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Biosynthesis of avenacins and phytosterols in roots of *Avena sativa* cv. *Image*

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Abstract

In keeping with the proposal that avenacin biosynthesis is restricted to the tips of primary roots of oat seedlings, the incorporation of radioactivity from *R*-[2-^{14}C]mevalonic acid (MVA) into avenacins and β-amyrin by serial sections of primary roots was found to be more-or-less restricted to root tip sections. Squalene synthase (SQS) (EC 2.5.1.21) and 2,3-oxidosqualene:β-amyrin cyclase (OSβAC) (EC 5.4.99) were also most active in these sections. The incorporation of radiolabel from *R*-[2-^{14}C]MVA into cycloartenol and 24-methylene cycloartanol by, and the 2,3-oxidosqualene:cycloartenol cyclase (OSCC) (EC 5.4.99) activity in, the various serial sections were consistent with phytosterol biosynthesis occurring in all the sections of the root with some tailing-off in the rate of synthesis in the more distal sections.

Keywords: Avena sativa; Poaceae; Biosynthesis; Saponins; Avenacins; Phytosterols

1. Introduction

Avenacins are antifungal saponins in which the aglycones are β-amyrin-derived pentacyclic triterpenoids esterified with either *N*-methylanthranilic acid or benzoic acid, and the sugar unit is a branched trisaccharide (Fig. 1). They are found exclusively in oat roots and have been implicated in the resistance of oat seedlings to the fungal soil pathogen *Gaeumannomyces graminis* var. *tritici*, the causal agent of 'take-all' disease of wheat and barley (Crombie and Crombie, 1986). Two *N*-methylanthranilic acid-containing avenacins (A-1 and B-1) and two benzoic acid-containing avenacins (A-2 and B-2) along with small amounts of desglucoavenacins A-1, glucoavenacin A-1 and the aglycone of avenacin A-1 (12,13-epoxyavenagenin A-1) have so far been isolated and characterised (Tschesche and Wulff, 1973; Begley et al., 1986). In primary roots, avenacins A-1 and A-2 are the major avenacins and account for some 90% of the total avenacin content of the roots (Crombie and Crombie, 1986). The concentration of avenacins is highest in the root tips of primary roots and this had led to the proposal that this is their site of biosynthesis (Goodwin and Pollock, 1954; Turner, 1960; Crombie and Crombie, 1986). Fluorescence microscopy has shown that the fluorescent avenacins (A-1 and B-1) are concentrated in the epidermal cells of the root (Osbourn et al., 1994).

The pentacyclic triterpenoid part of the avenacins, in common with the tetracyclic triterpenoid ring system of the phytosterols and their conjugates, is formed from mevalonic acid (MVA) by way of isopentenyl diphosphate (IPP), farnesyl diphosphate (FPP) and 2,3-oxidosqualene (Scheme 1). The avenacin and phytosterol pathways then diverge with the cyclization of

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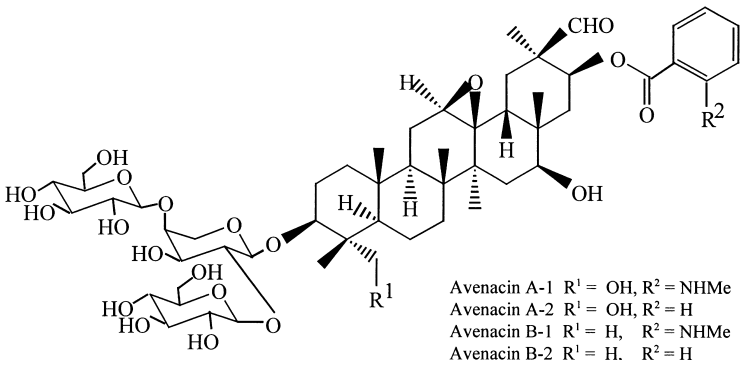


Fig. 1. Structures of the four oat root avenacins.

2,3-oxidosqualene to form the β-amyrin needed for avenacin biosynthesis and the cycloartenol needed for phytosterol biosynthesis. The formation of avenacins from β-amyrin requires: (i) extensive oxyfunctionalization of the pentacyclic triterpenoid nucleus of β-amyrin, (ii) esterification of the partly or fully functionalized β-amyrin nucleus with either *N*-methylanthranilic acid (avenacins A-1 and B-1) or benzoic acid (avenacins A-2 and B-2) and (iii) the sequential glycosylation of the 3β-hydroxy group of the appropriate 12,13-epoxyavenagenin or its progenitor.

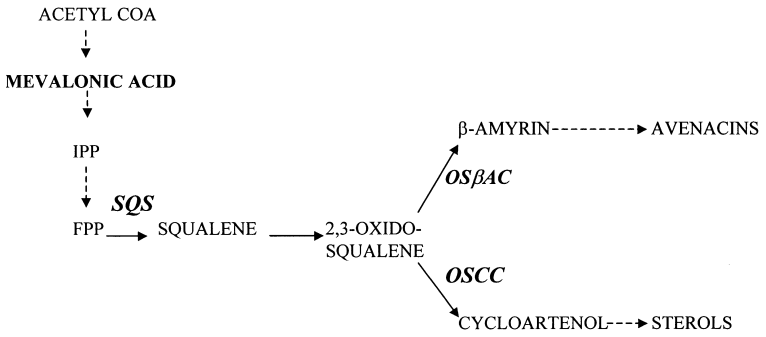
In this paper, as part of a larger study of the biochemistry and molecular biology of oat avenacins in disease resistance being undertaken at the Sainsbury Laboratory under the direction of two of the authors (AEO and MJD), we have investigated the biosynthesis of avenacins and phytosterols in sequential sections of the primary roots of *Avena sativa* cv. *Image*. These investigations were of two types: (a) radiochemical feeding experiments employing *R*-[2-^{14}C]MVA and (b) radiochemical measurement of IPP utilization and of SQS, OSCC and OSβAC activities in crude cell-free preparations.

A short report of a small part of this work has been published elsewhere (Trojanowska and Threlfall, 1999).

2. Results

2.1. Incorporation of R-[2-^{14}C]MVA into root terpenoids with time

Batches of finely chopped, whole roots (ca. 3–4 cm long) from 5-day-old *A. sativa* were incubated with *R*-[2-^{14}C]MVA for 3, 6, 9 or 12 h in order to determine the optimal incubation period ensuring both linearity of incorporation with time and good incorporation of radioactivity into the compounds of interest. After each of the time periods indicated above, a batch of roots was extracted with hot MeOH and the extracts analysed for the presence of radiolabelled compounds of interest. The incorporation of radioactivity into the MeOH extracts of the root tissue was found to increase in a more-or-less linear manner with time (Fig. 2). Furthermore, the same linearity was found for the two most heavily labelled sub-fractions (squalene, 2,3-oxidosqualene plus steryl esters, and dimethylsterols plus β-amyrin) isolated from the MeOH extracts (Fig. 3). Radio-TLC analysis backed up in some cases by radio-GC and radio-HPLC analysis established that radioactivity from *R*-[2-^{14}C]MVA was being incorporated into squalene, β-amyrin and the aglycone portion of the avenacins (poorly), cycloartenol, 24-methylene cycloartanol and des-



Scheme 1. Outline of the pathway for the biosynthesis of cyclic triterpenoids. SQS, squalene synthase; OSβAC, 2,3-oxidosqualene:β-amyrin cyclase; OSCC, 2,3-oxidosqualene:cycloartenol cyclase.

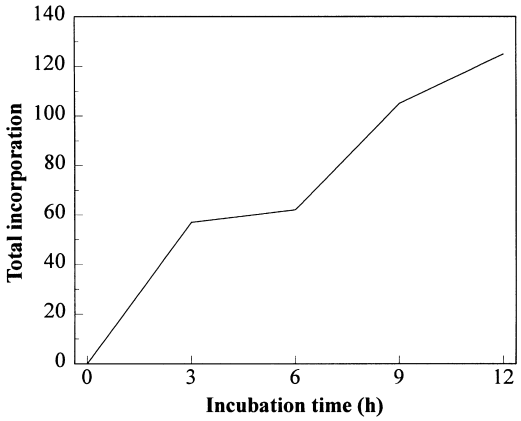
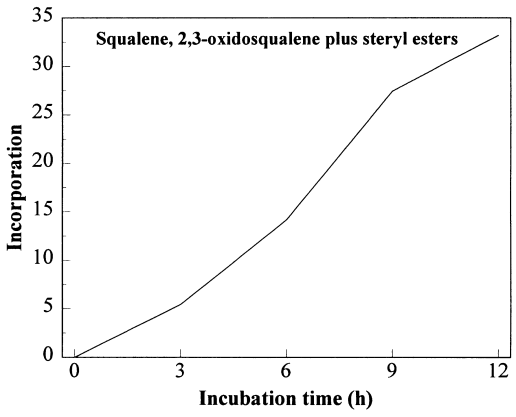


Fig. 2. Total incorporation of radioactivity (nCi g fr. wt. roots^{-1}) into methanol-soluble compounds by finely chopped oat roots incubated with *R*-[2-^{14}C]MVA for 3, 6, 9 or 12 h.



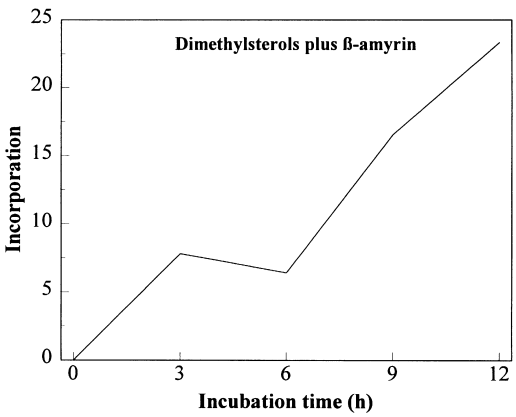


Fig. 3. Incorporation of radioactivity (nCi g fr. wt. roots^{-1}) into squalene, 2,3-oxidosqualene plus steryl esters, and dimethylsterols plus β-amyrin by finely chopped oat roots incubated with *R*-[2-^{14}C]MVA for 3, 6, 9 or 12 h.

methylsterols (poorly). A considerable amount of radioactivity was associated with the steryl ester fraction but on alkaline hydrolysis the recovered activity gave a diffuse band on TLC, no part of which was coincident with the free sterol zones. A compound with the TLC properties of the scopoletin glycoside described by Goodwin and Pollock (1954) was also highly labelled from *R*-[2-^{14}C]MVA. However, upon HPLC the radioactivity did not migrate with the scopoletin glycoside. The identities of the 'steryl esters' and 'scopoletin glycoside' were not pursued further.

In all of the feeding experiments to be reported herein, the incubations were performed for 9 h in the expectation that linearity of incorporation with time would hold for all of the compounds studied.

2.2. Incorporation of R-[2-^{14}C]MVA into root terpenoids by terminal 0.5 cm sections of washed and unwashed root tips

A washing step was included in this experiment in which one batch of finely chopped root tips was rinsed in buffer prior to incubation (washed root tips) whereas the other was not (unwashed root tips) (see Section 4), thus enabling a comparison to be made between the two sets of root tips and hence to elucidate whether or not any leeched avenacins were having an effect on the undamaged parts of the chopped root tissue. After incubating for 9 h, the roots were extracted with hot MeOH and the MeOH extracts analysed for radiolabelled compounds. The incorporation of radioactivity into methanol-soluble compounds (Table 1), after correction for the presence of unincorporated *R*-[2-^{14}C]MVA in the extracts (explanation given in Table 1), was greater in the extracts from the unwashed root tips (44.6%) than in the washed roots (31.7%). This demonstrated that the small amounts of avenacins and any other potentially phytotoxic compounds released from the freshly cut ends of the chopped tips were not having any obvious deleterious effects on the uptake and utilisation of the radiosubstrates by unwashed sections. Furthermore, the incorporation of *R*-[2-^{14}C]MVA into individual terpenoids or classes of terpenoids by chopped root tips was much higher on a fresh weight basis than by chopped roots. This was in keeping with the proposal that the tips of the roots were more metabolically active (in terms of terpenoid synthesis) than the rest of the root tissue.

2.3. Pattern of incorporation of radioactivity into terpenoids by (serial) 0.5 cm sections of roots fed with R-[2-^{14}C]MVA

Approximately 100 roots were used for the experiment and the weight of the dried, MeOH-extracted pellets ranged from 5–10 mg. Batches of root sections were each incubated with 5 μCi of *R*-[2-^{14}C]MVA for 9

Table 1
Incorporation of *R*-[2-^{14}C]MVA into triterpenoids by terminal 0.5 cm sections of washed and unwashed oat root tips. Values are expressed per flask

Incorporation from *R*-[2-^{14}C]MVA	Washed root tips		Unwashed root tips	
	31.7%		44.6%	
	μCi	% Extract	μCi	% Extract
Extract[a]	1.58	100	2.23	100
Squalene	0.42	26.6	1.10	49.5
Cycloartenol	0.10	6.6	0.12	5.4
24-Methylene cycloartanol	0.06	3.5	0.03	1.3
β-Amyrin	0.13	8.2	0.20	8.8
Methylsterols	Trace	Trace	Trace	Trace
Desmethylsterols	0.04	2.6	0.09	3.8
"Steryl esters"	0.32	20.1	0.30	13.4
Avenacins	0.04	2.5	0.03	1.3
"Scopoletin glycoside"	0.16	9.9	0.13	6.0
Total	1.27	79.9	2.00	89.6

[a] Corrected for the presence of unincorporated *R*-[2-^{14}C]MVA by subtraction of the radioactivity found to be associated with *R*-[2-^{14}C]MVA on TLC purification of the extract from the total radioactivity in the original extract.

h. They were then extracted with MeOH and analysed (see Section 4). The results of the experiment are summarised in Fig. 4. The greatest total incorporation of radioactivity was obtained with the sections from the root tip end. Thereafter, the total activity declined markedly in section 2 and continued to decline in each successive section. The radioactivity present in squalene was highest in the root tip section (section 1) and declined very markedly in section 2. Similar levels were then seen in the following root sections. β-amyrin, the first unique intermediate on the pathway leading from squalene to the aglycone portion of the avenacins, and the avenacins themselves, were both well labelled in the root tip section (section 1). The amount of label incorporated into these compounds declined markedly in sections 2 and 3, and fell to zero in sections 4–6. The incorporation of radioactivity into cycloartenol was highest in the root tip section (section 1). However, the incorporation of radioactivity into 24-methylene cycloartanol increased up to section 4 and then declined. The incorporation of radioactivity into 'steryl esters' and the unknown compound migrating with scopoletin glycoside was also highest in the root tip sections (section 1) (Fig. 5).

As part of this experiment, the amounts of avenacins were determined by HPLC using the incubated oat root sections and freshly cut root sections. In the sections from incubated root tissue (Fig. 6), the level of avenacin A-1 was highest in the root tip section (section 1) and decreased quite markedly in root sections 2 and 3, and was absent from root sections 4–6. The level of avenacin A-2 was highest in the root tip section (section 1) and decreased quite markedly in root sections 2 and was absent from root sections 3 and 4. The levels of avenacins A-1 and A-2 were comparable in the root tip section (section 1). A trace of avenacin B-2 was detected in the root tip section (section 1) only while avenacin B-1 was not detected in any of the root sections. The aqueous buffer medium in which the roots were incubated for 9 h was frozen, freeze-dried, dissolved in MeOH and analysed by HPLC. No avenacins were detected.

In freshly sectioned oat root tissue, the amounts of avenacins in the oat root sections reflected the pattern which was seen previously in the radiochemical experiment in Fig. 6. However, avenacins A-1 and A-2 were detected in all of the root sections. Avenacins A-1 and A-2 were not only similar in amounts but also showed a similar trend along the whole of the root (Fig. 7). In both cases, the levels were highest in the root tip section (section 1) and the levels decreased in root section 2. Root sections 2–4 showed comparable levels while a marked decrease was seen in root section 5 with a slight increase in root section 6. As previously seen,

Table 2
Ratio of ^{14}C-squalene to ^{14}C-2,3-oxidosqualene produced in [1-^{14}C]IPP utilization assays (Fig. 8)

Root section	Ratio
1	1.0:1.0
2	1.0:1.4
3	1.0:1.5
4	1.0:3.0
5	1.0:3.8
6	1.0:4.0

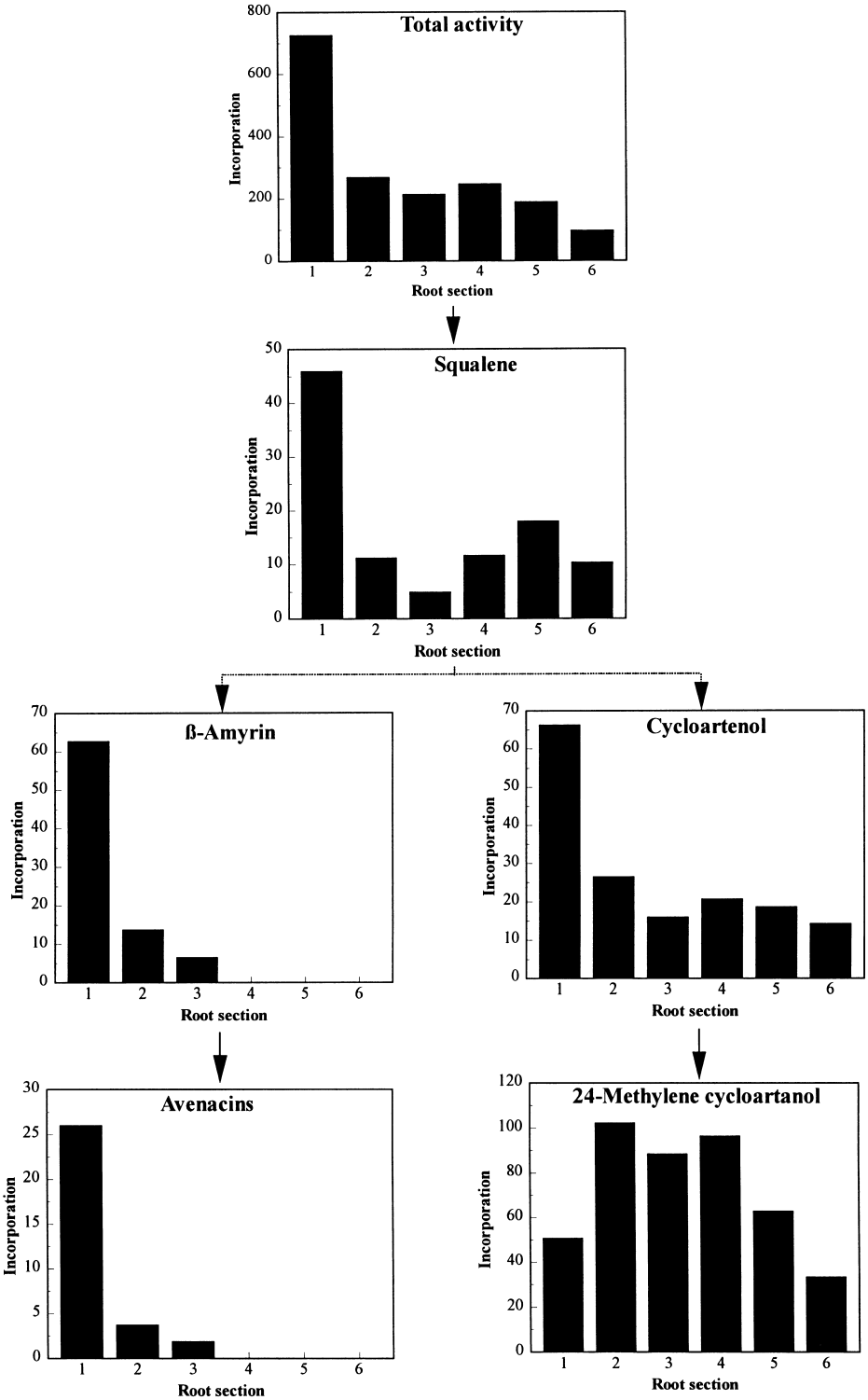


Fig. 4. Incorporation (nCi oat root section^{-1}) of radioactivity from *R*-[2-^{14}C]MVA into terpenoids by serial 0.5 cm sections of primary oat roots which had been incubated with *R*-[2-^{14}C]MVA for 9 h. The total activity values are corrected for the presence of unincorporated *R*-[2-^{14}C]MVA in the extracts (section 1 = root tip section).

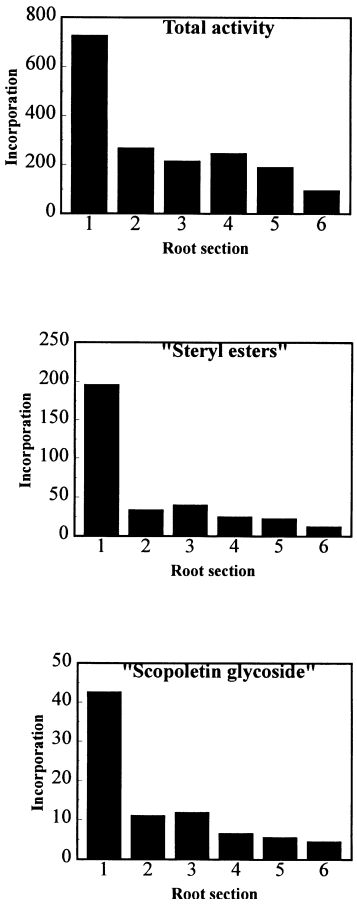


Fig. 5. Incorporation (nCi oat root section^{-1}) of radioactivity from *R*-[2-^{14}C]MVA into terpenoids, which ran, but were not chemically identical, with steryl esters and scopoletin glycoside, by serial 0.5 cm sections of primary oat roots which had been incubated with *R*-[2-^{14}C]MVA for 9 h. The total activity values are corrected for the presence of unincorporated *R*-[2-^{14}C]MVA in the extracts. (Section 1 = root tip section.)

avenacin B-2 was detected in small amounts in the root tip section (section 1) only while avenacin B-1 was not detected in any of the root sections.

2.4. SQS, OSCC and OSBAC activities in (serial) 0.5 cm sections of primary oat roots

These three enzyme activities were measured in crude cell-free preparations by standard radiochemical methods. The study also included a measurement of the relative rate of synthesis of squalene from [1-^{14}C]IPP.

Table 3
Ratio of ^{14}C-squalene to ^{14}C-2,3-oxidosqualene produced in SQS assays (see Fig. 9)

Root section	Ratio
1	1.0:1.4
2	1.0:1.7
3	1.0:2.1
4	1.0:2.5
5	1.0:3.2
6	1.0:7.0

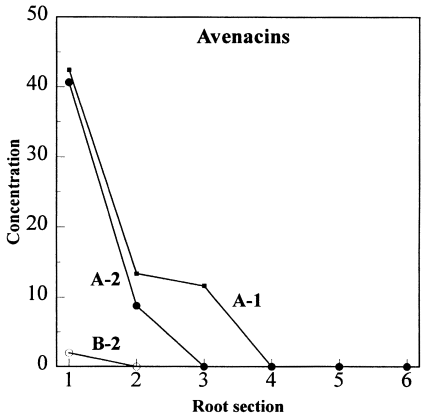


Fig. 6. Amounts (nmole oat root section^{-1}) of avenacins in serial 0.5 cm sections of primary oat roots which had been incubated with *R*-[2-^{14}C]MVA for 9 h. (Section 1 = root tip section.)

The incubations using [1-^{14}C]IPP showed that the relative rates of synthesis of ^{14}C-squalene (corrected for some conversion of squalene to 2,3-oxidosqualene) and of ^{14}C-2,3-oxidosqualene, decreased in a more-or-less linear manner along the root with the highest rates in the root tip section (section 1) and the lowest rates in root section 6 (Fig. 8 and Table 2).

The highest rate of SQS activity was found in the root tip section (section 1) and the rate decreased up the root away from the root tip (Fig. 9). A measure of the relative activity of squalene epoxidase (EC 1.14.99.7), the enzyme that converts squalene to 2,3-oxidosqualene, was possible due to the detectable amounts of ^{14}C-2,3-oxidosqualene produced in the assay mixture. The relative activity of this enzyme decreased very slowly up the root away from the root tip (Table 3).

It is noteworthy that in both the [1-^{14}C]IPP incubations and the SQS assays, the synthesis of 2,3-oxidosqualene relative to total squalene production (squalene plus 2,3-oxidosqualene) increased in a more-or-less linear manner from the root tip section (section 1) to section 6.

The formation of ^{14}C-2,3-oxidosqualene in these two assays was unusual, but not unknown (van der Heijden et al., 1989). TLC confirmed its identity.

The rate of synthesis of ^{3}H-ß-amyrin and ^{3}H-cycloartenol from (*S*)-2,3-oxido[3-^{3}H$_1$]squalene by the various root sections are shown in Fig. 10. OSCC activity was present in all root sections with the greatest activity found in root section 3. The highest level of OSßAC activity was found in the root tip section (sec-

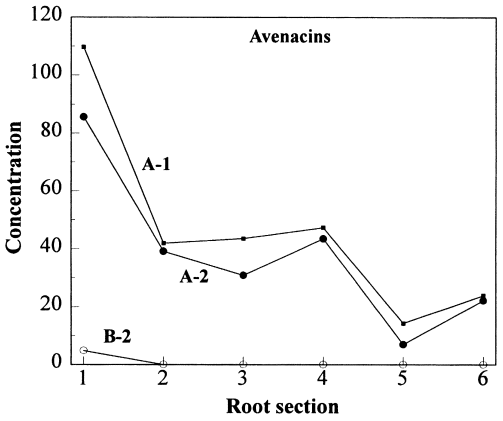


Fig. 7. Amounts (nmol oat root section^{-1}) of avenacins in serial 0.5 cm sections of primary oat roots. Roots were sectioned and extracted immediately with methanol and the avenacin band purified by TLC. (Section 1 = root tip section.)

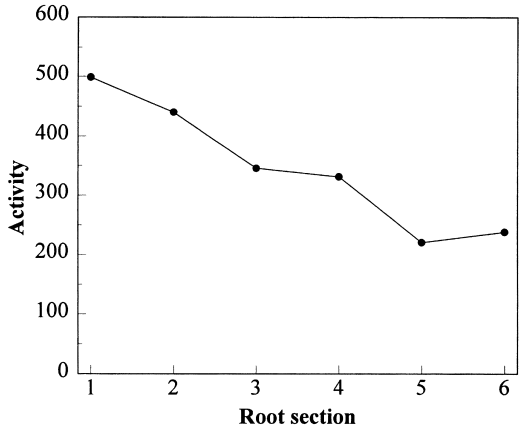


Fig. 9. SQS activity (pmol FPP utilised mg protein^{-1} h^{-1}) in cell-free preparations of serial 0.5 cm sections from primary oat roots incubated with [1-$^{3}H_2$]FPP.

tion 1) with the activity decreasing up the root away from the root tip. On repeating these assays with another batch of roots, essentially similar results were obtained.

3. Discussion

The incorporation of radioactivity from *R*-[2-^{14}C]MVA into the total methanol extracts by finely chopped whole roots, was found to increase in a more-or-less linear manner with time. This was true for periods of up to 9 h but after this time the rate sometimes slowed down, and this was reflected when the sub-fractions were analysed.

The incorporation of radioactivity into methanol extracts and their constituent terpenoids was much higher in finely chopped root tip tissue than in finely chopped whole roots, and suggested that the tips of the roots were metabolically more active (in terms of terpenoid synthesis) than the rest of the root tissue. This was in accordance with data from Crombie and Crombie (1986), who found that 3 to 4 day old roots contained the greatest amounts of all four of the avenacins and that the relative amounts decreased as the roots aged with the greatest amounts of avenacins in the root tips (2–3 mm).

It appeared that washing the root tips prior to incubation with *R*-[2-^{14}C]MVA reduced the overall incorporation of radioactivity into terpenoids, and also altered the pattern of incorporation to some extent.

Chromatographic analysis established that in the finely chopped root tip tissue, radioactivity from *R*-[2-^{14}C]MVA was being incorporated into squalene, cycloartenol, 24-methylene cycloartanol, β-amyrin,

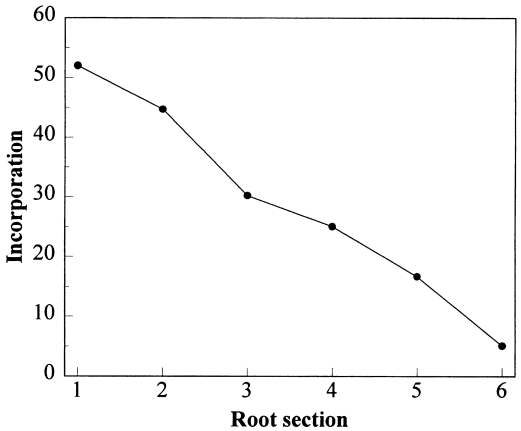


Fig. 8. Incorporation of [1-^{14}C]IPP (pmol mg protein^{-1} h^{-1}) into squalene plus 2,3-oxidosqualene by cell-free preparations of serial 0.5 cm sections of primary oat roots.

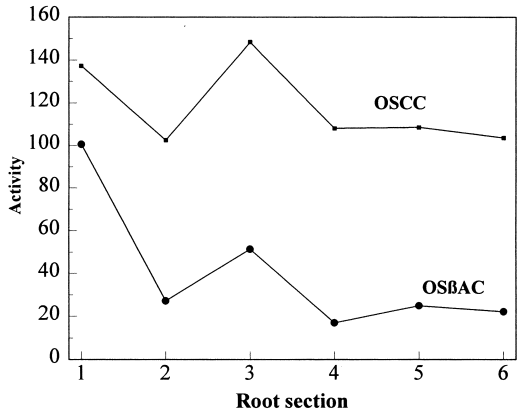


Fig. 10. OSCC and OSβAC activities (pmol mg protein^{-1} h^{-1} cell-free extract^{-1}) in cell-free preparations of serial 0.5 cm sections of primary oat roots.

avenacins (poorly), desmethylsterols (poorly) and two compounds, 'steryl esters' and 'scopoletin glycoside,' with the same TLC properties but not chemical properties, as steryl esters and scopoletin glycoside. These latter two compounds contained a considerable proportion of the radioactivity incorporated into the MeOH extracts.

The poor incorporation of radioactivity into avenacins and desmethylsterols was disappointing from the point of view of carrying out comparative studies to determine the location(s) of avenacin and desmethylsterol biosynthesis in oat roots. However, the very good incorporation of radioactivity into the first cyclised intermediate(s) on the avenacin (β-amyrin) and phytosterol (cycloartenol and 24-methylene cycloartanol) pathways meant that such studies were still possible.

The total incorporation of radioactivity into MeOH extracts and the cumulative incorporation of radioactivity from *R*-[2-^{14}C]MVA into squalene, cycloartenol, 24-methylene cycloartanol, β-amyrin and avenacins by the different root sections (Fig. 4) showed that (a) the root tip is the site of greatest terpenoid biosynthetic activity per se and (b) the flux of MVA through the intermediates of the cyclic triterpenoid pathways was highest in the oat root tip section (section 1) and declined slowly along the root before falling markedly in the two most distal root sections.

A similar trend was shown for the overall relative rates of ^{14}C-squalene and ^{14}C-2,3-oxidosqualene formation from [1-^{14}C]IPP by cell-free extracts with the highest rates being observed in the root tip section (section 1) and the lowest rates in root section 6 (Fig. 8).

SQS activity was highest in the root tip section (section 1) and gradually fell in subsequent root sections (Fig. 9). However, the relative rates of squalene epoxidase, as measured in the SQS assays, declined very slowly along the root.

These last two sets of results suggested that FPP synthase, the enzyme catalysing the two steps between IPP and FPP, has a role to play in determining the overall rate of terpenoid biosynthesis.

The results of the *R*-[2-^{14}C]MVA feeding experiment provided a clear cut demonstration that, as postulated by previous workers (Goodwin and Pollock, 1954), avenacin biosynthesis was more-or-less restricted to the root tips of primary oat roots. OSβAC, the enzyme which produces the β-amyrin needed for avenacin biosynthesis, was most active in the root tip section (section 1) and the activity decreased quite sharply in subsequent root sections. However, the OSβAC activity did not fall to zero in root sections 4–6 in contrast to the *R*-[2-^{14}C]MVA feeding experiment data. This observation indicated that either some, but not all, of the enzyme activities of the avenacin pathway had declined completely to zero in the more distal sections of the root or that the overall pathway was inoperative in the distal root sections due to the accumulative effect of the reduced activities of each of the enzymes on the avenacin pathway.

The situation with regard to sterol biosynthesis along the root was less clear-cut, due mainly to the very poor incorporation of radioactivity from *R*-[2-^{14}C]MVA into phytosterols (desmethylsterols) by all sections of the root. Nevertheless, the data from the *R*-[2-^{14}C]MVA feeding experiment was consistent with cycloartenol and 24-methylene cycloartanol synthesis and hence phytosterol synthesis occuring in all sections of the root with some tailing off in the rate of synthesis in the more distal root sections 5 and 6) (Fig. 4). This was reflected by the data from the OSCC assays, although there was no decline in OSCC activity in root sections 5 and 6. The results of the *R*-[2-^{14}C]MVA feeding experiments were in keeping with the expected cellular demand for phytosterols for membrane formation in the growing root tip and in the adjacent zone of cellular elongation.

The accumulation of radiolabelled 24-methylene cycloartanol observed in the *R*-[2-^{14}C]MVA feeding experiment suggested that the enzyme catalysing the conversion of 24-methylene cycloartanol to cycloeucalenol may have a regulatory role in phytosterol biosynthesis in oat roots.

Large amounts of radioactivity were incorporated from *R*-[2-^{14}C]MVA into 'steryl esters' and 'scopoletin glycoside.' However, as these compounds were clearly chemically unrelated to the sterols and avenacins, their exclusion from the above discussion was warranted. It is interesting, however, that they are very heavily labelled in the root tip section (section 1).

The failure to detect any radiolabelled steryl esters was somewhat unexpected given the regulatory role of dimethylsteryl esters in sterol biosynthesis in some plant tissues (Kemp et al., 1968; Grunwald, 1975; Goad, 1983). In this connection, it is interesting to note that ^{14}C-labelled cycloartenyl esters are present in root tissue obtained from young oat seedlings grown from seeds which have been imbibed with *R*-[2-^{14}C]MVA (Trojanowska and Threlfall, unpublished work).

The HPLC analytical data showed that, as expected from the studies of previous workers (Crombie and Crombie, 1986), the levels of the major avenacins (A-1 and A-2) in the freshly cut root sections were highest in the root tip section (section 1) and after declining by some 50–60% in root section 2, plateaued out in root sections 3 and 4 and then declined further in root sections 5 and 6 (Fig. 7). In the case of the sections which had been finely chopped and then incubated with *R*-[2-^{14}C]MVA for 9 h, a similar trend was seen (Fig. 6). However, the amounts present in root sections

1 and 2 were some 50–60% lower than those recorded for the fresh sections and both avenacins A-1 and A-2 were undetectable in root sections 4 (avenacin A-1) and 3 (avenacin A-2).

Crombie and Crombie (1986) had found in the terminal, 2–3 mm of 3-day old roots of *A. sativa* (var. Peniarth) a total of 12.76 mg g^{-1} dry wt. avenacins. In the experiments described in this paper, the 0.5 cm terminal root tips of freshly cut root sections of *A. sativa* (cv. *Image*) had a total of 40.98 mg g^{-1} dry wt. avenacins, while the finely chopped root tissue incubated with *R*-[2-^{14}C]MVA had a total of 9.87 mg g^{-1} dry wt. avenacins.

The lower levels of avenacins in the incubated tissue cannot be attributed to leaching, since no avenacins were detected in the incubation medium used in these experiments. The effect must, therefore, be attributed to catabolism of the avenacins by the chopped tissue to give products which, if still methanol soluble, were not detected in the TLC and HPLC assays used in this study, i.e. they did not give rise to fluorescent products or new TLC bands.

Although an undesirable effect, the loss of avenacins as a result of the incubation conditions, was not seen as a major cause for concern in these studies, since it was felt that (a) the labelling of the early cyclic intermediates on the avenacin and phytosterol pathways would be unaffected by these catabolic events and (b) as the catabolism was likely to be totally restricted to the in planta sequestered pools of avenacins, any newly synthesised avenacins would not be subject to catabolism.

4. Experimental

4.1. Chemicals and general methods

All solvents were of AnalaR grade. Et_2O was dried over Na/Pb alloy and redistilled (b.p. 34°C) over reduced iron. Lanosterol, squalene, dihydrocholesterol, stigmasterol, β-sitosterol, benzoic acid methyl ester, NADPH and glucose-6-phosphate dehydrogenase, Type XII from *Torula* yeast, were purchased from Sigma, Poole, Dorset, UK. Cycloartenol, α-amyrin and β-amyrin were purchased from Apin Chemicals, Abingdon, UK. *N*-methylanthranilic acid methyl ester was purchased from Lancaster Synthesis, Morecambe, UK. Mixtures of avenacins were kindly donated by the Sainsbury Laboratory.

R-[2-^{14}C]MVA lactone (53 μCi μmol^{-1}) and [1-^{14}C]IPP (55 μCi μmol^{-1}) were obtained from Amersham International, Amersham, Bucks, UK. *R*-[2-^{14}C]MVA lactone was converted to the sodium salt by removal of the toluene under a stream of nitrogen gas, and then adding 1 ml distilled water and a small amount of sodium hydrogen carbonate. [1-$^{3}H_2$]FPP (32 μCi μmol^{-1}) and (*S*)-2,3-oxido[3-$^{3}H_1$]squalene (30 μCi μmol^{-1}) in buffered aqueous solution were available from previous studies (Threlfall and Whitehead, 1988; Fulton, 1993). Protein estimation was performed using a modified method of Lowry (Peterson, 1977).

TLC was carried out on pre-coated silica gel G 20 × 20 cm aluminium backed plates (Merck) with a 2.5 cm concentrating zone at the base of each plate. For silver ion (Ag^+) chromatography, the plates were dipped in a solution of 10% (w/v) silver nitrate and then dried at 100°C for 1 h. Radioactivity on a developed plate was detected (prior to treating the plates with any reagent) by means of a radio-TLC scanner (Canberra Packard Bioscan Autochanger 4000, Bioscan System 200 Imaging Scanner). Compounds on a developed plate were located by examination of the plate under $UV_{254\ nm}$ light before and after spraying with 0.025% ANSA (Gitler, 1972) (non-destructive), by spraying with 1% anisaldehyde followed by heating (Waldi, 1965) (destructive), or by staining with iodine vapour. Compounds were recovered from the gel with Et_2O or MeOH (polar compounds). Radioactivity in solutions was measured by liquid scintillation spectrometry. Radioactive sterol and amyrin samples were analysed (after acetylation) by radio-Gas Chromatography (GC) (Brindle et al., 1988).

HPLC analysis of avenacins was performed on a Hewlett Packard Series 1050 HPLC with a Hewlett Packard 1040M Series II HPLC Detection System. A 25 cm C18 reverse phase spherisorb ODS3 column was used with aq. 75% MeOH as the solvent system at a flow rate of 0.5 ml min^{-1}. The runs were monitored at 6 wavelengths, corresponding to the main avenacin UV absorption bands of 223, 225, 255, 274, 281 and 356 nm. 356 nm also corresponded to the UV absorption of scopoletin. For radio-HPLC analysis of avenacins, the same conditions were used as above with samples being collected every 15 s for liquid scintillation analysis. For the calculation of amounts of avenacins, known amounts of benzoic acid methyl esters and *N*-methylanthranilic acid methyl esters dissolved in MeOH were injected onto the HPLC. The peak areas of these standards were then used to calculate the amounts of avenacins in the samples at 225 nm.

4.2. Growth of oat seeds and preparation of root material for feeding and cell-free experiments

Oat seeds (*A. sativa* var. *Image*) were surface-sterilised by being soaked in 10% sodium hypochlorite for 10 min, then rinsed in distilled water until free of chlorine. The seeds were then soaked overnight in distilled water. The soaked seeds were spread evenly (1–2 seeds thick) onto plastic meshes incorporated into a stainless steel frame (38 × 24 cm) to keep the mesh

taut. The meshes were then placed on top of trays (7 cm deep) filled with tap water. The seeds were covered with a layer of filter paper and dampened. The whole tray was covered with aluminium foil and incubated at 22°C and 55% humidity. At harvest after five days, the roots were 3–4 cm in length and were excised as near to the underside of the mesh as possible. The roots were lined up from the tips and either the terminal 0.5 cm from the root tip end excised with a razor blade or a jig was used to cut 6 × 0.5 cm sequential sections of oat roots.

4.3. Feeding experiments using R-[2-^{14}C]MVA

4.3.1. Incubation conditions

Roots (100; 3–4 cm in length), root tips (100; 0.5 cm in length) or sequential sections (6 × 0.5 cm) starting from the root tip of whole roots, were excised, chopped finely and placed in 10 ml conical flasks containing 1 ml 0.1 M KH_2PO_4 buffer at pH 7.0 and 5 μCi (95 nmol) *R*-[2-^{14}C]MVA. In one experiment, a set of chopped root tips was washed in buffer prior to the incubation step, while the other set had the rinsing step omitted. The flasks were shaken in a water bath at 23°C for 3, 6, 9 or 12 h (time course experiment) or 9 h (all other experiments). The contents of each flask were then filtered through a sintered glass funnel and washed with 5 ml distilled H_2O. The retained tissue was homogenised (with an Ultra Turrax homogenizer) with 2 × 5 ml hot MeOH and centrifuged at 4500 g for 10 min. The filtrates were pooled, taken to dryness in vacuo and the residue dissolved in MeOH (200 μl). Prior to analysis (see below), an aliquot (10 μl) was assayed for radioactivity.

4.3.2. Analysis of R-[2-^{14}C]MVA radiolabelled compounds

(i) *Initial separation by TLC*. Prior to analysis by TLC, some of the samples had mass markers added to them as carriers to help prevent degradation and also to aid in visualising the bands on the TLC plates. Typically, 100 μg each of cycloartenol, β-amyrin, α-amyrin, stigmasterol, squalene, sitosterol and cholesteryl oleate were added. The extract (100 μl) was then separated by TLC with cyclohexane–EtOAc (1:1) as the developing solvent. The developed plate was scanned for radioactivity, sprayed with ANSA and examined under UV light. The bands containing squalene and steryl esters (R_f 0.69), dimethylsterols and β-amyrin (0.56), monomethylsterols (0.54) and desmethylsterols (0.52) were scraped off the plate individually, eluted with Et_2O and taken up in cyclohexane (200 μl). The compounds on the origin (avenacins and *R*-[2-^{14}C]MVA) were eluted from the gel with MeOH and taken up in MeOH (200 μl). 1 μl of each of the samples was taken and counted. (ii) *Analysis of squalene and steryl esters* (R_f 0.69). The sample containing squalene and steryl esters was re-chromatographed (TLC) with petrol (40–60°C) as the developing solvent. The squalene (R_f 0.14) and steryl ester (0.00) bands were scraped off individually and eluted from the gel with Et_2O. The samples were dissolved in cyclohexane (200 μl). 1 μl of each was counted. The steryl ester sample was transferred to a screw topped glass tube, 5 ml 8% KOH in 80% ethanol added and the tube placed in a heating block at 70°C for 2 h. The tube was then cooled and the free sterols extracted with 3 × 5 ml hexane. The hexane layers were pooled, dried, transferred to vials, acetylated and analysed following the procedure for free sterols. (iii) *Analysis of the desmethylsterol- and dimethylsterol/β-amyrin-containing fractions*. After removal of the cyclohexane by evaporation, the fractions were each acetylated (200 μl Ac_2O and 1 drop of pyridine) overnight at room temperature. 3 ml ethanol were then added to destroy excess Ac_2O and the solvents removed under a stream of nitrogen. The acetylated samples were then dissolved in cyclohexane (200 μl) and separated by Ag^+-TLC with petrol–toluene (1:1) as the developing solvent. Acetylated markers were run at the same time. The developed plate was then scanned for radioactivity after which the acetylated markers were sprayed with anisaldehyde. Individual acetates were scraped off the plate, eluted with Et_2O and the samples taken up in cyclohexane (200 μl). Representative radioactive samples were further analysed by radio-GC. The R_fs and RR_ts (dihydrocholesteryl acetate) were as follows: cycloartenyl acetate, 0.31, 1.80; α-amyrin acetate, 0.44, 1.81; β-amyrin acetate, 0.44, 1.61; 24-methylene cycloartanol acetate, 0.21, 1.96. (iv) *Analysis of avenacins, steryl glycosides and acylated steryl glycosides* (R_f 0.00). 100 μl of the mixture of compounds recovered from the origin (of the preliminary TLC) was separated on TLC using $CHCl_3$–MeOH–H_2O (70:30:5.5) as the developing solvent. The area containing the avenacins (R_f 0.21–0.42), detected by their blue fluorescence under UV light, was scraped off the gel, eluted with MeOH, dissolved in MeOH (200 μl) and 1 μl counted. The extracts were then analysed by HPLC and radio-HPLC. The R_t values (min) were as follows: avenacins — A-1, 16.1; A-2, 12.0; B-1, 18.8; B-2, 13.6. (v) *Acid hydrolysis of avenacins to their corresponding pseudo aglycones*. Avenacins and a standard mixture of avenacins were acid hydrolysed to their corresponding pseudo aglycones (1 ml 1 M HCl, at 105°C for 2.5 h). The samples were cooled and extracted three times with Et_2O. The Et_2O layers were pooled, dried by passing through a bed of anhydrous sodium sulphate and the solvent removed under a stream of nitrogen. The extracts were taken up in MeOH (50 μl), analysed by TLC using $CHCl_3$–MeOH–H_2O (70:30:5.5) as the developing solvent, scanned for radioactivity and then

the plate examined under UV light (avenacin aglycones[2], R_f 0.86, 0.80, 0.74 and 0.70). (vi) *Calculation of total radioactive content of individual compounds in the original extracts*. This was carried out by back calculation starting from the amount of radioactivity present in the purified product.

4.4. Biosynthetic capacity (from IPP) and SQS activity of 0.5 cm sequential root sections

6 × 0.5 cm sections of oat roots were separately ground in cold mortars for 30 s with 0.2 g acid-washed sand g^{-1}, 0.1 g insoluble PVP g^{-1} and 1 ml extraction buffer g^{-1} fr. wt. of tissue. The extraction buffer comprised 0.1 M KH_2PO_4, 0.5 M sucrose, 10 mM Na_2EDTA, 10 mM 2-mercaptoethanol and 10 mM $MgCl_2$ at pH 7.5. The extracts were then squeezed through two layers of Miracloth into chilled Eppendorf tubes and centrifuged at 4500 g for 15 min. 50 μl of the filtrate from each oat root section was placed in a tube with 50 μl assay buffer (50 mM KH_2PO_4, 0.5 M sucrose, 1 mM Na_2EDTA, 1 mM 2-mercaptoethanol and 10 mM $MgCl_2$ g^{-1} cells at pH 7.5) and 2.5 μl NADPH-generating system (this was a freshly prepared solution of assay buffer containing 129 mM glucose-6-phosphate (sodium salt), 10.4 mM NADPH (sodium salt) and 1.5 units of glucose-6-phosphate dehydrogenase ml^{-1}). The tubes were allowed to equilibrate at 30°C for 5 min. 0.05 μCi [1-^{14}C]IPP (1 nmol) or 0.04 μCi [1-$^{3}H_2$]FPP (1 nmol) were added to each tube and the tubes incubated at 30°C for 30 min. The reactions were then quenched by addition of 5 ml $CHCl_3$–MeOH (1:2) and the $CHCl_3$-soluble lipids extracted by the procedure reported by Threlfall and Whitehead (1988). The extracts were dissolved in acetone (100 μl) and 10 μl assayed for radioactivity. Aliquots (40 μl) of the samples, along with standards of known concentration were subjected to TLC and double developed with cyclohexane–EtOAc (1:1) and petrol (40–60°C). The first solvent was allowed to run 10 cm up the plate and the plate was radioscanned prior to development (to the top of the plate) in petrol (40–60°C). The plate was re-radioscanned, sprayed with ANSA and the bands examined under UV light (R_f farnesol, 0.23; 2,3-oxidosqualene, 0.34; squalene, 0.52). The total incorporation of radioactivity into squalene and 2,3-oxidosqualene obtained by back calculation was then used to calculate the total squalene production ([1-^{14}C]IPP experiment) or SQS activity. In the latter case, the incorporation of radioactivity from 2 mol of [1-$^{3}H_2$]FPP into squalene was corrected for the loss of one ^{3}H atom during the condensation reaction.

4.5. OSCC and OSBAC activity along roots

Cyclohexane (50 μl) containing 2 nmol (*S*)-2,3-oxido[3-$^{3}H_1$]squalene was transferred to the bottoms of silanized vials. The solvent was removed under a gentle stream of nitrogen gas and the (*S*)-2,3-oxido[3-$^{3}H_1$]squalene resuspended with gentle agitation in 2 μl of cellosolve (ethylene glycol monomethyl ether). The mixture was made up to 100 μl by the addition of 98 μl of cell-free extracts (prepared, as above) and incubated for 1 h at 30°C. The reaction was terminated by the addition of 5 ml $CHCl_3$–MeOH (1:2) and the radiolabelled compounds extracted from the assay mixture, as above. Samples were dissolved in acetone (100 μl) prior to radio-TLC analysis. Aliquots (90 μl) of the extracts, along with standards of known concentration, were subjected to TLC with $CHCl_3$ as the developing solvent. The plate was then radioscanned, sprayed with ANSA and the bands visualised under UV light. Located compounds were recovered from the gel with Et_2O (unincorporated 2,3-oxidosqualene, R_f 0.27; cycloartenol and β-amyrin, 0.05). The cycloartenol and β-amyrin were dissolved in cyclohexane (100 μl), 10 μl counted and the remainder of the sample taken to dryness and acetylated overnight (for experimental details, see above). The acetylated sample was then subjected to Ag^{+}-TLC using petrol (40–60°C)–toluene (1:1) as the developing solvent, scanned for radioactivity and the plate either stained with I_2 vapour to locate bands of interest and allowed to fade, or the standards were sprayed with anisaldehyde while the rest of the plate was covered up. The areas of gel containing β-amyrin acetate (R_f 0.41) and cycloartenyl acetate (0.28) were then scraped off separately into scintillation vials and assayed for radioactivity. The total incorporation of radioactivity into each of these compounds (obtained by back calculation) was then used to calculate the OSβAC and OSCC activity, respectively.

[2] On acid hydrolysis, avenacins give rise to derivatives of the expected aglycone (Begley et al., 1986).

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