Application of Cell Culture for the Production of Bioactive Compounds from Sponges: Synthesis of Avarol by Primmorphs from Dysidea avara

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Application of Cell Culture for the Production of Bioactive Compounds from Sponges: Synthesis of Avarol by Primmorphs from *Dysidea avara*

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Among all metazoan phyla, sponges are known to produce the largest number of bioactive compounds. However, until now, only one compound, arabinofuranosyladenine, has been approved for application in humans. One major obstacle is the limited availability of larger quantities of defined sponge starting material. Recently, we introduced the in vitro culture of primmorphs from *Suberites domuncula*, which contain proliferating cells. Now we have established the primmorph culture also from the marine sponge *Dysidea avara* and demonstrate that this special form of sponge cell aggregates produces avarol, a sesquiterpenoid hydroquinone, known to display strong cytostatic activity especially against mammalian cells. If dissociated sponge cells are transferred into Ca^{2+}- and Mg^{2+}-containing seawater, they form after a period of two to three days round-shaped primmorphs (size of 1 to 3 mm). After longer incubation, the globular primmorphs fuse and form meshes of primmorphs that adhere to the bottom of the incubation chamber. Later, during incubation, freely floating mesh-primmorphs are formed. No bacterial rRNA could be detected in the primmorphs. We were able to prove that the primmorphs produce avarol. Levels (1.4 μg of avarol/100 μg of protein) close to those identified in specimens from the field (1.8 μg/100 μg) are reached. Avarol was extracted from the cells with EtOAc and subsequently purified by HPLC. The identification was performed spectrophotometrically and by thin-layer chromatography. Single cells apparently do not have the potency to produce this secondary metabolite. It is concluded that the primmorph model is a suitable system for the synthesis of bioactive compounds in vitro.

Among all metazoan phyla, Porifera (sponges) provide the largest number of bioactive compounds.[1−3] Despite the large number of compounds and the high variety of structurally different natural products, only a few of these secondary metabolites have been tested in clinical trials.[4] Arabinofuranosyladenine (ara-A) is the only secondary metabolite[4] approved to date for application in humans; it displays potent anti-herpes virus activity.[5,6] Other sponge metabolites, like avarol,[7−9] have been advanced to clinical evaluation for treatment of HIV-infected individuals (clinical trial of avarol: Fa. Merz [Frankfurt] − Clinics Mainz). Limited availability of larger quantities of a particular sponge species as starting material for extraction of the compounds is one of the major causes for the low attractiveness of such secondary metabolites for commercial development.

Four routes can be followed to obtain larger quantities of sponge secondary metabolites. The first of these is chemical synthesis; this promising approach has been successfully undertaken[10] but usually involves many steps and provides low yields. The second is cultivation of sponges in the sea or mariculture. Farming of sponges for the production of bioactive compounds has recently been started.[11−13] However, at present it cannot be judged if this direction has an applicable future. A third route is cultivation of sponge specimens in a bioreactor.[14] A fourth possibility, by analogy to the production of bioactive compounds from bacteria and fungi, is the production of secondary metabolites in bioreactors using sponge cells in culture. The first successful approach to show that sponge cells can proliferate and grow in vitro was recently started with the demosponge *Suberites domuncula*.[15−17] Earlier attempts appeared promising.[18,19] One crucial step toward a solution of this problem was the finding that single cells lose telomerase activity and, hence, their potency for (unlimited) cell division.[20] After aggregation the cells regain telomerase activity and, thus, their growth potential; these cells are able to differentiate in the aggregates. Such assemblies were termed primmorphs.[15−17]

It is well established that sponges contain microorganisms.[21] It was assumed that some of these are potential endosymbionts.[22] Recent studies using the demosponge *Halichondria panicea* revealed that the sequence of a bacterial 16S rRNA found in sponge specimens collected from extremely distant areas is identical. This finding strongly suggests that those bacteria live with the sponge either in a commensal or endosymbiotic relationship.[23] The presence of microorganisms in sponges gave rise to the question as to whether the bioactive compounds extracted from a given sponge stem from the host or these microorganisms. One rational solution to this question is the synthesis or biosynthesis of metabolites from sponges in a bioreactor under in vitro conditions.

In the present research, we have selected the demosponge *Dysidea avara* for the establishment of the primmorph system; we use primmorphs for the production of its bioactive compound, avarol.[7,8] Avarol has been reported to display strong bioactivity in vitro and in vivo,[24] particularly cytotoxic (in vitro),[8] antitumor (in vivo),[9] antibacterial,[25] and antiviral activity.[26,27] The major mode of action of avarol is its effect on the prostaglandin and leukotriene pathway.[28] The structure of avarol was elucidated by De Rosa et al.,[29] who identified it as a rearranged sesquiterpenoid hydroquinone. The enzymatic pathway by which

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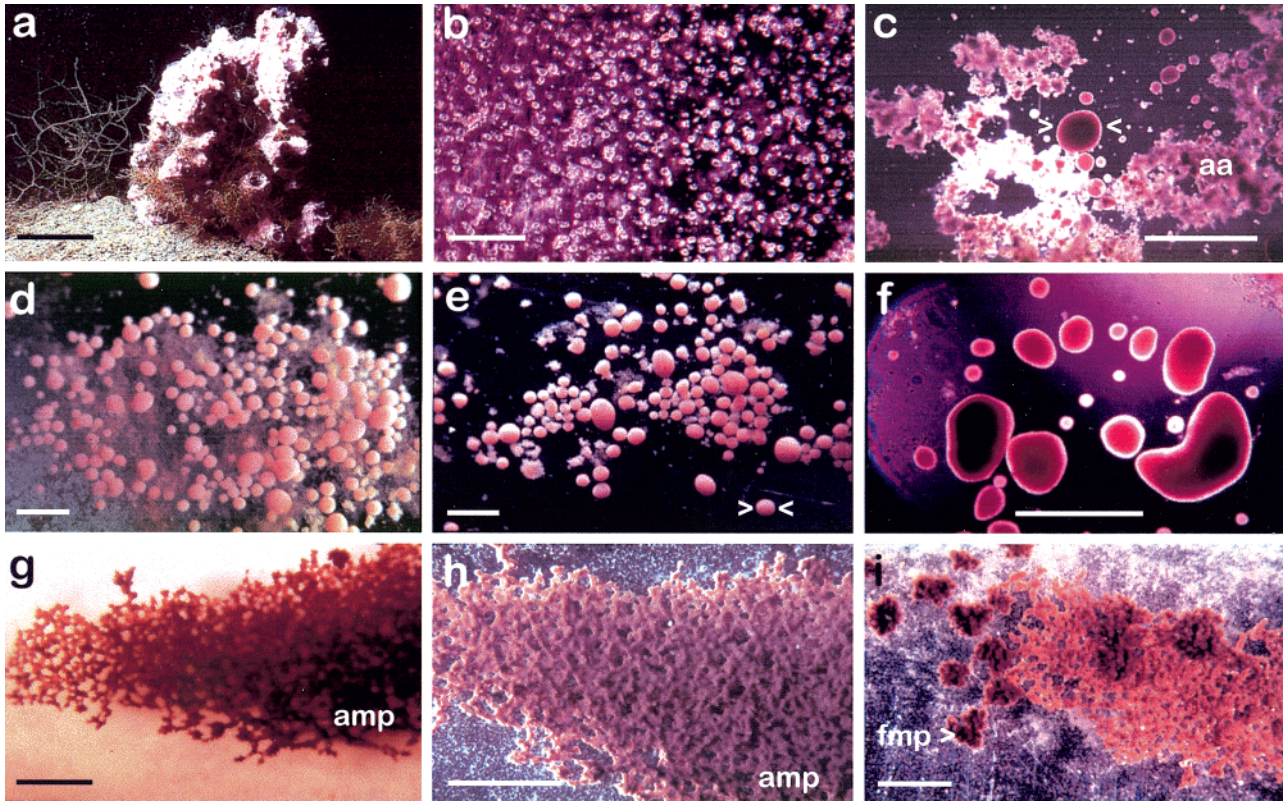

Figure 1. *D. avara*. a: a specimen living on the hard bottom (magnification × 0.2; bar corresponds to 50 mm). Re-aggregation of cells and formation of primmorphs: b: dissociated single cell suspension (× 50; bar: 200 μm); c: round-shaped primmorphs [> <] surrounded by amorphous cell aggregates [aa] (× 3; bar: 5 mm); d: round-shaped, floating primmorphs associated onto the amorphous cell aggregates (× 1.5; bar: 5 mm); e: round-shaped primmorphs (× 1.5; bar: 5 mm); f: large round-shaped primmorphs (× 3.5; bar: 5 mm); g and h: adherent mesh-primmorphs [amp] (× 10; bar: 1 mm [g]; × 15; bar: 1 mm [h]) that stick to the plastic dish; and i: adherent mesh-primmorphs with formation of floating mesh-primmorphs [fmp] at the rim (× 10; bar: 1 mm).

avarol is synthesized in the sponge is not known. It can be postulated that this bioactive compound can be produced by combining the isopentenyl pyrophosphate/isoprenoid and shikimate pathways of the host; but a potential synthesis by bacterial symbiosis cannot be excluded.[30]

We show in this report that the newly introduced primmorph system[16,17] can be used for the production of bioactive compounds from a marine sponge—illustrated here by the production of avarol from *D. avara* cells. It is demonstrated that avarol is synthesized only by cells that are assembled in primmorphs. Due to the lack of bacterial rRNA in the total RNA preparation from *D. avara* primmorphs, and in contrast to RNA from field specimens, it is concluded that the bioactive compound is produced by sponge cells.

Results and Discussion

Formation of Primmorphs by Cells from *D. avara*. Dissociated cells of a size between 12 and 18 μm (Figure 1b) were transferred into Ca^{2+}- and Mg^{2+}-containing seawater, which allows the formation of aggregates from dissociated single cells.[31] After two to three days, round-shaped primmorphs 1− 3 mm in diameter are formed. In the initial phase, the primmorphs are surrounded by amorphous cell aggregates (Figure 1c), which die during the following two days. Six days after transfer of the cells into seawater medium, only globular primmorphs are present (Figure 1d, e), which unite to form 5-mm large globular primmorphs (Figure 1f). From day 10, the primmorphs fuse with formation of mesh-primmorphs, which adhere to the plastic dish. Under the conditions used here, the adherent mesh-primmorphs reach sizes of 10−30 × 5−20 mm (Figure 1g−i). The interconnecting "stolons" (thickness, ca. 50 μm) leave a mesh pore size of about 150 μm. Usually beginning at day 12, some freely floating mesh-primmorphs are formed (Figure 1i). To date, mesh-primmorphs have been cultivated under the conditions described here for four weeks.

Synthesis of DNA. Previously we used the BrdU (5-bromo-2′-deoxy-uridine) incorporation protocol to demonstrate cell proliferation in primmorphs.[16,17] In the present routine study we applied the [methyl-^{3}H]thymidine ([^{3}H]-dThd) incorporation technique as a measure for DNA synthesis. The results revealed that single cells (time 0) show only a very low incorporation rate, with 107 ± 40 cpm/mg of protein. However, after transfer of the cells into Ca^{2+}- and Mg^{2+}-containing seawater for only 1 day, a significant increase of the incorporation rate (298 ± 50 cpm/mg protein) is observed. A further prolongation of the incubation to three or even 10 days yielded higher rates of 970 ± 60 cpm/mg and 835 ± 70 cpm/mg, respectively (Figure 2). From these results we conclude that, in contrast to single cells, the cells assembled in primmorphs from *D. avara* underwent DNA synthesis as described earlier for *S. domuncula*.[16,17]

Bacteria. It is known that *D. avara* specimens contain bacteria.[21] To trace the presence of bacteria, total RNA was extracted and analyzed by electrophoresis. The gel revealed that the RNA preparation obtained from a sponge specimen contained both 28S and 18S rRNA, two RNA species that are characteristic for eukaryotes (Figure 3 lane a). In addition, two bands are visible in this RNA preparation, which correspond to 23S and 16S; these species represent bacterial rRNA (Figure 3; lane a). In contrast, RNA obtained from primmorphs contains predominantly only the two eukaryotic 28S and 18S rRNA species (Figure 3;

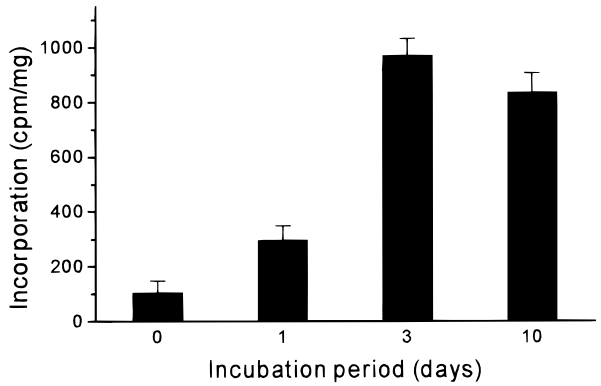

Figure 2. Incorporation of [^{3}H]dThd into the acid-insoluble fraction from cells of *D. avara*. Single cells in the dissociated state or those present in primmorphs were incubated with [^{3}H]dThd for 5 h. The acid-insoluble fraction was determined, and the incorporation values were correlated with the protein content in the sample. Cells remained either in the Ca^{2+}- and Mg^{2+}-free artificial seawater medium (time 0) or were transferred into Ca^{2+}- and Mg^{2+}-containing seawater for 1, 3, or 10 days to allow primmorph formation and then subjected to the incorporation assay.

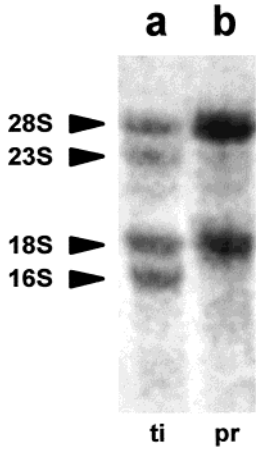

Figure 3. Total RNA isolated from tissue [ti] of a specimen of *D. avara* (lane a) or from primmorphs [pr] after 10 days in culture (lane b). (20 μg) was size-separated on a formaldehyde/agarose gel and visualized by ethidium bromide.

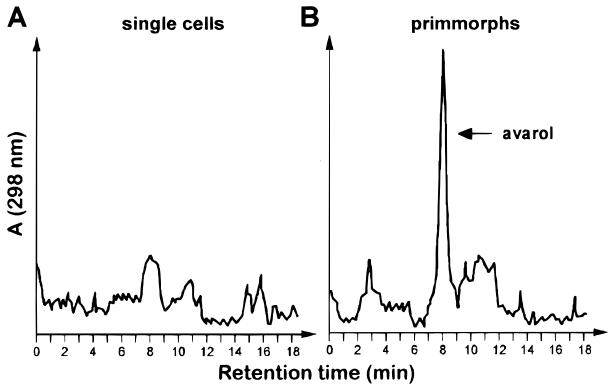

Figure 4. Separation of crude fractions, obtained after EtOAc extraction, by HPLC technique. **A**. Extracts from single cells, incubated for 3 days in CMFSW were analyzed. **B**. Extracts prepared from primmorphs after 6 days in seawater/marine broth medium. In a parallel run it was established that authentic avarol elutes from the column at 8 min (marked by an arrow).

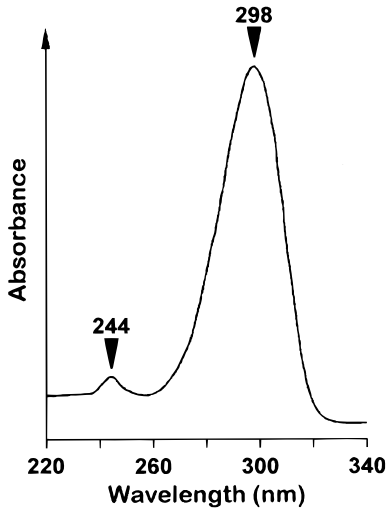

Figure 5. Absorption spectrum of avarol after separation of a crude extract (prepared from 6-day-old primmorphs) by HPLC. The fraction with a retention time of 8 min was analyzed spectrophotometrically. The two characteristic absorbance peaks (at 298 and 314 nm) are marked.

lane b), suggesting that the primmorphs from *D. avara* contain only trace amounts of bacteria.

Synthesis of Avarol by Cells from *D. avara* In Vitro. Avarol is known to be produced intracellularly in the host cells and is very likely released from vacuoles into the surrounding tissue.[30] To determine if cells, present in the single-cell state as well as in the primmorph state, produce avarol, they were extracted with EtOAc as described below. Subsequently, the crude extract was separated by the HPLC technique. It is found that avarol elutes from the column at a retention time of 8 min (Figure 4 B). In parallel runs it was established that authentic avarol appears at 8 min. The production of the second bioactive compound, avarone, which eluted later from the column (28 min), was not analyzed quantitatively in this study. In an earlier study it was reported that avarone is a minor component (compared to avarol) in *D. avara*.[7] The fractions containing avarol were additionally analyzed spectrophotometrically. The characteristic profile with the two absorbance peaks at wavelengths of 298 and 314 nm[7] is seen (Figure 5); in contrast, the maximal absorbance of avarone is at 246 nm.[7] Next, the crude extract was spotted onto a Si gel plate, and separation was performed with EtOH−$CHCl_3$. Two major spots were visualized, one of which had comigrated with authentic avarol (Figure 6). From previous studies it is known, that besides avarol, the oxidized derivative avarone is also present in the crude extract.[30,32] This compound migrates close to the solvent front (Figure 6). Finally, the fractions containing avarol were analyzed by IR spectroscopy and mass spectral measurements; identical data as described earlier were found[8] (data not shown). Using this same approach, samples from specimens of *D. avara*, collected from the field, were analyzed and found to contain 1.8 ± 0.3 μg of avarol per 100 μg of protein.

Single cells. Dissociated single cells were incubated in Ca^{2+}- and Mg^{2+}-free seawater for 0 to 3 days. Extracts were prepared and analyzed by HPLC and, subsequently, by spectrophotometer. The experiments revealed that the concentration of avarol is below <0.05 μg/100 μg protein (Table 1; Figure 4A).

Primmorphs. The round-shaped primmorphs were analyzed after incubation periods of 3 and 6 days; a considerable amount of avarol (Figure 4B), 0.4 ± 0.2 to 0.9 ± 0.4 μg/100 μg, could be measured (Table 1). This amounts to about 30% of the avarol content present in extracts from field samples. Even higher is the content of avarol measured in extracts obtained from adherent mesh-primmorphs; levels of 1.0 ± 0.6 μg/100 μg to 1.2 ± 0.5 μg/100 μg were determined (Table 1). Highest values were found

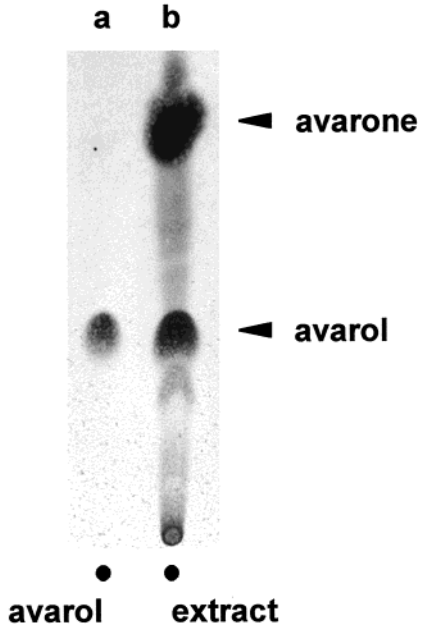

Figure 6. Separation of the crude EtOAc extract from 6-day-old primmorphs by TLC (lane b). In lane a, authentic avarol has been analyzed in parallel. The upper spot corresponds to avarone.

Table 1. Content of Avarol in Samples from Specimens Collected from the Field, Single Cells, and the Three Different Forms of Primmorphs[a]

starting material	incubation period (days)	avarol concentration (μg/100 μg of protein)
adult sponge "tissue"	field	1.8 ± 0.3
single cells	0	<0.05
	1	<0.05
	3	<0.05
primmorphs (round-shaped)	3	0.4 ± 0.2
	6	0.9 ± 0.4
primmorphs (adherent mesh-form)	10	1.2 ± 0.5
	12	1.0 ± 0.6
primmorphs (floating/adherent)	15	1.4 ± 0.5
	25	0.9 ± 0.5

[a] The samples were extracted with EtOAc; the extract was separated by HPLC, and in the fractions containing avarol, its concentration was determined. Five experiments were performed each; the means ± SD are given.

in assays of adherent mesh-primmorphs, containing also floating mesh-primmorphs with a content of 1.4 ± 0.5 μg/100 μg. This value represents around 80% of the amount of avarol that can be isolated from field samples.

Conclusions and Further Developments

One major prerequisite for a successful production of bioactive compounds from marine animals is the availability of a proliferating cell culture. With the establishment of the primmorph culture and the demonstration that this system can be used for the production of bioactive compounds in vitro, as illustrated here by avarol production using primmorphs from *D. avara*, a new direction in marine biotechnology has been opened. The next step must be scale-up of the cultures in larger fermentors. Furthermore, a series of improvements in the cell culture procedures is needed. Optimization of physical (e.g., pressure) and biochemical parameters has to be achieved. The first growth factors have already been cloned, which are very likely suitable for an optimization of the culture conditions (e.g., myotrophin or the endothelial-monocyte-activating polypeptide).[33]

In this context it should be noted that, in contrast to cells from bacteria[25] and mammals,[8,9] DNA synthesis in cells from *D. avara* is not inhibited by avarol concentrations below 5 μg/mL in vitro (data not shown). The "resistance" of cells from *D. avara* to avarol is also supported by the finding that the sensitive marker protein for stress, the p38 mitogen-activated protein kinase, does not respond with phosphorylation in cells from *D. avara* during incubation with avarol (data not shown).

Because, in the past few years, major progress in the elucidation of the sponge genome has been achieved,[34−36] our group is now involved in the isolation of the gene cluster from *D. avara* which codes for the enzyme pathway resulting in the synthesis of avarol.

Experimental Section

Materials. Natural seawater [sterile] (S9148), penicillin, and streptomycin were obtained from Sigma (Deisenhofen; Germany); [methyl-^{3}H] thymidine (dThd; specific activity of 19 Ci/mmole) from Amersham (Amersham; England). The compositions of Ca^{2+}- and Mg^{2+}-free artificial seawater (CMFSW) as well as of CMFSW containing EDTA (CMFSW-E) were described previously.[37]

Animal Material. Specimens of the marine sponge *D. avara* (Porifera, Demospongiae, Ceractinomorpha, Dendroceratida, Dysideidae) were collected in the Northern Adriatic near Rovinj (Croatia) from a depth of 15−25 m on the hard bottom (Figure 1a). The 10−25-cm large specimens were then kept in aquaria in Mainz (Germany) at a temperature of 16 °C.

Dissociation of Cells and Formation of Primmorphs. The procedure described previously for *S. domuncula* was applied for *D. avara*.[16,17] In brief, tissue pieces (4 to 5 cm^3) were dissociated in CMFSW-E under constant rotation. The cell suspension obtained was centrifuged and washed twice with CMFSW. Then the cells were resuspended in CMFSW supplemented with antibiotics (penicillin [100 IU/mL] and streptomycin [100 μg/mL]) and were placed into culture Petri chambers (Falcon; diameter of 9 cm). Primmorphs, a special form of aggregates, are formed from single cells after transferring them into seawater (supplemented with antibiotics and 0.1% (v/v) of marine broth 2216 [DIFCO]) following the procedure described previously.[16,17] The cultures were incubated at 16 °C, while permanently being gently shaken on a rocking platform. Under these conditions first globular primmorphs, later adherent mesh-primmorphs, and finally floating mesh-primmorphs are formed, as described above. For the studies to determine the production of avarol in cells in vitro, assays in 9-cm culture dishes were chosen, which contained 15 mL seawater/marine broth and had a cell concentration of about 2×10^6 cells/mL; incubation was performed for up to 3 days. The medium (always containing antibodies) was changed every second day.

Incorporation Studies. Assays of either single cells or primmorphs from *D. avara* (5 mL; 2 to 3×10^6 cells) were incubated with 25 μCi of the labeled DNA precursor [^{3}H]dThd for 5 h. Subsequently, the samples were analyzed for radioactivity in the acid-insoluble (DNA-) fraction as described.[38,39] The values for the radioactivity incorporated were correlated with the amount of protein from the cells used for the determination. Protein content was determined by two established methods[40,41] using bovine serum albumin as standard. The values obtained by the two methods differed by only 8%.

RNA Analysis. RNA was extracted from liquid-nitrogen-pulverized sponge tissue or from primmorphs with TRIzol Reagent (GibcoBRL) as recommended by the manufacturer. An amount of 20 μg of total RNA was electrophoresed through a formaldehyde [2.2 M] agarose gel [1%] and stained with ethidium bromide as described.[42] Subsequently, the RNA on the gel was visualized with a UV transilluminator.

Extraction and Analytical Methods. Single cells or primmorphs were added to two volumes of EtOH and homog-

enized with 10 strokes of a Dounce homogenizer. After 3 h of shaking, the slurry was extracted with EtOAc as previously described.[8] The EtOAc extract was dried over Mg_2SO_4 and evaporated to dryness.

The crude extract was analyzed as follows. TLC: an aliquot of the extract was spotted onto Si gel plates (Merck 60 F2540); separation was performed with EtOH−$CHCl_3$ (1:4) as solvent. The spots were visualized by 10% sulfuric acid (in EtOH) as previously described.[8] HPLC: a column containing 2 g of Nucleosil C_{18} was used and the separation performed with an analytical HPLC instrument (Gynkotek; model 250B/300CS). Elution was performed with MeOH−H_2O (9:1) as mobile phase, and the absorbance was recorded at 298 nm. The retention time of avarol was 8 min; the second active component, avarone, eluted from the column after 28 min. Purified avarol served as reference.[8] Spectrophotometric analysis: the measurements were performed with a UV/visible light spectrophotometer (Beckman; model DU64).

Determination of the Concentration of Avarol. The crude extracts were separated by HPLC as described above, and the fractions containing avarol (retention time: 8 min) were collected manually and evaporated to dryness. The solid material was dissolved in EtOH, and the concentration was determined spectrophotometrically on the basis of the extinction coefficient for avarol $E_{\lambda 298}$ of 3415 M^{-1} cm^{-1}.

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