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WITHANOLIDES OF WITHANIA SOMNIFERA L. DUNAL GROWING WILD IN EGYPT

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ABSTRACT

Three Withanolides (Substances A, B, and C) were isolated from the alcoholic extract of the Egyptian Withania somnifera wild plants. Substance A and substance C were identified by IR, NMR and Mass spectral data. Substance A appears to be a configurational isomer of Withanolide E and substance C is 4-hydroxy-derivative of substance A. Substance A is identified as 14,17,20-trihydroxy-5B,6B-epoxy-1-oxo-witha-2,24-dienolide (I) and substance C as 4,14,17,20-tetrahydroxy-5B, 6B-epoxy-1-oxo-witha-2,24-dienolide(II).

This is the first report of isolation and characterisation of substance C from Withania somnifera or the genus Withania. Substance B would be the subject of a further report.

INTRODUCTION

Withania somnifera L. (Dun) is a solanaceous plant, growing wild in Egypt[1], in the Nile Valley and Delta. The plant is used in Indian and African folk medicine against fever, as a sedative and hypnotic to induce sleep and as an antiseptic[2,3].

Withanolides of Withania somnifera L. Dunal growing in Egypt.

Literature survey, showed that the roots of the plant yielded a large number of alkaloids[3-10], while the leaves gave a large number of steroidal lactones, termed collectively withanolides[11-24]. Such compounds have been shown to possess antitumor[17,18] and antimicrobial activities[25]. Among these compounds, withaferin A, the first compound of this class to be isolated, caused significant retardation of growth of Ehrlich ascites carcinoma, sarcoma and mammary adenocarcinoma[18].

It was reported that specimen of W. somnifera from various areas yielded different withanolides and that 3 chemotypes grow wild in Israel[18]. These chemotypes differ in the substitution pattern of the steroidal lactones[18] A fourth chemotype (Indian I) has been shown to occur in North Western India[20].

Since 1965, about fifty withanolides have been isolated from different chemotypes of Withania somnifera[11-24], as well as several related species of the family Solanaceae e.g. Acnistus[26-27], Physalis[28], Datura[29] and Dunalia species[30]. In Addition, Withanolides have also been shown in the roots of Withania coagulans[31] and were also isolated from the roots of Indian variety of Withania somnifera.[32]

Investigation of the leaves of the Egyptian plant has yielded four crystalline compounds[25], that are still not identified. The authors gave M.P., I.R. and preliminary empirical formulae for their isolated compounds.

In view of the reported antitumor property and antibacterial activity of W. somnifera as well as the chemical variability of the specimens from different ecological habitats and the specimens

belonging to different chemotypes, we have undertaken the present investigation of the wild Egyptian plant to isolate and identify its contained withanolides, to determine its relationship to the 4 chemotypes already known, with the hope to identify any hitherto unknown components and if any, to examine their biological activity.

EXPERIMENTAL

Plant Material:

The material used in this investigation was the overground parts of Withania somnifera L. (Dunal), collected from the banks of the Nile, Kanater, Cairo, Egypt. Identity of the plant was confirmed by late Prof.Dr. Vivi Tackholm.

Authentic Reference Material

Witheferin A, withanolides D and E and Dihydrowithaferin A*

Preparation of Ether Extract of W. somnifera L((Dun):

The dried powdered herb of W. somnifera (650 g) was defatted by percolating with petroleum ether (60-80°C). The defatted powder was exhaustively extracted by percolation with ethanol 95%. The alcoholic extract was concentrated under reduced pressure to yield 60 g of a greenish-brown residue: 30g of this residue were dissolved in 100 ml of ethanol 95%, diluted with 100 ml of distilled water and partitioned with 5x200 ml of ether. The ether extracts were combined, washed with distilled water, dried with anhydrous sodium sulphate and evaported in vacuum to leave 5g of a greenish-brown soft residue.

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* Withaferin A and Withanolides D and E were kindly provided by Dr. Lavie, Dept. of Chemistry, Weizmann Institut, Rehovoth, Israel. Withaferin A and Dihydrowithaferin A were also provided by Dr.J. Rosazza, College of Pharmacy, Iowa State University., Iowa City, Iowa, U.S.A.

Withanolides of Withania somnifera L. Dunal growing in Egypt

Chromatographic Examination of the Ether Extract

The ether extract of W. somnifera was examined by tlc using silica gel G plates. Several solvent systems were tried including: chloroform-absolute ethyl alcohol (76:4), chloroform-ethanol 95% (95:5) and ethyl acetate-benzene (7:1)*, p-anisaldehyde/perchloric acid reagent** was used as spraying reagent. The plates were activated by heating at 120°C for 5 minutes; 12-13 spots appear, the majority of which were coloured blue to greyish-blue to violet.

Column Chromatographic Fractionation of the Ether Extract:

The ether extract of W. somnifera (5 g) was chromatographed over 100g silica gel G*** (Merck, Darmstadt, Germany) by gradient elution with mixtures of benzene-ethyl acetate(7:3), (5:5), (3:7), ethyl acetate and ethyl acetate-ethanol 95% (95:0.5). Thirty fractions (200 ml each) were collected and the pattern of chromatographic separation was followed by tlc examining each fraction and the similar fractions were combined.

Isolation of Substance A:

The residue obtained after distilling off the benzene-ethyl acetate fractions (5:5 & 3:7) was further purified by chromatography over silica gel G*** column. The residue(0.6g) was adsorbed on 1 g of silica gel G and the mixture was packed

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* Proved to be the most suitable

** 0.5 ml p-anisaldehyde, 50ml 60% perchloric acid, 10ml acetone and 40 ml distilled water.

*** To simulate conditions available in tlc.

on the top of a column of 15g of silica gel-G prepacked in a glass column (1 cmØ) by the wet technique using benzene. Fractionation was carried out by gradient elution with benzene, mixtures of benzene-ethyl acetate in the proportions 9:1,8:2, 7:3, 6:4, 4:6 and 2:8. Thirty four fractions (5ml each) were collected. Fractions 18 to 23 eluted with benzene-ethyl acetate (6:4 & 4:6) were evaporated under reduced pressure to yield a colourless residue (40 mg). Trials of crystallisation of this residue gave a crystalline product, m.p. 177.5°C(decomp.). Thin-layer chromatographic examination of this product using the solvent systems benzene-ethyl acetate (1:7) and chloroform-ethanol (9:1) revealed the presence of one spot identical with that of authentic withanolide E and of the R_f values 0.38 and 0.5 in the 2 systems respectively.

Isolation of Substance B:

The pale brown residue obtained after distilling off the ethyl acetate fraction of the column chromatographic fractionation of the ether extract, was washed with little benzene till the residue was nearly colourlesss. Trials of crystallisation of this residue yielded a colourless residue (15mg). Examination of this residue by tlc(using silica gel G plates), the solvent systems benzene-ethyl acetate (1:7),chloroform-ethanol (9:1) and p-anisaldehyde perchloric acid as spraying reagent, revealed the presence of one spot of the R_f values 0.25 and 0.48 in the 2 systems respectively. The compound was only obtained in trace amount. This would be chracterized in a further report.

Isolation of Substance C:

The ethyl acetate-ethanol(9.5:0.5) fractions obtained from the column chromatographic fractionation of the ether

extract, were collected and evaporated to dryness under reduced pressure. The pale brown residue (2g) was dissolved in hot ethanol, filtered and the filtrate was left to stand overnight. A crystalline substance was obtained, further recrystallised from hot ethanol to yield colourless needles, m.p. 277.5°C. Thin layer chromatographic examination of this crystalline compound using silica gel G plates, the solvent systems benzene-ethyl acetate (1:7), chloroform-ethanol (9:1) , revealed the presence of one spot of the R_f values 0.07 and 0.14 in the 2 systems respectively.

Characterization of the Substance A & C

Substance A:

M.P. 177.5°C (with decomposition): IR (KBr) 3400, 2930 , 2920, 2865, 1690 (broad), 1380, NMR (CD_3OD) ppm (δ):1.1(s,3H, 18-CH_3), 1.25 (s, 3H, 19-CH_3), 1.4(s, 21-CH_3), 1.85(s, 3H,27-3H, 27-CH_3), 1.95(s,3H, 28-CH_3), 3.22(t, 1H, 6-H), 5.9(d, 1H, 2-H) and 6.9 (1H, 3-H), Mass Spectrum (m/e): 450 (M^+-2x18). Thus molecular formula=$C_{28}H_{38}O_7$, 432(M^+- 3x18), 344, 326,299, 255, 237, 209, 207, 175, 169, 152, 135,125 (100%, base peak), 109, 91, 81, 79, 77, 71, 69, 67. This substance gave the same $R_f$as authentic withanolide E and both gave superimposable IR spectra; both gave identical fragmentation pattern in the Mass spectrum.

Substance C:

Major withanolide of the Egyptian plant, mp 277.5° IR(KBr) 3450, 2960, 2880, 1675(broad), 1450, 1420, 1380, 1330,1290, 1260, 1220, 1150, 1100, 1080, 1020, 960, 870, 810 and 770 cm^{-1}; NMR (CD_3OD) ppm (δ): 1.1 (s, 3H, 18-CH_3), 1.3 (s, 3H, 19-CH_3), 1.4 (s, 3H, 21-CH_3), 1.85 (s, 3H, 27-CH_3), 1.95 (s, 3H, 28-CH_3) 3.2 (t, 1H, 6-H), 3.6(d, 1H, 4-H), 4.6(m, 1H, 22-H), 5.8(d, 1H,

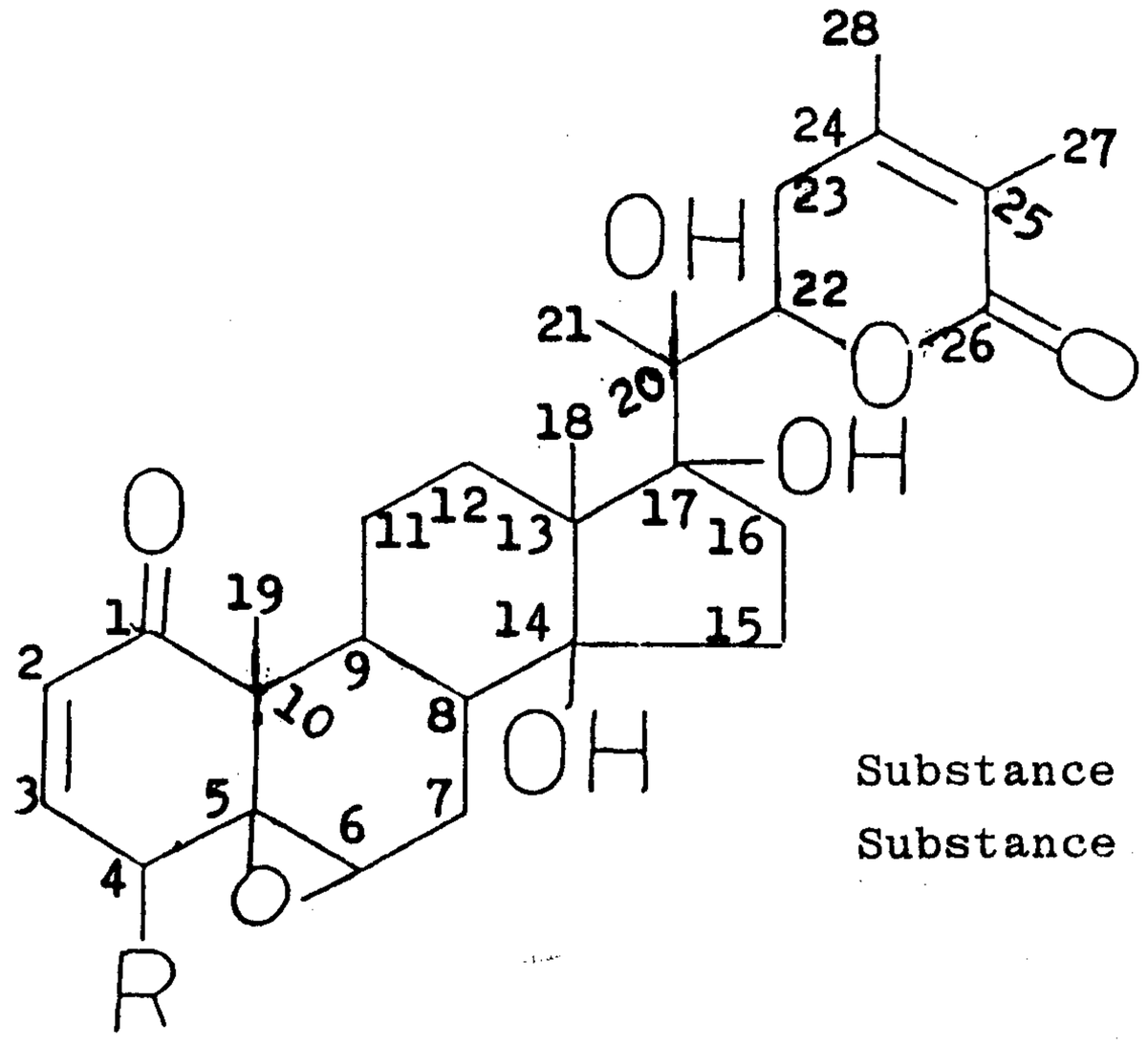
2-H, 6.7(m, 1H, 3-H); mass spectrum (m/e): 466, (M^+- 2x18). Thus Molecelar formula=$C_{28}H_{38}O_8$, 448 (M^+- 3x18), 430 (M^+- 4 x 18). 361, 343, 317, 299, 281, 244, 238, 213, 209, 207, 185, 170, 152, 125, (100%, base peak), 109, 97, 79, 67.

DISCUSSION

Three substances were isolated from Egyptian Withania somnifera L. (Dunal), substance A,B and C. Substance A was the least polar of the three compounds with R_f values 0.38 and 0.5 in the solvent systems benzene-ethyl acetat (1:7) and chloroform-ethanol (9:1) respectively. It corresponded in every respect with suthentic withanolide E, with superimposable I.R. and identical mass spectral fragmentation pattern; M^+ - 2x18 for both was at m/e 450. Moreover, NMR chemical shift data for the methyl group protons at C 18, 19, 21, 27 and 28 were identical in every respect with that of withanolide E. Comparing the NMR data obtained with those reported in the literature[20,22,28], confirms the substitution pattern of 14, 17,20-trihydroxy type.

The chemical shift values of C_{27} and 28 methyl groups confirm Δ^{24} double bond. However, the m.p. of the compound was different from that reported for withanolide E (m.p. 167-168°C)[21]. The 5,6-epoxide is confirmed from chemical shift of the 6-H giving a triplet at 3.22 ppm. Thus the structure of substance A (I) could be a configurational isomer of withanolide E. The aboslute configuration of the side chain at C_{17} remains to be determined.

Substance C was obtained as the major withanolide of the Egyptian plant. It behaved as the most polar withanolide with R_f values 0.07 and 0.14 in the solvent systems benzene-ethyl acetate (1:7) and chloroform-ethanol (9:1) respectively. Mass spectrum gave M^+ - 2x18 at 466, indicating the possibility of a hydroxy derivative of substance A. NMR spectral data confirmed such assumption. The chemical shift values of the methyl groups at 18, 19, 21, 27 and 28 were identical to those of withanolide E and substance A. Moreover, C_{22}-H appeared as multiplet at 4.6, indicating similar hydroxylation pattern to these two compounds at C_{17} and C_{20}[20,22]. The epoxide was also confirmed by chemical shift of C_6-H. The chemical shifts of 27-CH_3 and 28-CH_3 protons confirm the presence of a Δ^{24} double bond. The only difference in NMR spectra of substances A & C was the appearance of a doublet at 3.6 ppm corresponding to one proton indicating a 4-OH group. Thus substance C (II) is 4-hydroxy-substance A.



Substance A (R = H

Substance C (R =OH

Substance A and substance C show several similarities in the mass spectrum. Both gave $M^{+}-2x18$ as the highest m/e peak in electron impact mass spectrometer, both with a base peak at m/e 125, and with similar fragments obtained from the side chain at C_{17} according to reported fragmentation of similar withanolides[19,20,28], as shown in Fig. I.

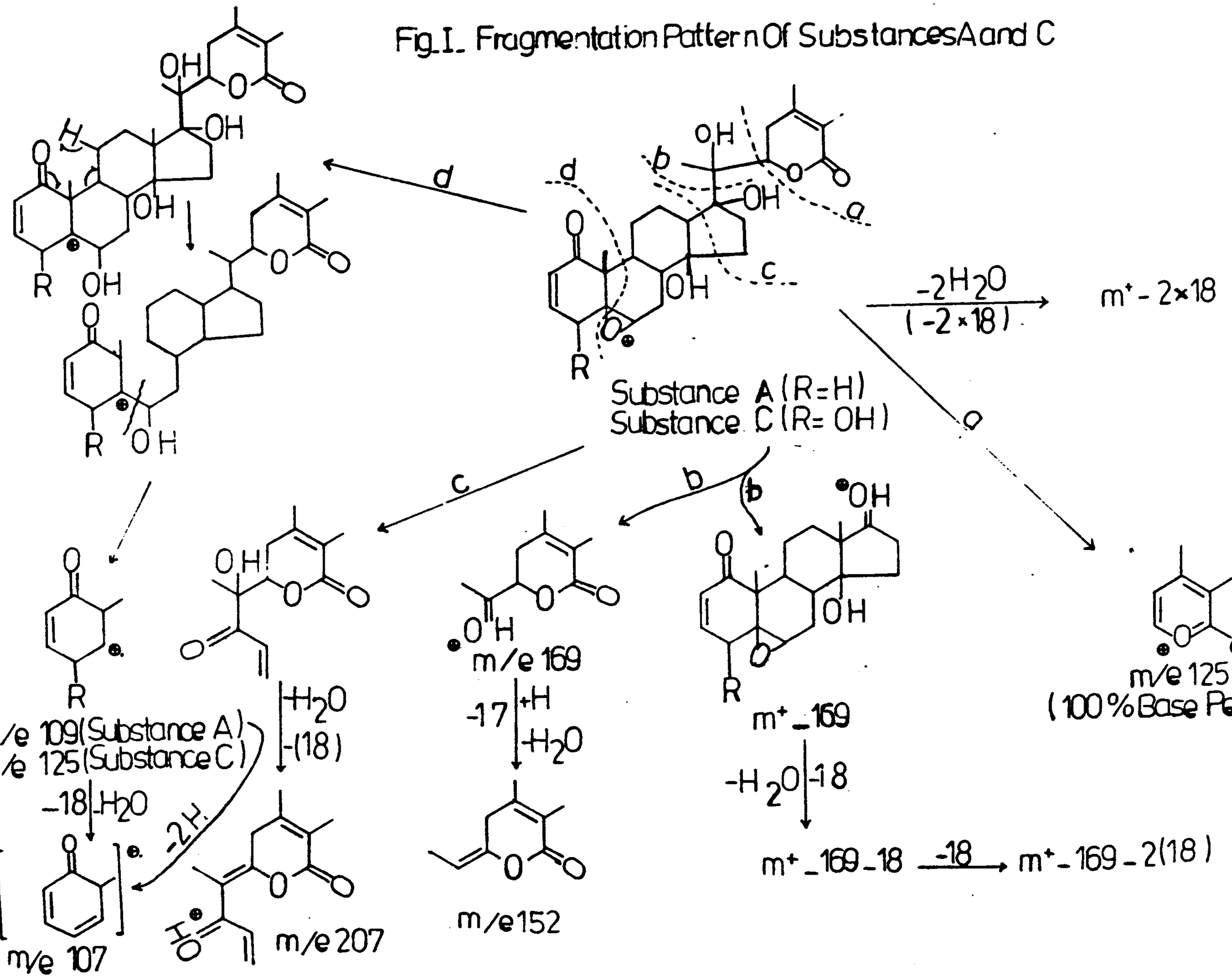
Thus substance A is 14,17,20-trihydroxy-5B, 6B-epoxy-1-oxo-witha-2, 24-dienolide(1). Substance C is 4,14,17,20-tetra-hydroxy-5B, 6B-epoxy-1-oxo-witha-2, 24-dienolide(II), which is isolated for the first time from Withania somnifera or the genus Withania

Kirson et al[28] reported the isolation of 4B hydroxy withanolide E from Physalis peruviana (Solanaceae), which possesses a 17 side chain similar to withanolide E. This compound was reported to have m.p. 197-198°[28] which is quite different from that found for substance C (m.p. 277.5°). Thus substance C could be regarded as a configurational isomer of 4B-hydroxy Withanolide E. The absolute configuration of the side chain at C_{17} remains to be determined.

It is worth to mention that substances A and C, characterized in this report, appear to correspond to compounds C and B respectively isolated by Khafagy and Ragab[25], since they show close m.p., similar I.R. data and somewhat related empirical formulae.

From the withanolide pattern of the withanolides isolated from the Egyptian plant, it may be concluded that the Egyptian plant belongs to a new chemotype which is intermediate chemotype II and chemotype III reported in Israel according to Abraham et al[18]

Fig.I. Fragmentation Pattern Of Substances A and C



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الويثانوليدات من نبات ويثانيا سومنيفيرا الذى ينمو بريا فى مصر

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تم فصل ثلاث مواد ويثانوليديه أ ، ب ، ج من الخلاصه الكحوليه لنبات ويثانيا سومنيفيرا الذى ينمو بريا فى وادى النيل والدلتا .

وقد تم التعرف على المادتين أ ، ج بواسطة التحليل الطيفى بالأشعة تحت الحمراء والرنين النووى المغناطيسى وطيف الكتله وتبين أن التركيب الكيميائى للماده أ هو ١٤،١٧،٢٠ ـ ثلاثى أيدروكسى ـ ٥ بيتا، ٦ بيتا ـ ايبوكسى ـ ١ ـ أوكسو ـ وثيا ـ ٢ ، ٢٤ ـ ثنائى انيوليد .
كما تبين أن الماده ج هى مشتق " ٤ ـ أيدروكسى " الماده أ .

وقد اتضح أن الماده ج قد تم فصلها لأول مره من نبات ويثانيا سومنيفيرا أو من جنس ويثانيا عامة .
أما الماده الثالثه ويثانوليد ب فسوف يتم وصف التعرف عليها فى دراسة لاحقه .

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