Phytochemistry, Vol. 28, No. 11, pp. 2981–2988, 1989.
Printed in Great Britain.

0031-9422/89 $3.00 + 0.00

REGULATION AND ENZYMOLOGY OF PENTACYCLIC TRITERPENOID PHYTOALEXIN BIOSYNTHESIS IN CELL SUSPENSION CULTURES OF *TABERNAEMONTANA DIVARICATA*

ROBERT VAN DER HEIJDEN,* DAVID R. THRELFALL,† ROBERT VERPOORTE* and IAN M. WHITEHEAD

Department of Applied Biology, School of Life Sciences, University of Hull, HU6 7RX, U.K.; *Division of Pharmacognosy, Center for Bio-Pharmaceutical Sciences, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands

(*Received* 27 *March* 1989)

Key Word Index—*Tabernaemontana divaricata*; Apocynaceae; cell suspension culture; cell-free synthesis; pentacyclic triterpenoid phytoalexins; elicitation; squalene synthetase; ursolic acids; oleanolic acids; monoterpene indole alkaloids.

Abstract—Treatment of growth-phase cell suspension cultures of *Tabernaemontana divaricata* with a *Candida albicans* elicitor preparation inhibited both growth and monoterpene indole alkaloid production within a few hours and caused a rapid accumulation of at least 10 pentacyclic triterpenoid phytoalexins. Cell growth was resumed at about the same time (36 hr after elicitor treatment) as phytoalexin accumulation ceased. Alkaloid production, however, was not resumed until some 72 hr after elicitation. Cell-free preparations obtained from cells 12 and 24 hr after elicitation efficiently catalysed the synthesis of squalene, squalene 2,3-oxide, α- and β-amyrin, uvaal and oleanal, ursolic and oleanolic acid, some monohydroxy derivatives of these acids, and dihydroxy derivatives of 3-*epi*-ifflaconic acid when incubated with either [1-^{14}C]IPP or [1-$^{3}H_2$]FPP as substrate. Preparations from unelicited cells synthesized squalene and small amounts of cycloartenol only. Squalene synthetase (EC 2.5.1.21) activity increased rapidly by approximately five-fold within 24 hr in response to the elicitor treatment and then declined slowly to basal levels. Feeding experiments with saturating levels (for sterol synthesis) of (*R*)-[2-^{14}C]MVA indicated that in elicited cultures the synthesis of phytosterols (and hence growth) was inhibited at the level of squalene 2,3-oxide : cycloartenol cyclase.

INTRODUCTION

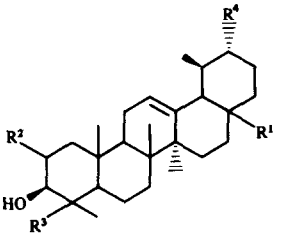
Tabernaemontana divaricata (L.) R. Br. ex Ruem. et Schult. is a rich source of indole alkaloids [1, 2]. These compounds are also produced by cell suspension of this plant [3]. It has been shown recently that cell suspension cultures of *T. divaricata* accumulate considerable amounts of pentacyclic triterpenoids in response to treatment with an elicitor preparation from the fungus *Candida albicans* [4, 5]. The major compounds which accumulate are ursolic acid (**4**), two monohydroxy derivatives of ursolic acid (**5**, **6**) and two dihydroxy derivatives of 3-*epi*-ifflaconic acid (**7**). It has been proposed that these acids may serve as phytoalexins [4, 5].

In this paper, we report on the effects of elicitor treatment on growth, monoterpene indole alkaloid production, pentacyclic triterpenoid phytoalexin accumulation and squalene synthetase activity in actively growing cultures of *T. divaricata*. We also report on the impressive biosynthetic capabilities of cell-free systems prepared from these cultures and, in addition, we provide evidence that squalene 2,3-oxide : cycloartenol cyclase activity, needed for phytosterol synthesis, is inhibited in response to elicitation.

RESULTS

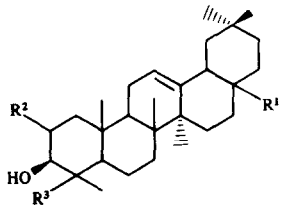
The experiments were performed with a cell line of *T. divaricata* which accumulates mainly the aspidospermatan alkaloids vallesamine and *O*-acetylvallesamine (major component) and the plumeran alkaloid vaophylline. This is a relatively simple alkaloid production profile [5] compared to the complex mixture of about 20 alkaloids reported for other lines [3].

All of the experiments were performed with five-day-old cell suspension cultures, i.e. cultures in the early growth phase. The cultures were homogenous in appearance and on elicitation with the *C. albicans* elicitor there was a rapid change (within two hours) in their colour from a very pale yellow to a dark yellow. This effect has also been observed in other elicited systems [6, 7].



	R¹	R²	R³	R⁴
1	Me	H	Me	Me
2	CH_2OH	H	Me	Me
3	CHO	H	Me	Me
4	CO_2H	H	Me	Me
5	CO_2H	OH	Me	Me
6	CO_2H	H	CH_2OH	Me
7	Me	OH	CH_2OH	CO_2H

and an isomer



	R¹	R²	R³
8	Me	H	Me
9	CH_2OH	H	Me
10	CHO	H	Me
11	CO_2H	H	Me
12	CO_2H	OH	Me
13	CO_2H	H	CH_2OH

†Author to whom correspondence should be addressed.

Effect of elicitation on growth (biomass production), alkaloid production, pentacyclic triterpenoid production and squalene synthetase activity

The biomass of the untreated cultures continued to increase for a further 72 hr from the start of the experiment and then declined slowly. Biomass production in the elicited cultures was inhibited for *ca* 24 hr after treatment of the cultures with elicitor and then resumed but at only approximately 50% the rate of the control cultures (Fig 1A).

The alkaloid content of the cells in the control cultures increased throughout the growth phase of the cells (Fig. 1B). The decrease in levels observed 96 hr after the start of the experiment (i.e. 9 days after subculture) was partly due to the catabolism of vaophylline [3] and excretion of *O*-acetylvallesamine. Alkaloid accumulation by the cells was inhibited in the elicited cultures for some 72 hr, after which time it was resumed but at a lower rate than in the control cultures (Fig. 1B). The rapid decrease in the alkaloid content of the cells, which occurs between six and 12 hr after elicitation, was matched almost exactly by the increase in the *O*-acetylvallesamine in the culture medium. The resultant elevated levels in the medium (4.28 μmol/l medium) then fell to basal levels (*ca* 2.1 μmol/l medium) within 18 hr. It is noteworthy that in a previous experiment, when the elicitor was added at the end of the growth phase, no excretion of alkaloids was observed [5].

Pentacyclic triterpenoid phytoalexins were either absent from, or were barely detectable in, control cultures. In the elicited cultures, the cell content of these terpenoids rose rapidly for some 36 hr and then showed little change until the end of the experiment (Fig. 1C). No pentacyclic triterpenoids were detected in the growth medium. The sterol content of the elicited cells (*ca* 7.6 μmol/g dry wt) remained unchanged despite the increased rate of squalene 2,3-oxide synthesis needed to bring about the rapid accumulation of the pentacyclic triterpenoids. The GC data from these experiments showed that ursolic acid (**4**) and each of its monohydroxy derivatives (**5**, **6**) was accompanied by a congener from which it was inseparable by TLC. The radiochemical studies performed with [1-^{14}C]IPP as a part of this experiment (see next section) indicated that these congeners were the corresponding β-amyrin-derived oleanolic acids (**11**–**13**) of the α-amyrin-derived ursolic acids. This was confirmed by comparison of GC and ^{1}H NMR data obtained for an authentic sample of the methyl ester of oleanolic acid (**11**) with those of the methyl esters of TLC purified ursolic/oleanolic

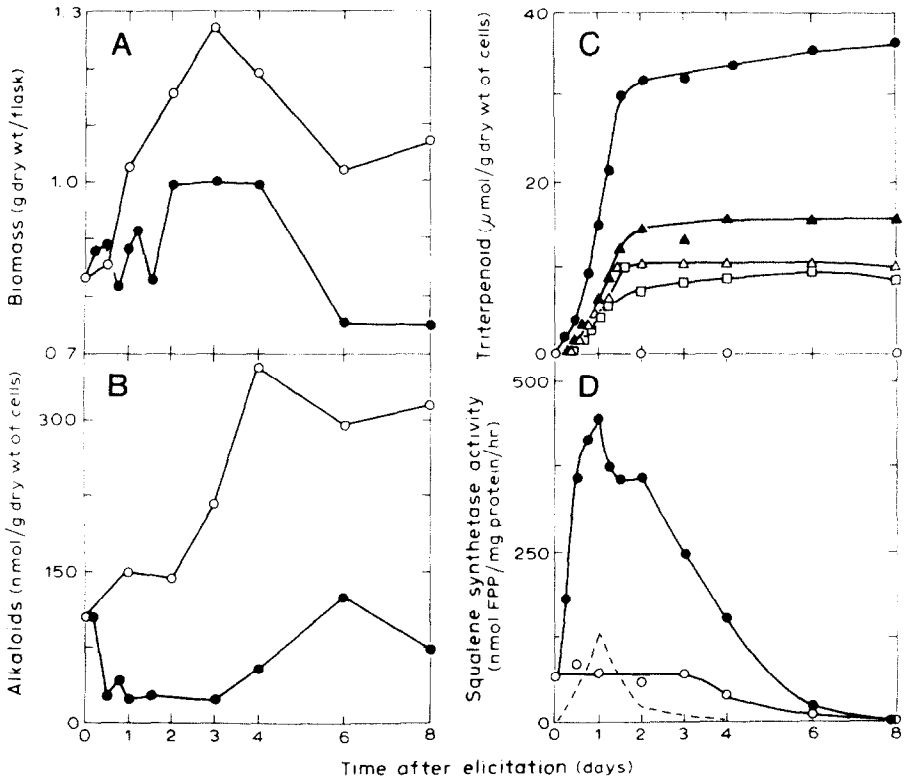


Fig. 1 Biomass production, alkaloid and pentacyclic triterpenoid accumulation, and squalene synthetase acitivity in unelicited and elicited cultures of *T. divaricata*. A. Biomass production. ○—○, unelicited cultures; ●—●, elicited cultures. B. Alkaloid content of cells. Symbols as A. C. Pentacyclic triterpenoid content of cells in elicited cultures. ○—○, Total in cells of unelicited cultures; ●—●, total in cells of elicited cultures; □—□, ursolic acid (**4**); △—△, monohydroxyursolic acids (**5** and **6**); ▲—▲, dihydroxy-3-*epi*-ifflaconic acids (**7**). Values shown on the graph include those of the corresponding congeners (**11**–**13**), for ratios see text. D. Squalene synthetase activity. Symbols as A.·- - -·, FPP incorporated into α- and β-amyrin in squalene synthetase assays performed on cells from elicited cultures.

acids from elicited cultures. In fact, the values (2.9:1) obtained from integration of the COOMe signals (δ3.605 and 3.625 respectively) in the ^{1}H NMR of the ursolic/oleanolic acid mixture extracted from the cultures corresponded closely to the values (2.8:1) obtained by integration of the peaks resolved by GC and demonstrated that either method was suitable for estimating the ratio of these two compounds. The ratios (as estimated by ^{1}H NMR) of monohydroxyursolic (**5**, **6**) to monohydroxyoleanolic acids (**12**, **13**) were 2.6:1. The GC and ^{1}H NMR data also indicated that the dihydroxy-3-*epi*-ifflaconic acids (**7**) were accompanied by congeners (**7**: congeners::3.5:1).

The squalene synthetase activity of the cells from control cultures remained constant for *ca* 96 hr and then declined rapidly (Fig. 1D). In the cells from elicited cultures, however, the activity of this enzyme increased rapidly over the first 24 hr to reach a level some five times higher than that in the cells of the corresponding control culture (Fig. 1D). This increase in squalene synthetase activity precedes pentacyclic triterpenoid phytoalexin accumulation by several hours (Fig. 1C and D). The activity then declined slightly over the next 24 hr, after which time it fell rapidly to the same levels as those measured in the corresponding control cultures. Despite the short incubation period (5 min) used for the squalene synthetase assays, α- (**1**) and β-amyrin (**8**) (3:1) were formed as assay products by the cell-free systems of cells from cultures elicited for 6–72 hr (Fig. 1D).

Table 1. Incorporation of radioactivity from [1-^{14}C]IPP into triterpenoid compounds by cell-free preparations of cells of unelicited and elicited cultures of *T. divaricata*

Triterpenoid	Time (hr) after elicitation				
	0	12	24	72	192
	Radioactivity (10^{-3} dpm)				
Lipid extract	178	198	241	238	113
Squalene	132	25	26	82	102
Squalene 2,3 oxide	11	4	3	20	trace
Cycloartenol	20	0	0	0	0
α-/β-Amyrin (**1**/**8**)*	0	24	43	131	0
Uvaal/oleanal (**3**/**10**)	0	24	64	0	0
Ursolic/oleanolic acids (**4**/**11**)*	0	21	45	0	0
Monohydroxy acids (**5**, **6**, **12**, **13**)	0	53	28	0	0
Dihydroxy acid (**7**)	0	34	17	0	0
Total in identified compounds (%)	92	93	94	98	90

Cell-free systems prepared from cells of elicited cultures at the time shown were incubated with [1-^{14}C]IPP (55 μCi/μmol, 4.44 × 10^5 dpm) and an NADPH generating system for 45 min at 30°.
*^{14}C-Labelled α-amyrin (**1**) : β-amyrin (**8**)::2.3:1; uvaal (**3**):oleanal (**10**)::4:1; ursolic acid (**4**):oleanolic acid (**11**)::3:1.

Biosynthetic capabilities of the cell-free system

This was investigated by incubating aliquots of the cell-free homogenates prepared from the cells of 0 (i.e. 5-day-old) and 192 hr control cultures and 12, 24, 72 and 192 hr elicited cultures (used for the assay of squalene synthetase activity, see above) with a non-saturating concentration of [1-^{14}C]IPP for a protracted period of time (45 min) in the presence of molecular O_2 and an NADPH-generating system. In the incubations containing homogenates from the 0 hr control culture and the 12, 24 and 72 hr elicited cultures, about 50% of the administered radioactivity (4.44 × 10^5 dpm) was recovered in the lipid extracts of the incubation mixtures whereas, in the 192 hr cultures the amounts recovered were much lower (elicited cultures 25%; control cultures 7%) (Table 1). In each case however, almost all of the radioactivity in the lipid extract was present in the triterpenoid compounds under study (Table 1).

Radio-TLC analysis of the lipid extracts (Fig. 2) followed by analysis (radio-TLC, radio-GC) of underivatized and derivatized samples of the ^{14}C-labelled compounds recovered from prep. TLC of the lipid extracts established that whereas the cell-free preparations from actively growing control cultures were capable of the synthesis from IPP of squalene and small amounts of cycloartenol, those from the cells of cultures which had been exposed to elicitor for 12 and 24 hr respectively were capable of the synthesis of squalene, squalene 2,3-epoxide, α- (**1**) and β-amyrin (**8**) (2.3:1), uvaal (urs-12-ene-3β-ol-28-al) (**3**) and oleanal (olean-12-ene-3β-ol-28-al) (**10**) (4:1), ursolic (**4**) and oleanolic acid (**11**) (3:1), the monohydroxy derivatives of each of these acids (**5**, **6**, **12**, **13**), and dihydroxy-3-*epi*-ifflaconic acids (**7** and congeners) (Table 1). The cell-free preparations from cells elicited for 72 hr, however, were only capable of the synthesis of the

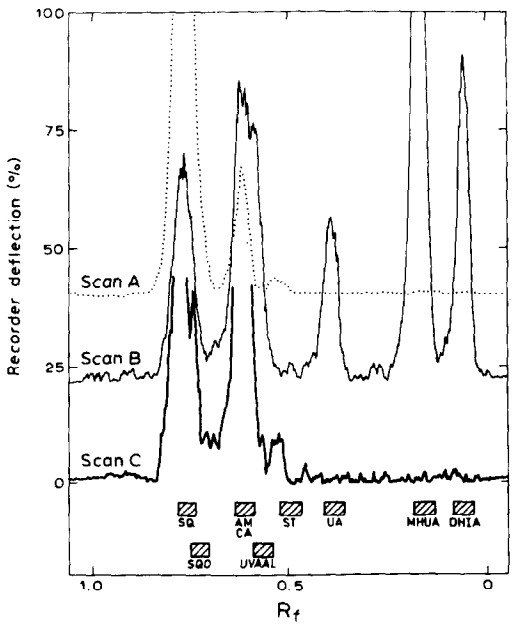


Fig. 2. Radioscans of a thin-layer chromatogram (system A) of the chloroform-soluble lipids isolated from cell-free homogenates which had been incubated with [1-^{14}C]IPP in the presence of an NADPH-generating system and air. Scans A, B and C show the ^{14}C-labelled compounds produced by cell-free homogenates prepared from cells of cultures which had been elicited for 0, 12, and 72 hr respectively. SQ, squalene; SQO squalene 2,3-oxide; CA, cycloartenol; AM α- (**1**) and β-amyrin (**8**); DMS, demethylsterols; UA, ursolic acid (**4**) plus oleanolic acid (**11**); MHUA; monohydroxy ursolic and oleanolic acids (**5**, **6** and **12**, **13**); DHIA, dihydroxy-3-*epi*-ifflaconic acids (**7**).

first four compounds while those from cells elicited for 192 hr synthesized squalene only (Table 1).

The synthesis from [1-^{14}C]IPP of all the compounds listed above was NADPH-dependent and, with the exception of squalene, O_2-dependent. When the [1-^{14}C]IPP in the incubations was replaced by [1-$^3H_2$]FPP (1 μCi; 40 μCi/μmol), the results were qualitatively identical. However, a much greater proportion of the total radioactivity incorporated was recovered in squalene, squalene 2,3-oxide and α- and β-amyrin.

Rate of accumulation of squalene and squalene-derived compounds in unelicited and elicited cells in the presence of exogenously supplied MVA

The results of the time-course study had shown that the increase in squalene synthetase activity and total triterpenoid content of the cells in elicited cultures was not accompanied by a measurable increase in the sterol content of the cultures. This suggested that either the rate of cycloartenol synthesis from squalene 2,3-oxide is already rate limiting in unelicited cells or the formation of cycloartenol from squalene 2,3-oxide in cells of elicited cultures is inhibited. To investigate this aspect further, the changes in the levels of squalene, squalene 2,3-oxide, sterols and pentacyclic triterpenoids were measured in control and elicited cultures administered a single dose of (*R*)-[2-^{14}C]MVA at what was considered [8] to be a saturating concentration (3.3 mM) for sterol synthesis (Fig. 3).

Squalene does not accumulate during normal metabolism in either unelicited or elicited cultures of *T. divaricata*. However, radio-TLC and GC analysis showed that there was a very rapid accumulation of squalene in unelicited cultures administered (*R*)-[2-^{14}C]MVA followed by a much slower conversion of the accumulated squalene into cycloartenol and 4-demethylsterols (Fig. 3A). In the elicited cultures, however, the (*R*)-[2-^{14}C]MVA was incorporated into pentacyclic triterpenoid phytoalexins (Fig. 3B), the levels of which were significantly higher than those in cultures elicited in the absence of (*R*)-[2-^{14}C]MVA. Only a very small amount of label was incorporated into squalene, cycloartenol and 4-demethylsterols. Specific radioactivity measurements established that over 90% of the carbon content of the squalene and ursolic acid (**4**) plus oleanolic acid (**11**) accumulated in the control and elicited cultures respectively was derived from the added MVA.

DISCUSSION

The elicitor-mediated induction of pentacyclic triterpenoid phytoalexin accumulation in growth-phase cells of *T. divaricata* cultures is accompanied by an inhibition of both growth and indole alkaloid accumulation. The accumulation of pentacyclic triterpenoids lasts for *ca* 36 hr at about which time growth is resumed. However, alkaloid accumulation remains inhibited for a further 36 hr.The total amount of pentacyclic triterpenoid phytoalexins produced by elicited cultures of *T. divaricata* is at least an order of magnitude greater than the respective levels of sesquiterpenoid/isoflavonoid phytoalexins produced by solanaceous/leguminous plant cell suspension cultures [6, 7, 9–11].

The results of the time-course study (Fig. 1), the [1-^{14}C]IPP incorporation experiments with cell-free systems (Fig. 2 and Table 1) and the (*R*)-[2-^{14}C]MVA feeding experiment (Fig. 3) are all consistent with the view that the accumulation of pentacyclic triterpenoid phytoalexins is dependent upon to the induction of squalene 2,3-oxide : α- and β-amyrin cyclase activities and, by implication, the enzyme activities needed to convert α- (**1**) and β-amyrin (**8**) to ursolic (**4**) and oleanolic (**11**) acid, the monohydroxy derivatives of these acids (**5**, **6** and **12**, **13**), and the dihydroxy derivatives of 3-*epi*-ifflaconic acid (**7**). It is assumed that the enzymes catalysing these reactions are formed by *de novo* synthesis and there is some evidence from protein inhibitor studies that this is indeed the case [3]. The biosynthetic capabilities of the cell-free systems prepared from elicited cells are impressive both in

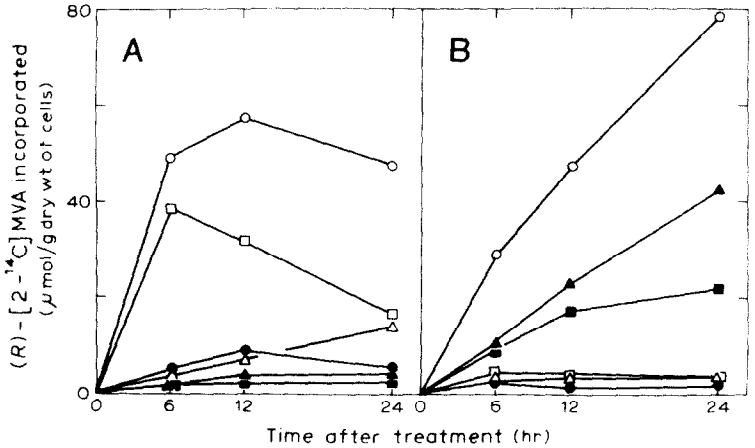


Fig. 3. Amounts of MVA incorporated into triterpenoids by cells of *T. divaricata* cultures which had been administered (*R*)-[2-^{14}C]MVA to a final concentration of 3.3 mM and then inoculated immediately with either the elicitor preparation (B) or SDW (A). ○—○, lipid extract; □—□, squalene; ●—●, cycloartenol; △—△, demethylsterols; ■—■, ursolic acid (**4**) and oleanolic acid (**11**); ▲—▲, monohydroxy ursolic and oleanolic acids (**5**, **6** and **12**, **13**) and dihydroxy-3-*epi*-ifflaconic acids and their congeners (**7**).

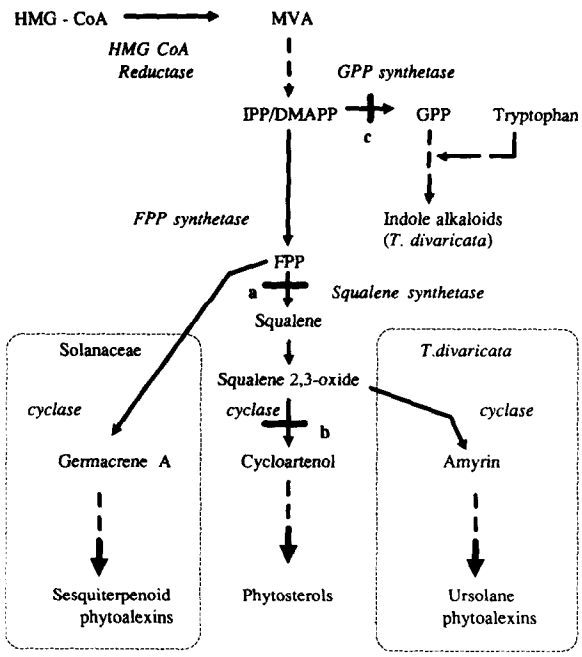
the number of steps catalysed and the amount of label from [1-^{14}C]IPP incorporated into the terminal products of the pathway (Fig. 2 and Table 1). The results indicate that the biosynthetic route from α-amyrin to the ursolic acids is: α-amyrin (**1**)→uvaal (**3**)→ursolic acid (**4**)→monohydroxyursolic acids (**5**, **6**). The analogous route in which α-amyrin is replaced by β-amyrin would be: β-amyrin (**8**)→oleanal (**10**)→oleanolic acid (**11**)→monohydroxyoleanolic acids (**12**, **13**). It is of interest that neither uvaol (**2**) nor oleanol (**9**) were detected in the cell-free incubations. This may indicate that the two-step conversion of α-amyrin to uvaal and of β-amyrin to oleanal is, in each case, catalysed by a single mixed function oxidase. No information was obtained about the biosynthetic route for the formation from α-amyrin of the dihydroxy-3-*epi*-ifflaconic acids.

The elicited cells lose the ability to synthesize pentacyclic triterpenoid phytoalexins some 36–48 hr after elicitation, as judged by the pentacyclic triterpenoid accumulation curve (Fig. 1B), the marked reduction in the amounts of α- and β-amyrin produced in the squalene synthetase assay when the assay is performed on cells which have been elicited for more than 24 hr (Fig. 1D), and the inability of cell-free homogenates from the cultures elicited for 72 hr to synthesize from IPP any pentacyclic triterpenoids other than amyrins (Fig. 2 and Table 1). It is noteworthy that the cell-free synthesis of α- and β-amyrin 72 hr after elicitation is not reflected by accumulation of these compounds in the cells. The time course study shows that the pentacyclic triterpenoid phytoalexins, unlike sesquiterpenoid phytoalexins [6, 7, 10], are not catabolized to any great extent and thus the rate of accumulation of these compounds in the cells is a direct measure of their rate of biosynthesis.

The very rapid induction followed by an equally rapid loss of some of the enzyme activities needed to synthesize pentacyclic triterpenoids from squalene 2,3-oxide is mimicked by the changes in the squalene synthetase activity in the elicited cells (Fig. 1C and Table 1). These changes in squalene synthetase activity probably represent a specific response designed to provide more substrate for pentacyclic triterpenoid phytoalexin biosynthesis, since the squalene synthetase activity of the unelicited cells as measured both directly (V_{max} about 1 μmol/hr/g dry wt of cells) and indirectly (initial rate shown in Fig. 2A, 1.4 μmol/hr/g dry wt of cells) is insufficient to provide the amounts of squalene needed for the maximum observed rate of synthesis of the pentacyclic triterpenoid phytoalexins in elicited cells [rate between 18 and 36 hr (calculated from Fig. 1B) = 1.7 μmol/g dry wt of cells].

The stimulation of squalene synthetase activity as a vital (regulatory) event in the elicitor-induced accumulation of pentacyclic triterpenoid phytoalexins in cells *T. divaricata* provides further support for the proposal [7, 12] that the observed inhibition of this enzyme's activity in cells of elicitor treated cultures of solanaceous plants, is a crucial and specific (regulatory) event which is designed to divert FPP away from the biosynthesis of phytosterols and into the biosynthesis of sesquiterpenoid phytoalexins (see Scheme 1).

The cell-free studies and the MVA feeding experiment both indicate that the formation of squalene 2,3-oxide from squalene is the first rate-limiting step on the pathway leading from MVA to phytosterols in unelicited cells and that an increase in the activity of the enzyme catalysing this reaction (squalene 2,3-epoxidase EC



Scheme 1. Summary of the major regulatory events which leads to the efficient channelling of the biosynthetic intermediates of the terpenoid pathway into terpenoid phytoalexin biosynthesis in *T. divaricata* and solanaceous plants. **a**, Enzyme inhibited in elicited solanaceous plants/stimulated in *T. divaricata*; **b**, this enzyme appears to be inhibited in elicited cultures of *T. divaricata*; **c**, this enzyme may be inhibited in elicited cultures of *T. divaricata*; dotted boxes = induced pathways. Note that in solanaceous systems, changes in the rate of catabolism of the phytoalexins are important events in determining the levels of individual phytoalexins which accumulate [7].

1.14.99.7) must take place in elicited cells. They also indicate that phytosterol biosynthesis is inhibited at the level of squalene 2,3-oxide: cycloartenol cyclase in elicited cells. In the absence of inhibition of this cyclase, a considerable amount of the extra squalene 2,3-oxide which is produced in elicited cells might well be utilized for the biosynthesis of cycloartenol rather than the α- and β-amyrins needed for pentacyclic triterpenoid phytoalexin biosynthesis. Evidence that cycloartenol formation from squalene 2,3-oxide can proceed in an unregulated manner in unelicited cells is provided by MVA feeding experiments with potato cell suspension cultures [6]. In the present study, however, the MVA feeding experiment did not yield such clear cut results due to the rate-limited formation of squalene 2,3-oxide from squalene in unelicited cells of *T. divaricata*. The inhibition of cycloartenol formation in elicited cultures would result in an inability of the cells to synthesize phytosterols which, in turn, would be a major factor contributing to the observed inhibition of growth.

The rapid cessation of indole alkaloid accumulation in elicited cells may result from the inhibition of the GPP synthetase which provides the geranyl-derived moiety of these alkaloids. Such a mechanism, which would further reduce the demand for intermediates of the terpenoid pathway, in this case IPP, would be significant in cultures and plant tissues producing large amounts of these alkaloids.

The results of this study and similar studies with potato and tobacco cell suspension cultures establish that some of the major regulatory events which bring about a rapid and efficient synthesis of terpenoid phytoalexins in elicited cultures are: the modulation of HMG-CoA reductase activity [12–15], and in the case of pentacyclic triterpenoid phytoalexins, squalene synthetase and squalene 2,3-epoxidase; the *de novo* synthesis of some (or all) of the specific enzyme activities needed for terpenoid phytoalexin production [6, 7, 12, 16, 17], and (significantly) the inhibition of any directly competing branch of the terpenoid pathway probably at the level of the first enzyme on the competing branch [6, 7, 12] (Scheme 1).

The phenomenon of substrate channelling in response to elicitation is the subject of an on-going research project in our laboratories which in addition to studying the events already identified as being important will attempt to establish if the biosynthesis of ubiquinone and dolichol, both of which require intermediates of the cytosolic pathways of terpenoid biosynthesis, are inhibited in elicited cell suspension cultures of *T. divaricata* and solanaceous plants.

EXPERIMENTAL

Radiochemicals and chemicals. [1-^{14}C]IPP (55 μCi/μmol) and (*R*)-[2-^{14}C]MVA lactone (55 μCi/μmol) were purchased from the Radiochemical Centre, Amersham. (*R*)-[2-^{14}C]MVA (9.23 μCi/mmol) was prepared by adding the appropriate amounts of (*R, S*)-MVA lactone (Sigma) and (*R*)-[2-^{14}C]MVA lactone (55 μCi/μmol) to an aq. soln of $NaHCO_3$. Ursolic acid (**4**) and uvaol (**2**) were purchased from Aldrich, Dorset. Oleanolic acid (**11**) was purchased from Apin Chemicals Ltd, Abingdon. Ruthenium (II)-tris-(triphenylphosphine)-dichloride [$Ru(Ph_3P)_3Cl_2$] was purchased from Thiokol Chemicals Ltd, Coventry. [1-^{3}H$_2$]FPP (40 μCi/μmol), squalene 2,3-oxide, cycloartenol and α- (**1**) and β-amyrin (**8**) were available from previous studies [6].

TLC was performed on (i) 0.5 mm Rhodamine 6G-impregnated silica gel G (or 0.5 mm silica gel G) developed with one of following solvents. A: EtOAc–cyclohexane (1:1); B: toluene–EtOAc (17:3); C: toluene–EtOAc (9:1); D: petrol (6p 40–60°)–Et_2O (4:1); E: diisopropyl ether–Me_2CO (5:2) and (ii) $AgNO_3$ (10%)–impregnated silica gel G (Ag^+-TLC) developed with either (F) toluene–petrol (1:1) or (G) MeOH–toluene (1:19). Compounds on a developed plate were located either by examination of the plate under UV_{254} light (Rhodamine 6G-impregnated plates) or by spraying with anisaldehyde–H_2SO_4 reagent (plus heating) [18]. Radioactivity on the developed plates was detected by means of a thin layer radiochromatogram scanner (Radio-TLC). The GC and radio-GC conditions used were the same as those used for the analysis of sterols and steryl esters in a previous study [6], except that following oven temp. programme was used: 280° for 5 min; ramp 280° to 320° over 5 min; 320° for 20 min. ^{1}H NMR were recorded in $CDCl_3$ at 270.17 MHz with TMS as int. std.

Synthesis of uvaal (*urs*-12-*ene*-3-*ol*-28-*al*). 10 mg of uvaol was dissolved in 2 ml C_6H_6 and stirred overnight with 34.6 mg Ru $(Ph_3P)_3Cl_2$ (1.6 mol excess) [19]. After removal of the solvent under a stream of N_2, the residue was taken up in Me_2CO and applied to a TLC plate. Development in system A and visualization under UV_{254} light gave two pink bands at R_f 0.5–0.7. The two compounds were recovered from the gel with Et_2O and re-TLC'd in system B to give unreacted uvaol (4.6 mg, R_f 0.21) and crude uvaal (R_f 0.34). The uvaal was further purified by TLC (system D) to give pure uvaal (amorphous solid, 3.8 mg, R_f 0.15). ^{1}H NMR: δ9.34 (1H, *d*, J = 1.15 Hz, –CHO).

Plant material and elicitation. The growth and maintenance of the *Tabernaemontana divaricata* cell suspension cultures and the preparation of the *Candida albicans* elicitor have been described previously [4]. In both of the experiments reported in this paper, each flask of a series of 250 ml conical flasks containing 50 ml of medium/flask was inoculated with 21 ml (*ca* 7 g fr. wt) of a 6-day-old suspension culture of *T. divaricata*. The necks of the flasks were closed with two layers of Al foil and the flasks placed in an illuminated orbital incubator (110 rpm, 22°, subdued light) for five days. After this time, the cultures were treated as follows. *Time course study*: twelve cultures were each inoculated with 1 ml (5 mg dry wt) of the *C. albicans* elicitor preparation. The remainder (8) of the cultures remained untreated. *MVA feeding experiment.* Out of a total of 10 cultures, 3 were each inoculated with 1 ml of the *C. albicans* elicitor preparation, three were each inoculated with (*R*)-[2-^{14}C]MVA (9.23 μCi/mmol) to a final concn of 3.3 mM and three were each treated with 1 ml elicitor and ^{14}C-MVA. The tenth culture remained untreated.

Analysis of cultures from time course study. Each of the cultures for analysis (one flask/treatment) was harvested by filtration through Miracloth and the retained cells washed with 300 ml H_2O. The initial filtrate plus the first 30 ml of the H_2O wash was stored at −10° for subsequent analysis of alkaloid content by a method described elsewhere [3, 20]. The washed cells were weighed and 4 g taken for the preparation of a cell-free homogenate (see below). The remainder of the cell mass was 'snap-frozen' in liq. N_2 and lyophilized. The lyophilized cells were weighed and this value was used to calculate the total dry wt based on the total fr. wt obtained. The lyophilized material was then assayed for alkaloid content (200 mg dry wt of cells/analysis) by the method referred to above and for triterpennoid content. To determine the latter, a sample (200 mg) of the lypohilized cells was extracted by a method similar to that described in ref. [4]. The extract obtained was methylated (CH_2N_2) and acetylated (Ac_2O–pyridine), and then subjected to analytical GC. The RR_t values (MeAc ursolic = 12 min) of the derivatized pentacyclic triterpenoids under these conditions were as follows: (steryl acetates, 0.5–0.79); MeAc-congener of ursolic acid (later identified as oleanolic acid), 0.94; MeAc-ursolic acid, 1; $MeAc_2$-monohydroxyursolic acids, 1.20 (congeners) and 1.27; $MeAc_3$-dihydroxy-3-*epi*-ifflaconic acids, 1.41, 1.59 (congeners) and 1.72. The amounts of the triterpenoids present were estimated by comparison with the peak area given by a stock solution of MeAc-ursolic acid. In a previous study, the pentacyclic triterpenoids produced in elicited cultures of *T. divaricata* were identified (TLC, MS, ^{1}H NMR) as ursolic acid (**4**), 2- and 23-monohydroxyursolic acid (**5** and **6**), 2,23-dihydroxy-3-*epi*-ifflaconic acid (**7**) and an isomer of 2,23-dihydroxy-3-*epi*-ifflaconic acid [3]. However, GC analysis of methylated and acetylated extracts of elicited cells of *T. divaricata* and of similarly derivatised TLC (system E) purified pentacyclic triterpenoids (equivalent to I-V of [4]) from these extracts indicated that the pentacyclic triterpenoid composition of the cultures was more complex (see RR_t data above) than reported in ref. [4]. The demonstration by GC and ^{1}H NMR (see Results) of the presence of oleanolic acid (**11**) in TLC (system E and then A) purified samples of ursolic acid established that at least three series of pentacyclic triterpenoids were being produced in response to elicitation: one based on ursolic acid (major components, **4**–**6**); one based on 3-*epi*-ifflaconic acid (**7**) and one based on oleanolic acid (congeners, **11**–**13**).

Analysis of cultures from ^{14}C-*MVA feeding expt.* At the times shown (Fig. 3), the cells of each of the cultures for analysis were harvested by the procedures described above. The washed cells

were then weighed, 1 g lyophilized for dry wt determination, and the remainder analysed for ^{14}C-labelled triterpenoids. The wet cell mass was extracted by the method described in [4]. The resultant extract was taken up in 1 ml $CHCl_3$–MeOH (1:1), 10 μl assayed for radioactivity and 300 μl derivatised and subjected to GC analysis as described in the previous section. The GC traces were then used to estimate the amounts of squalene (present in MVA-treated cultures, *RR*, 0.16), sterols and pentacyclic triterpenoid acids. A further aliquot (40 μl) was then subjected, along with marker compounds, to TLC in system A (see Fig. 2 for R_f values) and a further 40 μl to TLC in system B (squalene, R_f 0.78; squalene 2,3-oxide, 0.7; cycloartenol and α- and β-amyrin, 0.4; 4α-methylsterols, 0.33; demethylsterols, 0.27; ursolic oleanolic acid, 0.13; monohydroxyursolic acids, 0.04; dihydroxy-3-*epi*-ifflaconic acid and its isomer, 0.01). The developed plates were radioscanned and the bands of gel corresponding to squalene, squalene 2,3-oxide, cycloartenol plus α- and β-amyrins, 4α-methylsterols, demethylsterols, ursolic plus oleanolic acid, these acids monohydroxy derivatives and dihydroxy-3-*epi*-ifflaconic acid and its isomer respectively were scraped into scintillation vials and assayed for radioactivity. The counts obtained were then used in conjunction with the GC data (see above) to calculate the amounts of the above compounds formed from the added ^{14}C-MVA. No significant amounts of radioactivity were ever found to be associated with squalene 2,3-oxide, monomethyl sterols or any compounds other than those described above. The unused portions of the extracts were run on TLC (system A) and the areas of gel containing squalene, cycloartenol and/or α/β-amyrins, and ursolic–oleanolic acid were eluted with Et_2O. The cycloartenol and/or α-/β-amyrin-containing fractions (after the addition of carrier cycloartenol and α- and β-amyrin) were then acetylated (Ac_2O–pyridine) and the ursolic acid–oleanolic acid fractions both methylated (CH_2N_2) and acetylated (Ac_2O–pyridine). Radio-Ag^+-TLC [system F or G (for squalene)] and/or radio-GC (310° isothermal) of the squalene-containing fractions and the derivatized fractions established that all of the radioactivity in the squalene-containing fractions co-chromatographed with squalene (R_f 0.21), the radioactivity in the cycloartenol and/or α-/β-amyrin-containing fractions co-chromatographed with the marker cycloartenol (α-amyrin acetate R_f 0.37, R_t (min) 11.0; β-amyrin acetate 0.37, 9.8; cycloartenyl acetate R_f 0.32,; 24-methylenecycloartanyl acetate R_f 0.26) and the radioactivity in the ursolic acid/oleanolic acid-containing fraction was distributed between the two acids in a manner which refelcted the amounts of the two acids present in the extracts. Finally, the radio-GC data was used to calculate the sp. radioactivity of the ^{14}C-labelled squalene and MeAc-ursolic acid.

Assay of squalene synthetase acitivity and biosynthetic capacity of cell-free systems. The 4 g sample of freshly harvested cells along with 0.8 g acid-washed sand and 0.4 g insoluble PVP (Sigma) was transferred to a pre-cooled (−10°) pestle and mortar and ground for 30 sec in 2 ml ice-cold 0.1 M K-Pi buffer, pH 7.5, containing 1 M sucrose, 2 mM EDTA and 10 mM 2-mercaptoethanol. The homogenate was filtered through Miracloth and the filtrate centrifuged at 4 500 g for 15 min at 0°. The 4 500 g superatant was designated cell-free preparation. The squalene synthetase assay mixture (total vol 1 ml) contained 0.2 ml cell-free preparation (0.1–0.3 mg protein) and 0.8 ml 0.05 M K-Pi-buffer, pH 7.5, containing 0.5 M sucrose, 1 mM EDTA and 1 mM 2-mercaptoethanol, 10 μmol $MgCl_2$ (added last to avoid pptn), 0.25 μmol NADP, 3.2 μmol G-6-P, 0.8 IU G-6-P dehydrogenase and 25 nmol [1-$^3H_2$]FPP (40 μCi/μmol). The [1-$^3H_2$]FPP was added after the incubation mixture had been pre-equilibrated at 30° for 5 min. The assay mixture was then incubated at 30° for either 5 or 10 min, after which time the incubation was terminated by the addition of 5 ml $CHCl_3$–MeOH (1:2). The $CHCl_3$-soluble lipid was extracted from the $CHCl_3$–MeOH-treated reaction mixture by the procedure described in a previous paper [7]. The lipid extract was taken up in 100 μl Me_2CO containing 100 μg each of squalene and farnesol and 10 μl assayed for radioactivity. The remainder of the soln, unless analysed immediately, was stored at −20°. The incubation mixture used to determine the biosynthetic capacities of the cell free preparations contained (total vol. 1 ml) 0.5 ml cell-free preparation and 0.5 ml 0.05 M K-Pi-buffer, pH 7.5, containing 0.5 M sucrose, 1 mM EDTA and 1 mM 2-mercaptoethanol, 10 μmol $MgCl_2$, 0.25 μmol NADP, 3.2 μmol G-6-P, 0.8 IU G-6-P dehydrogenase and 3.6 nmol [1-^{14}C]IPP (55 μCi/μmol). Each incubation was performed in duplicate and the reactions were terminated after 45 min as described above.

(*a*) *Estimation of squalene synthetase activity.* As radio-TLC analysis (systems A and B) showed that the radioactivity in the lipid extracts from the squalene synthetase assays was associated only with squalene (major labelled compound), squalene derived compounds and farnesol, the total radioactivity content of each extract minus the radioactivity in the farnesol was used in the calculation of squalene synthetase activity. In the calculation, allowance was made for the loss of one ^{3}H atom in the formation of 1 molecule of ^{3}H-labelled squalene from two molecules of [1-$^3H_2$]FPP.

(*b*) *Characterization of* ^{14}*C-labelled compounds produced in cell-free incubations containing* [1-^{14}C]*IPP.* Radio-TLC (systems A and B) of aliquots of the $CHCl_3$-soluble lipids established that all of the radioactivity present in each extract was distributed between compounds which had the same R_f values as some or all of the marker compounds of the expected products, except in the case of some of the lipid extracts from the incubations containing cell free preparations from elicited cells which also contained an unknown ^{14}C-labelled compound (Fig. 2). The remainder of each extract was bulked with its duplicate and, after the addition of carrier amounts of squalene, squalene 2,3-oxide, cycloartenol, α- and β-amyrin, ursolic acid and oleanolic acid, subjected to prep TLC (system A, double developed to resolve α- and β-amyrin from the unknown compound). Each radioactive band on the gel along with its coincident carrier compound(s) was scraped off the plate and eluted with Et_2O. The unknown compound(s) was also recovered in the same way. Radio-Ag^+-TLC [systems F and G (for squalene)] and/or radio-GC (300° isothermal) analysis of the recovered fractions, after acetylation (Ac_2O) of the fractions containing cycloartenol and α-/β-amyrin and acetylation and methylation (CH_2N_2) of the fractions containing ursolic acid and oleanolic acid, confirmed that the radioactivity present in the extracts of the incubations containing cell-free preparations from unelicited cultures was present only in squalene, squalene 2,3-oxide and cycloartenol whereas that present in the extracts of the incubations containing cell-free preparations from elicited cells was present in squalene, squalene 2,3-oxide, α- and β-amyrin (2.25:1), ursolic and oleanolic acid (3:1), the monohydroxy derivatives of these acids, and dihydroxy-3-*epi*-ifflaconic acid and its isomer (R_f and R_t values given in the section describing the analysis of cultures from the ^{14}C-MVA feeding experiment). In the course of these characterization studies, it became apparent that the unknown radioactive compound(s) formed in the incubations containing cell-free preparations of elicited cells were most probably uvaal (**3**) and oleanal (**10**), the corresponding aldehydes of ursolic acid and oleanolic acid respectively. This was confirmed by the demonstrations that: (a) the radioactivity associated with these compounds cochromatographed with synthetic uvaal on TLC in system A (see Fig. 2); (b) after treatment of the compounds with

$NaBH_4$ the radioactivity co-chromatographed with authentic uvaol (R_f 0.52) on TLC in system A; (c) after acetylation of the reduction product(s) the bulk of the radioactivity had the same R_t on GC as acetyl uvaol (11 min), the remainder being eluted just prior to acetyl uvaol (9.6 min), cf. behaviour of α- and β-amyrin and of ursolic acid and oleanolic acid.

Acknowledgements—The authors wish to thank Mrs Margaret Huffee for expert technical assistance, our colleagues in the Department of Chemistry for obtaining NMR and MS data and the AFRC and British Council (Amsterdam) for financial support.

REFERENCES

1. van Beek, T. A., Verpoorte, R., Baerheim Svendsen, A., Leeuwenberg, A. J. M. and Bisset, N. G. (1984) *J. Ethnopharmac.* **10**, 1.
2. van Beek, T. A., van Gessel, M. A. J. T. (1988) in *The Alkaloids: Chemical and Biological Perspectives* (Pelletier, S. W., ed.) Vol. 6, p. 75. Wiley, New York.
3. van der Heijden, R., Hermans-Lokkerbol, A. de Kool, L. P. J., Lamping, P. J., Harkes, P. A. A. and Verpoorte, R. (1988) *Planta Med.* **54**, 393.
4. van der Heijden, R., Verheij, E. R., Schripsema, J., Baerheim Svendsen, A., Verpoorte, R. and Harkes, P. A. A. (1988) *Plant Cell Reports* **7**, 51.
5. van der Heijden, R., Verpoorte, R. and Harkes, P. A. A. (1988) in *Manipulating Secondary Metabolism in Culture* (Robins, R. J. and Rhodes, M. J. C., eds), p. 217. Cambridge University Press, Cambridge.
6. Brindle, P. A., Kuhn, P. J. and Threlfall D. R. (1988) *Phytochemistry* **27**, 133.
7. Threlfall, D. R. and Whitehead, I. M. (1988) *Phytochemistry* **25**, 2567.
8. Goad, L. J. (1983) *Biochem. Soc. Trans.* **11**, 548.
9. Whitehead, I. M., Ewing, D. F. and Threlfall, D. R. (1988) *Phytochemistry* **27**, 1365.
10. Chappell, J., Nable, R., Fleming, P., Andersen, R. A. and Burton, H. R. (1987) *Phytochemistry* **26**, 2259.
11. Hargreaves, J. A. and Selby, C. (1978) *Phytochemistry* **17**, 1099.
12. Vögeli, U. and Chappell, J. (1988) *Plant Physiol.* **88**, 1291.
13. Ôba, K., Kondo, K., Doke, N. and Uritani, I. (1985) *Plant Cell Physiol.* **26**, 873.
14. Stermer, B. A. and Bostock, R. M. (1987) *Plant Physiol.* **84**, 404.
15. Chappell, J. and Nable, R. (1987) *Plant Physiol.* **85**, 469.
16. Whitehead, I. M., Threlfall, D. R. and Ewing, D. F. (1989) *Phytochemistry* **28**, 775.
17. Coolbear, T. and Threlfall, D. R. (1985) *Phytochemistry* **24**, 1963.
18. Waldi, D. (1965) in *Thin Layer Chromatography* (Stahl, E., ed.), p. 485. Springer Berlin.
19. Tomioka, H., Takai, K., Oshima, K. and Nozaki, H. (1981) *Tetrahedron Letters* **22**, 1605.
20. van der Heijden, R., Lamping, P. J., Out, P. P., Wijnsma, R. and Verpoorte, R. (1987) *J. Chromatog.* **396**, 287.