Phytochemistry, 1976, Vol. 15, pp. 937–939. Pergamon Press. Printed in England.

BIOSYNTHESIS OF STEROIDAL WITHANOLIDES IN *WITHANIA SOMNIFERA*

WILLIAM J. S. LOCKLEY, HUW H. REES and TREVOR W. GOODWIN
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool, L69 3BX England

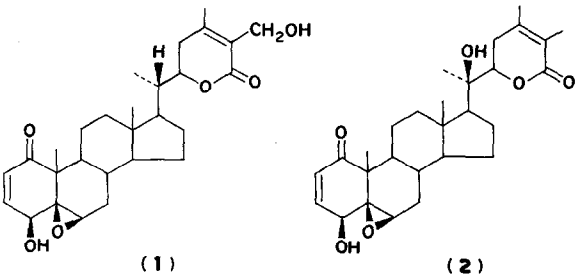
(*Received* 14 *October* 1975)

Key Word Index—*Withania somnifera*; Solanaceae; biosynthesis; isolation; withanolides; 24-methylene-cholesterol.

Abstract—Administration of 24-methylene-cholesterol-[28-^{3}H] to *Withania somnifera*, yielded [^{3}H] radioactivity in the isolated withaferin A and withanolide D, whereas administered 24-(*R,S*)-methyl-cholesterol-[28-^{3}H] was not incorporated into these compounds. 24-Methylene-cholesterol is, therefore, proposed as a sterol precursor of the withanolides. A novel procedure is described for the isolation of withanolides from *W. somnifera*. This method in conjunction with an improved procedure for administration of labelled sterols and mevalonolactone produces a greatly increased yield of labelled withanolides.

INTRODUCTION

About 40 steroidal lactones structurally related to withaferin A (**1**) and withanolide D (**2**) have so far been isolated from various chemotypes of the plant *Withania somnifera*. The structures and biological properties of many of these withanolides [1,2] have already been examined. However, little is known concerning their biosynthesis.



(**1**) (**2**)

In a previous communication, the major sterols of *W. somnifera* were identified as campesterol, 24-methyl-cholesta-5,24-dien-3β-ol, sitosterol, stigmasterol and 28-isofucosterol [3]. We have subsequently identified minor and variable amounts of 24-methylene-cholesterol (usually $<2\%$ of the total sterol). In view of the C_{28} carbon skeleton present in the withanolides, the C_{28} sterols, campesterol, 24-methyl-cholesta-5,24-dien-3β-ol and 24-methylene-cholesterol must be regarded as possible sterol precursors of the withanolides, provided that withanolide biosynthesis does not diverge from the major sterol biosynthetic pathway before the 4-desmethyl sterol stage.

The isolation of comparatively large amounts of 24-methylene-cholesterol from *in vitro* callus cultures of *W. somnifera* which contained no detectable withanolides, may be particularly significant [4]. If this absence of withanolides implies that withanolide biosynthesis is not occurring in the cultures, then the accumulation of 24-methylene-cholesterol could be ascribed to its role as a sterol precursor of the withanolides. We now report investigation of this possibility utilising [28-^{3}H] labelled 24-methylene-cholesterol and 24-(*RS*)-24-methyl-cholesterol. Since both sterols had identical specific radioactivities, direct comparison of their incorporation into withanolides could be made.

RESULTS

24-Methylene-cholesterol-[28-^{3}H] was prepared via a Wittig condensation between methylenetriphenylphosphorane-[methylene-^{3}H] and 24-oxo-cholesteryl acetate [4]. Subsequent hydrogenation of a portion of the 24-methylene-cholesterol-[28-^{3}H] using Wilkinson's catalyst [5] yielded 24-(*RS*)-24-methyl-cholesterol-[28-^{3}H]. 24-Methylene-cholesterol-[28-^{3}H] and 24-(*RS*)-24-methyl-cholesterol-[28-^{3}H], both in admixture with 3-(*RS*)-mevalonolactone-[2-^{14}C], for reference purposes were administered to young leaves of *W. somnifera* plants over a two week period (experiments I and II, respectively).

Radioactive withaferin A and withanolide D from plants of experiment I were extracted by the method of Lavie *et al.* [6] and purified by TLC, recrystallisation and by the formation of their 4-oxo-derivatives [7]. The extraction of withanolides from plants of experiment II utilised a novel procedure. Fresh stalks and leaves from the plants were allowed to stand 18 hr in ether, and the ether decanted and evaporated. The resulting residue, which consisted mainly of withanolides, contained only small amounts of chlorophylls, carotenoids and lipids, and was amenable to direct purification by TLC. The isolated withanolides were then further purified by recrystallisation and derivative formation. We have found the above procedure to be both quantitatively more efficient and considerably more convenient than published methods.

In order to maximise the conversion of labelled precursors into the withanolides, the incorporation of 3-(*RS*)-mevalonolactone-[2-^{14}C] was studied using a different administration procedure. A greatly enhanced conversion into withanolides was obtained when young plants (*ca* 25 cm high) were cut off about 10 cm above

Table 1. Incorporation of radioactive substrates into withanolides in *Withania somnifera*

	Substrate	$^3H/^{14}C$ Radioactivity ratio of 4-oxo-withanolide[3] Withaferin A	Withanolide D	% Incorporation of isotope: Withaferin A ^3H	Withaferin A ^{14}C*	Withanolide D ^3H	Withanolide D ^{14}C*
Experiment I	24-Methylene-cholesterol-[28-^{3}H] (20 μCi) + 3-(*RS*)-Mevalonolactone-[2-^{14}C] (10 μCi) (Leaf administration)	0.65	1.54	0.19	1.15	0.03	0.08
Experiment II	24-(*RS*)-Methyl-cholesterol-[28-^{3}H] (20 μCi) + 3-(*RS*)-Mevalonolactone-[2-^{14}C] (10 μCi) (Leaf administration)	0	0	0	17.86	0	0.72
Experiment III	24-Methylene-cholesterol-[28-^{3}H] (10 μCi) + 3-(*RS*)-Mevalonolactone-[2-^{14}C] (3 μCi) (Stem administration)	3.70	5.04	3.89	6.73	2.79	3.35

* ^{14}C Incorporations are based on the biological utilisation of only one epimer of 3-(*RS*)-mevalonolactone-[2-^{14}C].

the soil and the stems placed in an aqueous solution of 3-(*RS*)-mevalonolactone-[2-^{14}C] containing Tween 20. Using this procedure, up to 60% conversion of the biologically active form of mevalonolactone into withaferin A was obtained within 2 days of administration.

In view of this greatly increased incorporation, 24-methylene-cholesterol-[28-^{3}H] in admixture with 3-(*RS*)-mevalonolactone-[2-^{14}C], for reference purposes, was administered to *W. somnifera* utilising the improved procedure (experiment III). After purification, the biosynthesised withanolides showed an increased incorporation of ^{3}H from the methylene sterol. The results from experiments I, II and III are summarised in the Table 1.

DISCUSSION

The incorporation of ^{3}H radioactivity from 24-methylene-cholesterol-[28-^{3}H]-into withaferin A and withanolide D demonstrates that 24-methylene cholesterol can serve as a biosynthetic precursor of these compounds in *W. somnifera*. Moreover, the accumulation of this sterol in tissue cultures of *W. somnifera* which apparently did not biosynthesise withanolides [4] implies that 24-methylene-cholesterol is a normal precursor of the withanolides *in vivo*. Since no ^{3}H label was present in the withanolides isolated from *W. somnifera* administered with 24-(*RS*)-24-methyl-cholesterol-[28-^{3}H], i.e. a mixture of campesterol-[28-^{3}H] and dihydrobrassicasterol-[28-^{3}H], campesterol itself seems unable to function as a precursor of the withanolides. Whilst 24-methylene-cholesterol can serve as a precursor of withanolides but campesterol cannot, it remains possible, however, that other C_{28} 4-desmethyl-sterols such as 24-methyl-cholesta-5,24-dien-3β-ol are involved in withanolide biosynthesis either prior to or after 24-methylene-cholesterol. Preliminary attempts to investigate the intermediacy of 24-methyl-cholesta-5,24-dien 3β-ol yielded results from which clear conclusions could not be drawn. No ^{3}H label was found in the withanolides isolated from *W. somnifera* administered with 24-methyl-cholesta-5,24-dien-3β-ol-[28-^{3}H], which suggested the compound was not a precursor. However, we observed an extensive loss of ^{3}H from the administered sterol upon reisolation from the plant [8]. In view of this loss of ^{3}H, 24-methyl-cholesta-5,24-dien-3β-ol cannot be excluded as a possible sterol precursor of the withanolides. It also remains possible that the withanolides biosynthetic pathway may branch from the normal sterol pathway before the 4-desmethyl sterol stage.

A comparison of the incorporations of 3-(*RS*)-mevalonolactone-[2-^{14}C] administered via the leaves and via the stem shows an increased conversion into withanolides using stem administration. This procedure in conjunction with the direct ether extraction method described enables the biological preparation of ^{14}C-labelled withanolides in acceptable yields. Furthermore, the administration of 24-methylene-cholesterol-[28-^{3}H] to *W. somnifera* via the stem greatly increased the incorporation of this sterol into the withanolides *vis a vis* administration to the leaves. Since the conversion of the reference 3-(*RS*)-mevalonolactone-[2-^{14}C] in this particular plant (experiment III) was low (*ca* 10% of the maximum we have obtained), much greater incorporation of 24-methylene-cholesterol into the withanolides should be feasible by this procedure.

It is noteworthy that the $^3H/^{14}C$ radioactivity ratios, a measure of the relative incorporation of sterol to mevalonolactone, for withanolide D and withaferin A biosynthesised from 3-(*RS*)-mevalonolactone-[2-^{14}C] and 24-methylene-cholesterol-[28-^{3}H] were not identical, and that the ratio of withanolide D was consistently higher than for withaferin A in both experiments I and III. This may represent a compartmentalisation of the biosynthetic sites and/or precursor pools of the two withanolides, or may be due to differing isotope effects in the biosynthetic steps leading to withaferin A and withanolide D.

EXPERIMENTAL

Trivial names. Dihydrobrassicasterol [(24*S*)-24-methyl-cholest-5-en-3β-ol]; campesterol [(24*R*)-24-methyl-cholest-5-en-3β-ol]; 24-methylene-cholesterol [24-methyl-cholesta-5,24(28)-dien-3β-ol]; sitosterol, [24*R*)-24-ethyl-cholest-5-en-3β-ol]; 28-isofucosterol [24-ethylcholesta-5,*Z*-24(28)-dien-3β-ol]; stigmasterol [(24*S*)-24-ethyl-cholesta-5,22-dien-3β-ol]; withanolide D [4β, 20α-dihydroxy-1-oxo-5β,6β-epoxy-22*R*-witha-2,24-dienolide]; withaferin A [4β, 27-dihydroxy-1-oxo-5β,6β-epoxy-22*R*-witha-2,24-dienolide].

Plants and radiochemicals. Withania somnifera plants were supplied by the University of Liverpool Botanical Gardens, Ness, Cheshire and contained withaferin A and withanolide D as the major withanolides. 3-(*RS*)-mevalonolactone-[2-^{14}C] (9.5 mCi/m mol) and MeI [^{3}H] (2.2 Ci/m mol) were supplied by the Radiochemical Centre, Amersham, Bucks.

Radioassays. Samples disolved in 10 ml of a dioxan scintillation soln containing 15 g of 5-(4-biphenyl)-2-(4-t-butyl-phenyl)-1-oxa-3,4-diazole (Butyl PBD) and 100 g of naphtha-

lene per l. of dioxan were radioassayed using a 3 channel scintillation spectrometer.

24-*Methylene-cholesterol*-[28-^3H]. MeI-[^{3}H] (25 mCi, 2.2 Ci/mmol) was cooled to Me_2CO/dry ice temp and MeI (15 mg) in C_6M_6 (250 μl) added. After the further addition of triphenylphosphine (55 mg) in C_6H_6 (100 μl), the mixture was allowed to warm to room temp. and kept 18 hr. Volatile material was removed under a stream of dry N_2 and THF (250 μl) added, followed by *n*-butyllithium in hexane (15% w/w, 250 μl). The mixture was allowed to stand until all the phosphonium salt had dissolved to yield a deep red soln when 24-oxo-cholesteryl acetate (38 mg) in THF (250 μl) was added. The resulting gel was kept for 30 min and then dissolved in Et_2O (30 ml) and the ethereal soln thoroughly extracted with H_2O (4 × 5 ml) and then dried. After filtration and evaporation of the Et_2O, the residue was subjected to preparative TLC on Si gel GF_{254} developed with C_6H_6 to yield 24-methylene-cholesterol-[28-^{3}H] (300 μCi, 54 mCi/mmol) and 24-methylene-cholesteryl-acetate (160 μCi, 54 mCi/mmol).

24-(RS)-24-*Methyl-cholesterol*-[28-^3H]. 24-Methylene-cholesterol (80 μCi, 54 mCi/mmol) in C_6H_6 (5 ml) was hydrogenated in the presence of Wilkinson's catalyst (6 ml) [5] for 16 hr, and the solvent then removed under vacuum. The sterol was isolated by preparative TLC and development with $CHCl_3$ to yield 24-(*RS*)-24-methyl-cholesterol (66 μCi, 54 mCi/mmol).

Preparation of 4-*oxo-derivatives of withanolides.* 4-Oxo-withaferin A [7] and 4-oxo-withanolide D were prepared by identical procedures. The withanolide (5 mg) was dissolved in $CHCl_3$–EtOAc (1:1, 2 ml) and active MnO_2 (50 mg) added. The mixture was stirred for 1 hr and then filtered. Evaporation of the filtrate followed by preparative TLC and development with C_6H_6–EtOAc (2:3) gave the 4-oxo-derivative (*ca* 4 mg).

Administration of labelled sterol via the leaves, and extraction of withanolides. Radioactive 24-methylene-cholesterol (20 μCi, experiment I) or 24-(*RS*)-24-methyl-cholesterol (20 μCi, experiment II) was mixed with 3-(*RS*)-mevalonolactone-[2-^{14}C] (10 μCi) in C_6H_6 soln and evaporated to dryness. A soln of Tween 20 in EtOH (10 mg/ml; 1 ml) was added, and the mixture shaken well. The resulting soln was applied every 24 hr to the young leaves of three *W. somnifera* plants (*ca* 25 cm in height) over a 2 week period. Plants from experiment I were extracted by the method of ref. [6], whereas those from experiment II were cut off above the soil and allowed to stand 18 hr in Et_2O (700 ml). After decanting and evaporating the Et_2O, the residue (300 mg) was purified by preparative TLC developed with C_6H_6–EtOAc (1:4) and the isolated withaferin A and withanolide D further purified by TLC after development in MeOH–$CHCl_3$ (1:14) followed by recrystallisation from aq MeOH. Purified withanolides were converted into the 4-oxo-derivative prior to the final radioassay.

Administration of 3-(RS)-*mevalonolactone*-[2-^{14}C] *via the stem and extraction of withanolides.* One plant of *W. somnifera* (*ca* 25 cm in height) was severed about 10 cm above the soil and the cut end of the stem crushed. The cutting was then placed in a soln of 3-(*RS*)-mevalonolactone-[2-^{14}C] (50 μCi) in H_2O (2 ml) until all the soln had been absorbed (*ca* 1 hr). H_2O was then added as necessary and the plant allowed to stand for 2 days, after which it was placed in Et_2O (200 ml) and kept 18 hr at room temp. The Et_2O was decanted, evaporated and the residue subjected to preparative TLC using C_6H_6–EtOAc (1:4), to yield crude withaferin A-[^{14}C] and withanolide D-[^{14}C]. Further TLC purification after development with MeOH–$CHCl_3$ (1:14) yielded crystalline withaferin A-[^{14}C] (13.5 μCi, 23 mg) and withanolide D-[^{14}C] (1.5 μCi, 3 mg).

Administration of 24-*methylene-cholesterol*-[28-^{3}H] to W. somnifera *via the stem* (*experiment III*). 24-Methylene-cholesterol-[28-^{3}H] (10 μCi) and 3-(*RS*)-mevalonolactone-[2-^{14}C] (3 μCi) were mixed in C_6H_6 soln and the C_6H_6 removed under N_2. Tween 20 in MeOH (4% v/v, 0.2 ml) was added and the solvent evaporated. After addition of H_2O (1.5 ml) the resulting suspension was sonicated. The soln was administered via the stem to one plant following the previous procedure, and the withanolides isolated and purified as in experiment II.

Acknowledgements—We are grateful to the Science Research Council for financial support, Mr. J. K. Hulme, Ness Botanical Gardens, University of Liverpool for supplying *W. somnifera*, and Professor D. Lavie for authentic samples of withanolides.

REFERENCES

1. Lavie, D., Glotter, E. and Shvo, Y. (1965) *J. Chem. Soc.* 7517.
2. Lavie, D., Kirson, I. and Glotter, E. (1968) *Israel J. Chem.* **6,** 671.
3. Lockley, W. J. S., Roberts, D. P., Rees, H. H. and Goodwin T. W. (1974) *Tetrahedron Letters* 3773.
4. Yu, P. L. C., El-Olemy, M. M. and Stohs, S. J. (1974) *Lloydia* **37,** 593.
5. Birch, A. J. and Walker, K. A. M. (1966) *J. Chem. Soc.* (C) 1894.
6. Kirson, I., Glotter, E., Abraham, A. and Lavie, D. (1970) *Tetrahedron* **26,** 2209.
7. Kupchan, S. M., Anderson, W. K., Bollinger, P., Doskotch, R. W., Smith, R. M., Renauld, J. A. S., Schnoes, H. K., Burlingane, A. L. and Smith, D. H. (1969) *J. Org. Chem.* **34,** 3858.
8. Lockley, W. J. S., Rees, H. H. and Goodwin, T. W. unpublished results.
9. Attenburrow, J., Cameron, A. F. B., Chapman, J. H., Evans, R. M., Hems, B. A., Jansen, A. B. A. and Walker, T. (1952) *J. Chem. Soc.* 1094.