# Differential Inhibition by Mevinolin of Prenyllipid Accumulation in Radish Seedlings

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We have studied in intact radish seedlings the effects of mevinolin (at concentrations of 0.25 to 5 μM), a specific inhibitor of HMG-CoA reductase, and, therefore, of mevalonate biosynthesis, on the production of various isopentenoids and prenyllipids. Whereas the content of free desmethyl sterols was decreased steadily, only depending on the concentration of inhibitor present in the parts of seedlings investigated separately (*e.g.* roots, hypocotyls, and cotyledons), the effect on ubiquinone accumulation was different. Irrespective of the part of seedlings being analyzed, the maximal inhibition reached was 50%. Plastidic pigment accumulation, however, as well as that of chloroplast quinones (plastoquinone and phylloquinone), appeared even to be enhanced at low inhibitor concentrations and was not significantly lowered by application of 5 μM mevinolin. α-Tocopherol showed a similar profile in the dose response to compounds known to be exclusively synthesized in the plastid.

The results indicate a differential accessibility of the mevalonate synthesizing enzymes presumably present in the cytoplasm, mitochondria and plastids in respect to the inhibitory action of mevinolin. If prenyllipid formation in the different cell compartments solely depended on cytoplasmic mevalonate biosynthesis, all prenyllipids should be affected to the same extent as the sterols, which are exclusively synthesized by cytoplasmic enzymes.

## Introduction

Mevalonic acid is needed for the biosynthesis of the different plant prenyllipids such as sterols (cytoplasmic biomembranes), ubiquinones (mitochondria) as well as chlorophylls, carotenoids and plastoquinone (chloroplasts). The biosynthesis of mevalonate, catalyzed by the enzyme HMG-CoA reductase, appears as a major control point of the isopentenoid pathway not only in animals [1–4] but also in plants [5–14] and fungi [15–18]. Our studies are concerned with elucidating the regulation of mevalonate formation in plants and studying the flux of mevalonate into the various major and minor classes of prenyllipids and other isopentenoid compounds.

In recent years it has been shown that the fungal metabolite mevinolin [19] specifically inhibits a wide variety of eukaryotic and prokaryotic HMG-CoA reductases [19–22]. We have introduced mevinolin as a molecular probe to study how an inhibited mevalonate formation effects the development of an intact plant.

Mevinolin proved to be a specific inhibitor of root growth in etiolated and light-grown seedlings [20, 23–25]. A significant inhibition of root elongation growth is already obtained at low concentrations between $2.5 \times 10^{-8}$ and $2.5 \times 10^{-7}$ M. In the root and hypocotyl tissue of radish seedlings mevinolin treatment primarily affects the accumulation of sterols [24, 25]. We have expanded these studies and now describe the effect of mevinolin on the biosynthesis and accumulation of other prenyllipids such as the mitochondrial ubiquinones, the chlorophylls, carotenoids and plastidic prenylquinones. In addition, we report on the possibilities of using this drug in studies of the compartmentation of isopentenoid biosynthesis in plants.

*Abbreviations:* HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MVA, mevalonic acid; IPP, isopentenyl pyrophosphate; Q-9, Q-10, ubiquinone; PQ-9, plastoquinone

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## Materials and Methods

Radish seeds (*Raphanus sativus* L. cv. Saxa Knacker) were submersed in an aerated water bath for one hour and then placed onto a sprouting tray. 800 ml of water, or water supplied with increasing

amounts of mevinolin (sodium salt) was used to permit germination and subsequent development. The trays were placed under Fluora white light (2.5 W/m², 14 h light/10 h dark cycles) at 25 °C and 65% relative humidity for a period of six days. Water was added to overcome evaporation and to keep the inhibitor concentration constant. Plants were harvested and cut with a razor blade to yield cotyledons, hypocotyls and roots. The plant material was macerated in the presence of 100% acetone and the lipids were transferred into petrol ether (b.p. 50–70 °C). For the determination of chlorophyll a and total carotenoid content, spectra of the diluted petrol ether extracts were recorded and calculations were made using the new equations introduced by Lichtenthaler and Wellburn, 1983 [26]. For further purification and quantification of prenyllipids aliquots of the total lipid extracts were subjected to TLC on silica gel plates [*cf.* 27]. Separation of plastoquinone and phylloquinone from other lipids was achieved in a solvent system $CH_2Cl_2 : CHCl_3$ 3 : 2 (v : v), $R_f$ values 0.8 and 0.75, respectively. Parts of the plates containing reference substances were sprayed with 0.5% rhodamine B in abs. ethanol and the compounds were visualized under $UV_{254}$ as blue-pink spots on an orange background. Bands containing the quinones were scraped and the silica gel was eluted with ether. For quantification of plastoquinone and phylloquinone the compounds were redissolved in acetonitrile and separated either by adsorption HPLC (LiChrosorb S160, 5 µm, 0.5% dioxan in *n*-hexane, p = 70 b) or by reversed phase – HPLC (Nucleosil $C_8$, 5 µm, 2% $H_2O$ in MeOH, p = 140 b), detection both at $\lambda = 250$ nm [28, 29]. α-Tocopherol and ubiquinone were separated by silica gel TLC in the solvent system $CH_2Cl_2 : CHCl_3$ 1 : 1 (v/v) with $R_f$ values of approximately 0.5 and 0.65 respectively, and standards were visualized with rhodamine B. α-Tocopherol was determined spectrophotometrically, as described in detail [27]. The total ubiquinone eluted from the silica gel was dissolved in a defined volume of *n*-hexane and further purified by the aid of adsorption HPLC (LiChrosorb S160, 5 µm, 15% dioxan in *n*-hexane, p = 110 b, detection at $\lambda = 275$ nm) [28, 29]. Separation of ubiquinone homologues was achieved by reversed phase HPLC (Nucleosil $C_8$, 5 µm, 2% $H_2O$ in MeOH), which yielded a linear dependency of the log $R_t$ as a function of the number of isoprene units in the side chain. The different prenylquinones were identified by UV-VIS spectrophotometry before and after reduction with $KBH_4$ [27]. For separation by means of RP-HPLC, the compounds were injected in acetonitrile solution. Reference chromatograms of pure standards were used for quantification of biological samples. Free 4-desmethylsterols were quantified after TLC on silica gel plates (petrol ether b.p. 50–70 °C 84 ml + 15 ml acetone, $R_f = 0.25$). Plates were sprayed with saturated $SbCl_3$ in water-free $CHCl_3$ and after a short heating period at 100 °C, the pink spots indicating desmethylsterols (with pure stigmasterol as a standard) were scanned with a densitometer at 550 nm with separate standard curves being made for each plate. Anthocyanins were extracted and determined as described [30].

## Results

Under the conditions in which mevinolin is applied to the seedlings it has to be taken up by the roots exposed to the watery solution of the inhibitor. If there is any inhibitory effect on isopentenoid biosynthesis, the roots can be expected to react on the inhibitor more clearly than the upper parts of the seedlings comprised of hypocotyls and cotyledons. Exactly this is demonstrated by comparison of the sterol (Fig. 1) as well as of ubiquinone content in different parts of the seedlings upon treatment with mevinolin. At a maximum concentration of 5 µM, mevinolin reduces the content of free 4-desmethylsterols in roots to about 20 per cent of the control. Already at 0.625 µM, sterol content is reduced by about 50 per cent, thus indicating a very fast response of sterol accumulation to inhibition of MVA biosynthesis. These effects are less dramatic in hypocotyls, and especially in cotyledons. This result implies that there might exist a gradient in the concentration of mevinolin present in upper parts of the seedlings. Whereas the accumulation of free sterols reflects such a gradient, total ubiquinone content remained stable at the maximal inhibitor concentration, regardless of what part of the seedling was analyzed (Fig. 1). This effect seems to be specific to plant cells, since similar observations were made in the case of cell suspension cultures of *Silybum marianum*, where sterol accumulation could even be suppressed to a level of about 5% of the controls, whereas ubiquinone content was reduced by a maximum value of only 50% [43]. In radish seedlings

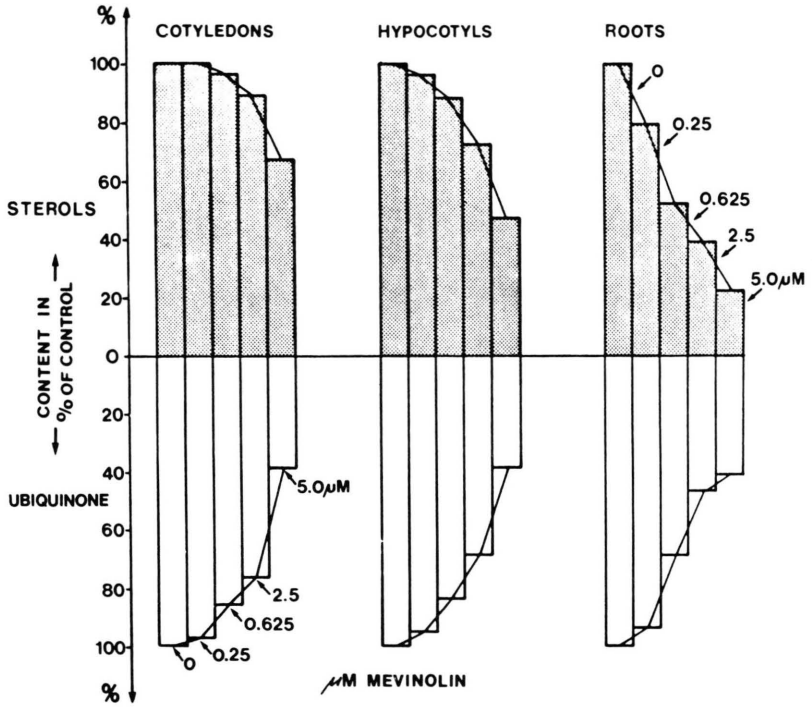


Fig. 1. Inhibition by mevinolin of free desmethylsterol and ubiquinone accumulation in 6 day-old, light-grown radish seedlings. Mean of three independent determinations.

Table I. Development of fresh and dry weight and of sterol content in different parts of radish seedlings grown in light for 6 days in the presence of mevinolin[a].

| Plant part | g fresh weight | g dry weight | free sterols μg/100 parts | % of controls |
|---|---|---|---|---|
| Cotyledons[b] | | | | |
| 0 μM mevinolin | 4.7 | 0.62 | 1 640 | 100 |
| 0.25 μM mevinolin | 4.9 | 0.66 | 1 647 | 100 |
| 0.625 μM mevinolin | 4.6 | 0.63 | 1 569 | 96 |
| 2.5 μM mevinolin | 3.9 | 0.63 | 1 453 | 89 |
| 5.0 μM mevinolin | 3.4 | 0.63 | 1 091 | 67 |
| Hypocotyls[c] | | | | |
| 0 μM mevinolin | 3.9 | 0.23 | 593 | 100 |
| 0.25 μM mevinolin | 4.2 | 0.25 | 571 | 96 |
| 0.625 μM mevinolin | 4.1 | 0.26 | 521 | 88 |
| 2.5 μM mevinolin | 3.3 | 0.25 | 426 | 72 |
| 5.0 μM mevinolin | 2.2 | 0.18 | 276 | 47 |
| Roots[c] | | | | |
| 0 μM mevinolin | 2.8 | 0.15 | 522 | 100 |
| 0.25 μM mevinolin | 2.5 | 0.14 | 416 | 79 |
| 0.625 μM mevinolin | 2.2 | 0.12 | 274 | 52 |
| 2.5 μM mevinolin | 1.8 | 0.11 | 200 | 39 |
| 5.0 μM mevinolin | 1.3 | 0.09 | 112 | 22 |

[a] Mean values of three independent determinations.
[b] 100 Cotyledon pairs analyzed per single experiment.
[c] 100 Hypocotyls or roots per analysis.

Table II. Effect of mevinolin treatment on the accumulation of total ubiquinone (Q-9 + Q-10) in radish seedlings grown in light for 6 days in the presence of mevinolin [a]

| Plant part | Total ubiquinone µg/100 parts | % of controls | % Q-9 of total Q-9 + Q-10 | Ratio Q/sterols |
|---|---|---|---|---|
| Cotyledons [b] | | | | |
| 0 µM mevinolin | 61.10 | 100 | 8.6 | 0.037 |
| 0.25 µM mevinolin | 59.2 | 97 | 8.8 | 0.036 |
| 0.625 µM mevinolin | 52.7 | 86 | 10.1 | 0.034 |
| 2.5 µM mevinolin | 46.8 | 77 | 11.2 | 0.032 |
| 5.0 µM mevinolin | 23.7 | 39 | 12.5 | 0.022 |
| Hypocotyls [c] | | | | |
| 0 µM mevinolin | 12.2 | 100 | 7.2 | 0.021 |
| 0.25 µM mevinolin | 11.6 | 95 | 8.6 | 0.019 |
| 0.625 µM mevinolin | 10.3 | 84 | 10.5 | 0.022 |
| 2.5 µM mevinolin | 8.5 | 69 | 12.8 | 0.020 |
| 5.0 µM mevinolin | 4.8 | 39 | 14.3 | 0.018 |
| Roots [c] | | | | |
| 0 µM mevinolin | 17.0 | 100 | 6.3 | 0.033 |
| 0.25 µM mevinolin | 15.9 | 94 | 9.5 | 0.038 |
| 0.625 µM mevinolin | 11.8 | 69 | 11.4 | 0.043 |
| 2.5 µM mevinolin | 8.0 | 47 | 13.0 | 0.040 |
| 5.0 µM mevinolin | 7.0 | 41 | 16.6 | 0.064 |

[a] Mean values of three independent determinations.
[b] 100 Cotyledon pairs analyzed per single experiment.
[c] 100 Hypocotyls or roots per analysis.

mevinolin treatment caused a shift towards the synthesis of Q-9 at the expense of Q-10, the predominant homologue in radish (Table II).

As expected, only the fresh weight, but not the dry weight of cotyledons was affected, whereas in hypocotyls and more clearly in roots, fresh weight and to a lower extent dry weight was diminished upon mevinolin treatment (Table 1). It appears that sterols, as they are mainly affected, might be involved in physiological processes governing water uptake and cell elongation growth.

On the other hand, even though a clear inhibition of sterol accumulation in cotyledons by mevinolin can be achieved at higher concentration as compared to the roots, there was however, no inhibition of chlorophyll and carotenoid biosynthesis under these conditions (Table III). The ratio of chlorophyll *a* to chlorophyll *b* also remained constant over the range of mevinolin concentrations tested. At low mevinolin concentrations the development of leaf area as well as of chlorophyll *a* + *b* and carotenoid content was even enhanced as compared to untreated controls (Table III). Mevinolin also appeared to induce a time shift in the developmental pattern, which might reflect secondary responses of its action on the phytohormone balance as discussed elsewhere [25]. Since this effect on the leaf area was time-dependent (Table III), it led us to conclude that cotyledons become increasingly susceptible to the inhibitory effects of mevinolin at later stages of development. However, there is even a stimulating effect on pigment accumulation that becomes more evident in ageing cotyledons.

Mevinolin-induced inhibition of acetyl-CoA utilization by the multibranched isopentenoid pathway probably accounts for the promotion of anthocyanin accumulation at the expense of sterols (Fig. 2) in hypocotyls of treated plants. This effect also becomes evident at a later stage of development. The synthesis of other isopentenoid compounds of plastidic origin such as phylloquinone and plastoquinone appeared to be slightly enhanced at low inhibitor concentrations and hardly inhibited even at 5 µM (Table IV). α-Tocopherol (Table IV), which is considered to be synthesized in the chloroplast as well as in the cytoplasm [31–35], does not react like sterols, which are clearly cytoplasmic products, but rather like phylloquinone, known to be exclusively synthesized in the plastid [34–37].

## Discussion

As demonstrated earlier [23–25] mevinolin is a potential inhibitor of plant growth and was shown

Table III. Time course study of mevinolin effects on cotyledons of light-grown radish seedlings.

| Day of germination | Mevinolin [μM] | Leaf area | | Chlorophylls $a+b$ | | $a/b$ | Carotenoids $x+c$ | | Free sterols | | Ubiquinone | | Plastoquinone | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | [cm²] | [%] | [μg/100] | [%] | | [μg/100] | [%] | [μg/100] | [%] | [μg/100] | [%] | [μg/100] | [%] |
| 3 | 0 | 0.64 | 100 | 2 610 | 100 | 3.48 | 580 | 100 | 933 | 100 | 55 | 100 | 127 | 100 |
| | 0.625 | 0.78 | 121 | 2 450 | 95 | 3.26 | 540 | 93 | 853 | 92 | 52 | 94 | 113 | 89 |
| | 1.25 | 0.84 | 132 | 2 410 | 93 | 3.37 | 520 | 89 | 853 | 92 | 54 | 98 | 115 | 91 |
| | 2.5 | 0.72 | 113 | 2 420 | 93 | 3.51 | 490 | 83 | 789 | 85 | 51 | 93 | 111 | 87 |
| | 5.0 | 0.70 | 109 | 2 180 | 84 | 3.44 | 460 | 79 | 773 | 83 | 50 | 92 | 117 | 92 |
| 4 | 0 | 0.73 | 100 | 3 420 | 100 | 3.59 | 690 | 100 | 1 332 | 100 | | | | |
| | 0.625 | 0.89 | 122 | 4 000 | 117 | 3.47 | 660 | 96 | 1 208 | 91 | | | | |
| | 1.25 | 0.86 | 118 | 3 790 | 111 | 3.31 | 630 | 91 | 1 083 | 82 | | | | |
| | 2.5 | 0.74 | 96 | 2 900 | 85 | 3.69 | 730 | 106 | 997 | 75 | | | | |
| | 5.0 | 0.70 | 96 | 2 900 | 85 | 3.69 | 730 | 106 | 958 | 72 | | | | |
| 5 | 0 | | | 3 670 | 100 | 3.51 | 690 | 100 | | | | | | |
| | 0.625 | | | 4 280 | 117 | 3.55 | 790 | 114 | | | | | | |
| | 1.25 | | | 4 190 | 114 | 3.61 | 770 | 112 | | | | | | |
| | 2.5 | | | 4 040 | 110 | 3.43 | 720 | 105 | | | | | | |
| | 5.0 | | | 4 280 | 117 | 3.33 | 770 | 111 | | | | | | |
| 6 | 0 | 0.86 | 100 | 3 970 | 100 | 3.53 | 740 | 100 | 1 640 | 100 | 61 | 100 | 154 | 100 |
| | 0.625 | 0.97 | 113 | 4 930 | 124 | 3.60 | 830 | 111 | 1 647 | 100 | 59 | 97 | 157 | 102 |
| | 1.25 | 0.81 | 94 | 4 670 | 118 | 3.58 | 830 | 113 | 1 569 | 96 | 53 | 86 | 192 | 124 |
| | 2.5 | 0.76 | 89 | 4 770 | 120 | 3.51 | 840 | 114 | 1 453 | 89 | 47 | 77 | 158 | 103 |
| | 5.0 | 0.75 | 88 | 4 470 | 113 | 3.37 | 770 | 104 | 1 091 | 67 | 24 | 39 | 137 | 89 |

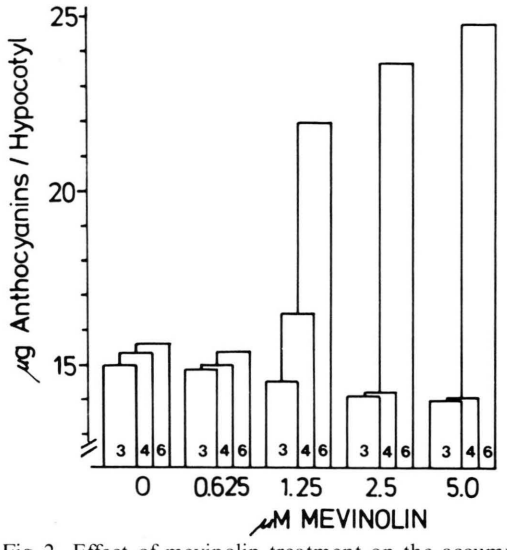


Fig. 2. Effect of mevinolin treatment on the accumulation of anthocyanins in hypocotyls of light-grown radish seedlings. 100 Hypocotyls per condition were analyzed at day 3, 4, and 6 of germination.

to inhibit sterol synthesis in radish seedlings [24, 25]. Since we aimed our studies towards investigating what type of final product of the multibranched isopentenoid pathway might primarily depend on an intact MVA biosynthesis, we have expanded our studies to other isopentenoid compounds than sterols functional in plant cells. The results presented in this communication confirm previous results and discussions on the role and compartmentation of MVA biosynthesis [25]. The drastic inhibition of sterol biosynthesis by mevinolin clearly demonstrates MVA biosynthesis to be rate-limiting, as has already been demonstrated in the case of animal cells (*cf.* [1–4, 38]). This seems also to be true for ubiquinone, but apparently not for other compounds investigated here. Even though conclusive evidence is available to indicate an independent MVA-synthesizing machinery being present in plant mitochondria [6, 8, 24, 39, 40] possibly regulated differentially from that assayed in the ER [40], HMG-CoA reductase activity is clearly inhibited, which led us to conclude that mevinolin can easily penetrate the mitochondrial envelope. Of course, we do not exclude the possibility that mitochondrial MVA utilization – and thereby ubiquinone biosynthesis – might additionally be linked to cytoplasmic MVA synthesis, depending on the need of the organelle for additional IPP units (*cf.* [41]). The mevinolin-induced shift in the Q-pattern towards homologues containing shorter

Table IV. Effect of mevinolin on plastidic prenylquinones[a].

| Plant part | Plastoquinone [μg/100] | [%] | PQ-9/sterols | Phylloquinone [μg/100] | [%] | α-Tocopherol [μg/100] | [%] |
|---|---|---|---|---|---|---|---|
| Cotyledons[b] | | | | | | | |
| 0 μM mevinolin | 154 | 100 | 0.94 | 23.9 | 100 | 252 | 100 |
| 0.25 μM mevinolin | 157 | 102 | 0.93 | 26.7 | 112 | 259 | 103 |
| 0.625 μM mevinolin | 192 | 124 | 0.122 | 25.0 | 104 | 268 | 106 |
| 2.5 μM mevinolin | 158 | 103 | 0.109 | 21.6 | 90 | 240 | 95 |
| 5.0 μM mevinolin | 137 | 89 | 0.125 | 20.2 | 85 | 212 | 84 |
| Hypocotyls[c] | | | | | | | |
| 0 μM mevinolin | 14.0 | 100 | 0.024 | 2.2 | 100 | | |
| 0.25 μM mevinolin | 16.8 | 120 | 0.029 | 2.6 | 118 | | |
| 0.625 μM mevinolin | 19.0 | 135 | 0.037 | 3.1 | 141 | | |
| 2.5 μM mevinolin | 15.5 | 113 | 0.036 | 3.5 | 159 | | |
| 5.0 μM mevinolin | 13.5 | 98 | 0.049 | 2.1 | 96 | | |

[a] Mean values of three independent determinations.
[b] 100 Cotyledon pairs analyzed per single experiment.
[c] 100 Hypocotyls per analysis.

isopentenoid side chains (Table II) might reflect the ability of mitochondria to adjust the usage of isopentenyl units to the available substrate inside or outside of the organelle, thereby maintaining a basic rate of synthesis needed for a functional respiratory electron transport. The slight increase of phylloquinone and, to a somewhat lower extent, of plastoquinone, upon mevinolin treatment at concentrations below 2.5 μM be explained by an increased availability of acetate to be routed towards plastidic isopentenoid biosynthesis. This would require that the acetate from the cytoplasm penetrates the chloroplast envelope. The inability of mevinolin to prevent pigment accumulation in chloroplasts, which also depends on MVA synthesis, favors the assumption that plastids contain their own independent enzyme system for MVA production [12, 25, 39, 40, 42]. Since it was demonstrated [25] that mevinolin treatment of radish seedlings results in a senescence retardation, *e.g.* the maintenance of a better photosynthetic activity, this might not only be explained by mevinolin-induced changes in the phytohormone balance, but moreover, by a relatively higher amount of compounds functional in the thylakoid electron transport chain.

## Conclusion

The results indicate that mevinolin can be successfully used as a powerful tool in studies on regulation and compartmentation of isopentenoid biosynthesis. So far it appears that at least sterol accumulation and, to a lower extent, also ubiquinone synthesis depend on an efficient HMG-CoA reductase activity. However, we do not exclude that later enzymic steps in the branched pathway might be tuned for fine control of substrate flow depending on the developmental and functional needs of the plant.

Future studies have to be aimed towards elucidating the importance of MVA biosynthesis in the production of further functional compounds such as polyprenols [44, 45], playing a key-role in cell wall biosynthesis and in the glycocylation of proteins, or isopentenoid phytoalexins [46, 47] which are involved in defense against infective microorganisms.

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