$
1c, 2c $R^1 = R^2 = R^3 =$ OTAC

OTAC = —O.CO.NH.CO.CCl_3

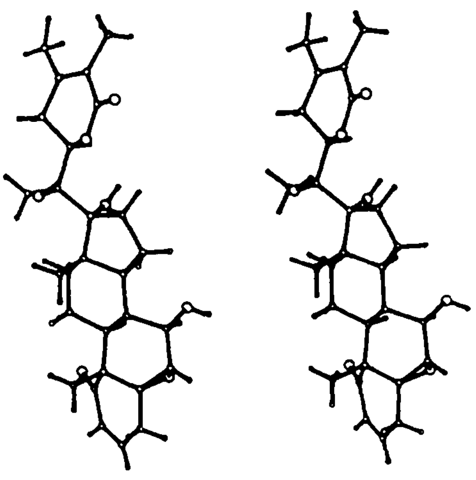


Fig. 1. Stereoscopic view of compound **1a**.

due to the exchange to 7-OD, thus giving the expected double doublet in the shape of a triplet due to the similar $J = 5.0$ values of both protons (Table 2). At high field the signals corresponding to Me-27 and Me-28 and to H-22 were characteristic for the α-β unsaturated lactone side chain. The position of the H-22 signal at δ4.51 indicates the presence of a 17-OH group. The pattern of this signal (*dd*) and the shape and location of the Me-21 as a singlet were indicative of a 20-OH group. The high field signal of Me-18 (δ0.83) was typical for a normal β side chain stereochemistry.

Trichloroacetyl isocyanate (TAI) is a very well known reagent for determining the number and position of the hydroxyl groups in the molecules, through the formation of the carbamate ester derivative (–$OCONHCOCCl_3$, –OTAC). This imidic proton absorbs at very low field (δ8–9), enabling its detection in the ^1H NMR spectrum. The use of TAI derivatives on withanolides has been previously reported [14, 15]. This reagent provides useful chemical information concerning the location of the adjacent protons to the OTAC derivative, the more so when the reaction is followed at certain time intervals. Taking advantage of the X-ray structure determination, a better understanding of the behaviour of this reagent could be reached. The reaction was carried out by adding a few drops of TAI into the NMR tube containing **1a** dissolved in $CDCl_3$.

It is known that the less hindered hydroxyl group reacts completely during the first minutes of the reaction. Thus, in **1a** the 7-OH group reacts after 3 min and shows an imidic proton at δ8.40. At this stage, the total disappearance of the signal belonging to the H-7 at δ3.91 with the concomitant appearance of one at δ5.03 (multiplet) due to the shift induced by the deshielding effect of the adjacent 7-OTAC, was observed. The epoxidic H-6 was deshielded from δ3.36 to δ3.55, however, the shape of the H-6[*t*(*br*)] and the H-7 (*m*) together with the ratio of the signals suggested that this reaction was incomplete.

After two hr, a complete picture of the formation of the 7-OTAC and 20-OTAC appeared (**1b**), showing a clear doublet for H-6 at δ3.58, and a signal for the H-7 at δ5.03. The formation of the 20-OTAC induced a shift of the adjacent Me-21 by 0.8 ppm; a shift to low field of 0.15 ppm was observed for both Me-18 and H-22. The imidic proton of 7-OTAC remained unaffected at the same position (δ8.40), while the 20-OTAC appeared as a clear singlet at δ8.65. Due to the influence of the 7-OTAC, the H-8 appeared now very clearly at δ1.65 as a *ddd* ($J = 12, 12, 5.0$). By irradiating the signal of H-7, that of H-6 was converted into a singlet and the one of H-8 into a triplet (*dd*, $J = 12.0$; 12.0). This J value was explained by the large dihedral angle formed between H-8β with H-9α and H-14α (Table 2).

It has been reported that the formation of the 17α-OTAC is very slow due to steric hindrance [14]. In withanolide Y, the formation of the 17-OTAC was completed after 72 hr (**1c**), singlet signals at δ8.40, 8.63 and 8.71, corresponding to 7-OTAC, 20-OTAC and 17-OTAC respectively.

Withanolide T (**2a**) differs from Y only in the relative position of the epoxide and the hydroxyl groups in ring B (5α-OH-6α,7α-epoxide), and was selected for comparison studies. Surprisingly, **2a** reacted in a rather different way to **1a**. After 3 min two imide protons signals appeared at δ8.59 and 8.52, which correspond to 20-OTAC and 5-OTAC. H-22 was shifted from δ4.52 to 4.90 and Me-

Table 1. ${}^1H$ NMR data of compounds **1a** (withanolide Y), **1b**, **1c**, **2a** (withanolide T), **2b** and **2c** (270 MHz, $CDCl_3$, TMS as int. standard)

H	**1a**	**1b**	**1c**	**2a**	**2b**	**2c**
2	5.98 *dd* (10.0; 3.0)	5.97 *dd*	5.98 *dd*	5.83 *dd* (10.0; 2.0)	5.9 *dd* (10.0; 2.5)	5.88 *dd* (10.0; 2.5)
3	6.72 *ddd* (10.0; 5.0; 2.5)	6.73	6.74	6.58 *ddd* (10.0; 4.0; 2.0)	6.56	6.57
4α	1.93 *dd* (20.0; 5.0)					
4β	3.10 *dt* (20.0; 2.5)	3.10	3.10		3.65 *dd* (20.0; 5.5)	3.65 *dd* (20.0; 5.0)
5-OTAC					8.48 *s*	8.47 *s*
6	3.36 *d* (5.0)	3.58 *d*	3.58 *d*	3.03 *d* (4.0)	3.43 *d*	
7	3.91 *ddd* (11.0; 5.0; 5.0)	5.03 *dd* (5.0; 5.0)	5.03 *dd*	3.3 *dd*	3.27	
7-OTAC		8.40 *s*	8.40 *s*			
8β-ax.	1.98 *ddd* (12.0; 12.0; 5.0)	1.65 *ddd* (12.0; 12.0; 5.0)	1.65 *ddd* (12.0; 12.0; 5.0)			
17-OTAC			8.71 *s*			8.88 *s*
18-Me	0.83 *s*	0.98 *s*	0.98 *s*	0.91 *s*	0.95 *s*	1.03 *s*
19-Me	1.33 *s*	1.35 *s*	1.35 *s*	1.17 *s*	1.22 *s*	1.23 *s*
20-OTAC		8.65 *s*	8.63 *s*		8.56 *s*	8.76 *s*
21-Me	1.27 *s*	2.05 *s*	2.06 *s*	1.28 *s*	1.84 *s*	2.01 *s*
22	4.51 *dd* (13.5; 3.5)	4.66 *dd*	4.68 *dd*	4.52 *dd* (13.0; 3.5)	4.90 *dd*	
27-Me	1.87 *s*	1.84 *s*	1.84 *s*	1.87 *s*	1.86 *s*	1.84 *s*
28-Me	1.94 *s*	1.93 *s*	1.93 *s*	1.95 *s*	1.96 *s*	1.94 *s*

Coupling constants (Hz) are in parentheses.

Table 2. Dihedral angles and *J* (Hz) for relevant protons of **1a**

Protons	Dihedral angle*	*J* (Hz)
H_2–H_3	5.56	10.0
H_3–$H_4\beta$	−78.01	2.5
H_3–$H_4\alpha$	37.68	5.0
$H_6\beta$–$H_7\beta$	−21.26	5.0
$H_7\alpha$–$H_7\beta$	62.65	11.0
$H_7\beta$–$H_8\beta$	−50.23	5.0
$H_8\beta$–$H_9\alpha$	−165.00	12.0
$H_8\beta$–$H_{14}\alpha$	179.67	12.0
$H_{22}\alpha$–$H_{23}\alpha$	58.99	3.5
$H_{22}\alpha$–$H_{23}\beta$	171.56	13.5

*Dihedral angles are given according to the right-hand rule [13].

18, Me-19 and Me-21 to δ0.96, 1.22 and 1.87, respectively. The formation of 5-OTAC was related to the appearance of the H-4 β at δ3.65. After 30 min the reaction of both, the 5- and 20-OTAC was complete, showing two imide signals with full proton size at δ8.48 and 8.56, respectively (**2b**) (Table 1). It was only after 75 hr that the reaction involving the three OH-groups was complete (**2c**), signals corresponding to 5-OTAC, 20-OTAC and 17-OTAC at δ8.47, 8.76 and 8.88.

Comparing the OTAC signals of **1c** with **2c**, one can see that in the latter they are deshielded relative to the former though the relation between them is preserved. 17-OTAC is at lower field than 20-OTAC, which in turn is lower than 5- or 7-OTAC. The same relation is valid for 17- and 20-OTAC in withanolide J and G [14]. The fact that the 20-OTAC imidic proton in **1b** was very slightly shifted by the formation of the 17-OTAC can be explained by the antiparallel position of the 17- and 20-OH groups in **1a** (Fig. 1).

It has been shown earlier that the substitution patterns of the hydroxyl and epoxy groups in the hybrids are based on the dominant or recessive characters prevailing during the hybridization process [10]. 4β-Hydroxyl and 5β,6β-epoxy groups as well as the 20-OH are formed through characters which are dominant, whereas the 14α-OH and 27-OH are derived through recessive ones [12].

In withanolides, the formation of 6,7α- and 6,7β-epoxides is possibly by way of a 1-oxo-2,5-diene intermediate, though the former predominates, probably due to the directing effect of the 5α-OH. Like the 5,6-epoxywithanolides, the β-orientation is favoured almost exclusively, even in the absence of a 4β-OH directing group (*cf.* withanolide E) [8–10, 12]. The formation of the 5α,6α-epoxide with the presence of a directing 7α-OH group in **1a** is recessive in character and biosynthetically could be derived from a 1-oxo-2,5-diene-7α-OH type of intermediate. In fact, this type of substitution is found in a compound belonging to the Indian I type, compound IX in ref. [16], and is present as a minor component of the plant. Thus, when crossing chemotype III (Israel) by Indian I (Delhi), withanolide Y appeared with the dominant character of the 20-OH group and without the recessive 27-OH group for the side chain, whereas in ring D, a 17α-OH group was observed. The latter is dominant with respect to the 17β-OH group in the hybrid which is in very low occurrence and probably originates from a recessive gene. The 5α,6α-epoxy-7α-OH substitution pattern of **1a** is unique so far in the whole group of withanolides. The 5α,6α-epoxide system, to the best of our knowledge, was only reported in physalin J as a minor component of *Physalis angulata* and *P. lancifolia* [17].

From the X-ray structure (Fig. 1) one can observe that ring A has carbons 1–4 and 10 in a plane whereby C-5 lies below it. Due to the 5α,6α-epoxide, ring B adopts a distorted chair conformation in which C-8 is above the plane. Ring C is a regular chair while ring D has an envelop shape. The δ-lactone is almost flat with C-22 below the ring.

EXPERIMENTAL

Mps (Fisher-Johns apparatus) are uncorr; Analytical TLC: silica gel F_{254}; LC: silica gel G 60 (E. Merck) flash type of column eluted with EtOAc–CH_2Cl_2–MeOH (8:2:0.2, same solvent system for TLC); ^1H NMR: 270 MHz, $CDCl_3$, TMS as int. standard; X-ray: single crystal analysis using 3-dimensional intensity data, collected on a computer controlled Enraf-Nonius CAD-4 diffractometer [λ(Cu$K\bar{\alpha}$) = 1.5418] by ω-2θ technique ($\theta < 70°$) at room temp.

Isolation procedure. The general isolation procedure has been reported elsewhere [11, 12]. The mother liquor residues (3.5 g) were chromatographed over silica gel using a gradient generated with mixtures of CH_2Cl_2, EtOAc and MeOH. The fraction eluted with CH_2Cl_2–EtOAc (1:1) (45 mg) was found by TLC (EtOAc–CH_2Cl_2–MeOH, 8:2:0.2) to contain three principal compounds: withanone; 14β-OH-withanone and **1a**. It was rechromatographed by flash chromatography (EtOAc –CH_2Cl_2–MeOH, 8:2:0.2), purified and crystallised to give *ca* 9 mg **1a**.

Withanolide Y (**1a**). (20*R*,22*R*)-5α-6α-epoxy-7α-17α,20-trihydroxy-1-oxo-witha-2,24-dienolide, mp 270–273° from EtOAc–CH_2Cl_2 (1:2, solvent) and hexane–toluene (1:1, vapour phase) in a closed system. IR $\nu_{\max}^{\text{KBr}}$ cm^{-1} 3590, 3508, 3458, 2963, 1685, 1384, 1331, 1138, 1116, 1046; MS(HR) *m/z* (rel. int.): 468.2474 $[M - H_2O]^+$ (2.3), 453.2271 $[M - H_2O - CH_3]^+$ (2.5), 450.2418 $[M - 2H_2O]^+$ (2.2) 435.2115 $[M - 2H_2O - CH_3]^+$ (1.3), 361.1960 $[M - C_7H_9O_2$; δ-lactone$]^+$ (5.8), 325.1881 [M − δ-lactone − $2H_2O]^+$ (6.3), 317.1747 $[M - C_9H_{13}O_3$; side chain$]^+$ (5.4), 307.1689 [M − δ-lactone − $3H_2O]^+$ (4.1) 299.1612 [M − side chain − $H_2O]^+$ (13.3), 263.1479 [M − side chain— $3H_2O]^+$ (5.6), 256.1471 $[C_{17}H_{20}O_2]^+$ (9.7), 238.1374 $[C_{17}H_{18}O]^+$ (20.4), 225.1292 $[C_{16}H_{17}O]^+$ (12.8), 211.1117 $[C_{15}H_{15}O]^+$ (9.8), 171.0818 $[C_{12}H_{11}O]^+$ (20.9), 170.0945 [side chain + H$]^+$ (55.9), 152.0857 [side chain + H − $H_2O]^+$ (100), 138.0698 $[C_8H_{10}O_2]^+$ (23.5), 125.0620 $[C_7H_9O_2$; δ-lactone$]^+$ (44.3), 124.0910 [side chain + H − H_2O − CO$]^+$ (22.1), 121.0658 $[C_8H_9O]^+$ (14.1), 109.0647 $[C_7H_9O]^+$ (75.7), 107.0889 $[C_8H_{11}]^+$ (11.5), 91.0590 $[C_7H_7]^+$ (18.0), 81.0734 $[C_6H_9]^+$ (15.3), 79.0579 $[C_6H_7]^+$ (15.3), $C_{28}H_{38}O_7$ requires: 484.2607.

Crystal data. Monoclinic, $a = 6.40$ (1), $b = 17.25$ (1), $c = 11.30$ (1) Å space group $P2_1$ and $Z = 2$. The structure was solved by direct methods and refined using 2794 unique reflections with $F_0 > 3\sigma$ (F_0). The non-hydrogen atoms were treated with anisotropic temp. factors. All hydrogens were found from a difference Fourier map and refined with isotropic temp. factors. Block-matrix least-squares refinement converged to $R = 0.034$. The final difference Fourier map revealed only randomly distributed electron density (maximum peak of 0.20 eÅ^{-3}). Crystallographic data are available as supplementary material.

REFERENCES

1. Glotter, E., Kirson, I., Lavie, D. and Abraham, A. (1978) in *Bioorganic Chemistry* (van Tamelen, E. E., ed.) Vol. 2, p. 57. Academic Press, New York.
2. Kirson, I. and Glotter, E. (1981) *J. Nat. Prod.* **44**, 633.
3. Gamoh, K., Hirayama, M. and Ikekawa, N. (1984) *J. Chem. Soc. Perkin Trans.* I, 449.
4. Hirayama, M., Gamoh, K. and Ikekawa, N. (1982) *J. Am. Chem. Soc.* **104**, 3735.
5. Lavie, D. (1980/1981) *Mem. Inst. Butantan* **44/45**, 439.
6. Shohat, B., Kirson, I. and Lavie, D. (1978) *Biomedicine* **28**, 18.
7. Budhiraja, R. D., Sudhir, S. and Garg, K. N. (1984) *Planta Medica* **50**, 134.
8. Glotter, E., Kirson, I., Abraham, A. and Lavie, D. (1973) *Tetrahedron* **29**, 1353.
9. Abraham, A., Kirson, I., Lavie, D., and Glotter E. (1975) *Phytochemistry* **14**, 189.
10. Kirson, I., Abraham, A. and Lavie, D. (1977) *Israel J. Chem.* **16**, 20.
11. Nittala, S., Vande Velde, V., Frolow, F. and Lavie, D. (1981) *Phytochemistry* **20**, 2547.
12. Nittala, S. and Lavie, D. (1981) *Phytochemistry* **20**, 2741.
13. Klyne, W. and Prelog, V. (1960) *Experientia* **16**, 521.
14. Kirson, I. and Gottlieb, H. E. (1980) *J. Chem. Res.* S, 338: M, 4275.
15. Ramaiah, P. A., Lavie, D., Budhiraja, R. D., Sudhir, S. and Garg, K. N. (1984) *Phytochemistry* **23**, 143.
16. Kirson, I., Glotter, E., Lavie, D. and Abraham, A. (1971) *J. Chem. Soc.* (C) 2032.
17. Row, L. R., Sarma, N. S., Reddy, K. S., Matsuura, T. and Nakashima, R. (1978) *Phytochemistry* **17**, 1647.