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In vitro formation of the anthranoid scaffold by cell-free extracts from yeast-extract-treated *Cassia bicapsularis* cell cultures

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ABSTRACT

The anthranoid skeleton is believed to be formed by octaketide synthase (OKS), a member of the type III polyketide synthase (PKS) superfamily. Recombinant OKSs catalyze stepwise condensation of eight acetyl units to form a linear octaketide intermediate which, however, is incorrectly folded and cyclized to give the shunt products SEK4 and SEK4b. Here we report *in vitro* formation of the anthranoid scaffold by cell-free extracts from yeast-extract-treated *Cassia bicapsularis* cell cultures. Unlike field- and *in vitro*-grown shoots which accumulate anthraquinones, cell cultures mainly contained tetrahydroanthracenes, formation of which was increased 2.5-fold by the addition of yeast extract. The elicitor-stimulated accumulation of tetrahydroanthracenes was preceded by an approx. 35-fold increase in OKS activity. Incubation of cell-free extracts from yeast-extract-treated cell cultures with acetyl-CoA and [2-^{14}C]malonyl-CoA led to formation of torosachrysone (tetrahydroanthracene) and emodin anthrone, beside two yet unidentified products. No product formation occurred in the absence of acetyl-CoA as starter substrate. To confirm the identities of the enzymatic products, cell-free extracts were incubated with acetyl-CoA and [U-$^{13}C_3$]-malonyl-CoA and ^{13}C incorporation was analyzed by ESI-MS/MS. Detection of anthranoid biosynthesis in cell-free extracts indicates *in vitro* cooperation of OKS with a yet unidentified factor or enzyme for octaketide cyclization.

1. Introduction

Plant anthranoids are well known for their purgative properties due to the 1,8-dihydroxyanthrone structure (Dewick, 2009). Anthranoids form a subclass of anthracene derivatives and include anthrones, dianthrones, and anthraquinones, commonly encountered as glycoconjugates. Plant anthranoids are widely distributed in the families Rhamnaceae, Asphodelaceae, Polygonaceae, and Fabaceae (formerly Caesalpiniaceae), which provide a number of laxatives.

Biosynthesis of the anthranoid scaffold in plants is catalyzed by octaketide synthase (OKS), which is a member of the superfamily of type III polyketide synthase (PKS) enzymes. PKSs generate an amazing array of natural products by catalyzing iterative condensation of acetyl units from malonyl-CoA into a growing polyketide chain (Austin and Noel, 2003). The high degree of product diversity is due to variation of (i) the starter substrate, (ii) the number of acetyl additions, and (iii) the mechanism of ring formation. The starter for OKS is an acetyl unit, which is stepwise condensed with seven molecules of malonyl-CoA to give a linear octaketide intermediate (Fig. 1; Dewick, 2009). Three aldol-type condensations then lead to formation of tetrahydroanthracenes, such as atrochrysone (**3**, route B). If a carbonyl of the linear octaketide intermediate undergoes reduction involving NADPH, the product is germichrysone (**1**; route A). The polyketide folding mode in higher plants is mode F, which was also found in fungi, whereas bacteria use mode S, which refers to *Streptomyces* (Bringmann et al., 2006).

Tetrahydroanthracenes can dehydrate to yield anthrones, such as chrysophanol and emodin anthrone (**5**,**7**), which in turn can undergo oxidation of the central cyclohexadienone ring to give anthraquinones, such as chrysophanol and emodin (**8**,**10**). In *Aspergillus terreus*, fixation of molecular oxygen into C-10 is catalyzed by a membrane-bound emodin anthrone oxygenase (Chen et al., 1995). When two anthrone molecules undergo oxidative coupling, the product is a dianthrone. In some plant families, such as Rubiaceae, the isochorismate/2-oxoglutarate/mevalonate pathway is used to produce anthracene derivatives which, however, lack the 1,8-dihydroxyanthrone scaffold and hence the purgative potential (Leistner, 1985; Han et al., 2001).

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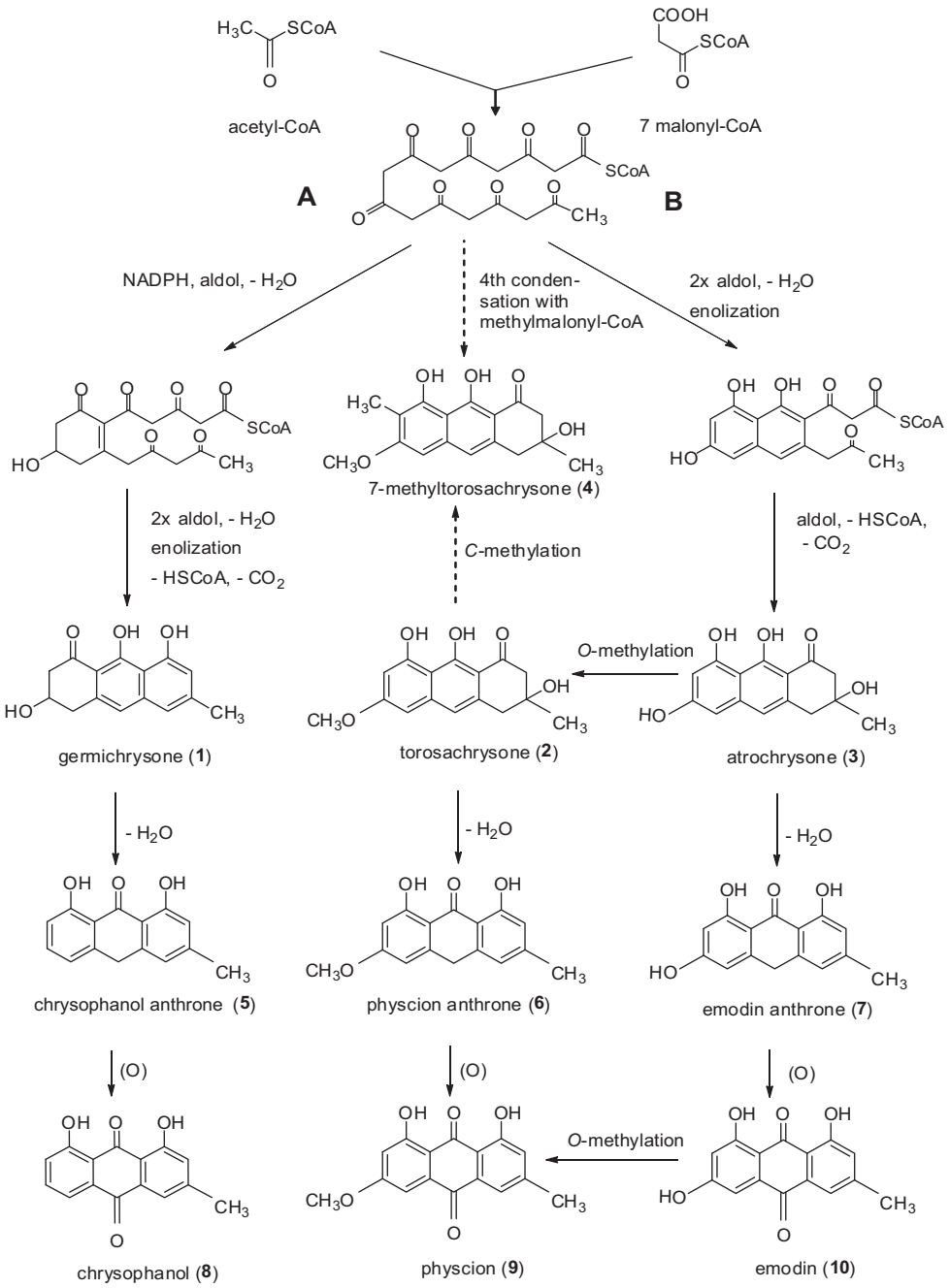

Fig. 1. Proposed biosynthesis of tetrahydroanthracenes and anthraquinones in *C. bicapsularis* cell cultures. Route A involves reduction of a carbonyl at the octaketide stage, which does not occur in route B. Dotted lines illustrate alternative pathways that may lead to formation of 7-methyltorosachrysone.

OKS-encoding transcripts from *Aloe arborescens* (Asphodelaceae) and *Hypericum perforatum* (Hypericaceae) were cloned and heterologously expressed, demonstrating that both recombinant enzymes formed a linear octaketide which, however, was incorrectly folded and cyclized to give two aromatic polyketides known as SEK4 and SEK4b (Fig. 2; Abe et al., 2005a; Karppinen et al., 2008; Mizuuchi et al., 2009). Thus, both PKSs were incapable of accomplishing the *in vivo* reaction under *in vitro* conditions, raising the question of whether an additional factor or enzyme may be involved in anthranoid biosynthesis. Very recently, a polyketide cyclase which functions cooperatively with a tetraketide synthase in cannabinoid biosynthesis has been identified in *Cannabis sativa* (Gagne et al., 2012). Here, anthranoid biosynthesis was studied in newly established cell cultures of *Cassia bicapsularis* L. This species is a glabrous shrub or small tree attaining 3–4 m in height with bright yellow flowers in dense terminal racemes (Bailey, 1961). The anthraquinones physcion (**9**), emodin (**10**), and emodin-8-*O*-glucoside were isolated from leaves, beside flavonoids, triterpenes, and sterols (Abdel-Rahman, 2006). We now report *in vitro* anthranoid biosynthesis in cell-free extracts from yeast-extract-treated *C. bicapsularis* cell cultures upon incubation with [2-^{14}C] and [U-$^{13}C_3$]malonyl-CoA in the presence of acetyl-CoA.

2. Results

2.1. Establishment of C. bicapsularis cell cultures

Seeds of *C. bicapsularis* were germinated and the seedlings used to establish callus and cell suspension cultures. Cell cultures were grown in a modified Murashige and Skoog (MS) medium. Typical

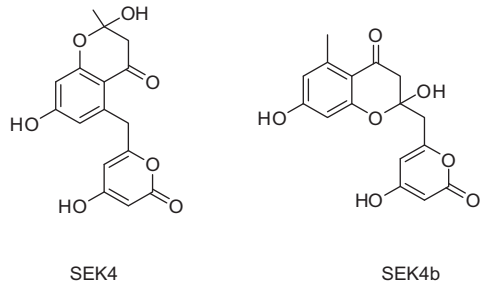

Fig. 2. *In vitro* products of recombinant octaketide synthases.

growth curves were observed (Fig. 3). After a 2-day-lag phase, both the fresh weight (fr. wt) and the dry weight (dry wt) increased linearly up to day 22. Thereafter, the dry wt remained constant until day 36, whereas the fr. wt slightly decreased.

2.2. Accumulation of anthranoids

The residue of a methanolic extract from 7-day-old cell cultures was partitioned between dichloromethane and water. The aqueous phase was freed from sugars on an Amberlite XAD-16 column and subjected to preparative HPLC, yielding three yellow tetrahydroanthracene (THA) compounds. Germichrysone (**1**, Fig. 1), torosachrysone (**2**), and 7-methyltorosachrysone (**4**) were identified by UV spectroscopy, mass spectrometry, and ^{1}H and ^{13}C NMR spectroscopy (Table S1, Fig. S1). The spectra obtained matched the literature data (Kitanaka and Takido, 1984; Kitanaka et al., 1985; Delle Monache et al., 1991). However, there were some differences in the chemical shifts due to the different solvents used (DMSO-d_6, $CDCl_3/CD_3OD$ as opposed to $CDCl_3$ in the present work). Furthermore, a number of discrepancies in the ^{13}C NMR signal assignments occurred which, however, did not hamper the identification of the compounds. The assignment of the ^{13}C chemical shifts for **1** given in Table S1 agrees with that derived from biosynthetic ^{13}C-labeling studies (Ko et al., 1995). Close ^{13}C chemical shifts in Table S1 are still considered interchangeable because of the different solvents used ($CDCl_3$ vs. acetone-d_6). The dichloromethane-soluble fraction was subjected to silica gel column chromatography, yielding three anthraquinone (AQ) compounds. The UV spectroscopic properties of chrysophanol (**8**), physcion (**9**), and emodin (**10**) agreed with those of authentic reference compounds.

2.3. Effect of elicitors and salt stress on anthranoid accumulation

Seven-day-old cell cultures were treated with three different concentrations of chitosan, methyl jasmonate, and yeast extract (YE), which were previously used to enhance secondary product formation (Monir and Proksch, 1989; Gundlach et al., 1992; Villegas and Brodelius, 1990). Cell cultures were also subjected to salt stress, which was reported to stimulate anthraquinone production in *Cassia acutifolia* cell cultures (Nazif et al., 2000). While chitosan, methyl jasmonate, and sodium chloride were poorly active in *C. bicapsularis* cell cultures, YE increased formation of both THAs and AQs (Fig. 4). Since the stimulatory effect of YE was dependent on the amount of elicitor added, a series of increasing YE concentrations were tested (Table 1). The optimum YE concentration was 4 g/l, which stimulated formation of compounds **1**, **2**, and **4** to similar extents and led to maximum THA and AQ contents. Compared to control cell cultures which received the corresponding volume of water only, the THA and AQ levels in YE-treated cell cultures were increased 2.5- and 2.8-fold, respectively. Regarding the ratio of the THA and AQ levels, the THA content was approx. 7 times higher than the AQ content in both treated and control cell cultures. The optimum YE concentration (4 g/l) was then added to differently old cell cultures within the linear growth phase (Fig. 5). The most responsive stage for both THA and AQ formation was day 7, i.e. the early growth phase. No differential stimulation of THA and AQ formation in dependence on cell culture age was observed. High responsiveness to elicitors of young cell cultures was previously observed with cultivated cells of some other species (Ketchum et al., 1998; Lu et al., 2001; Chong et al., 2005). Finally, changes in THA and AQ accumulation and dry weight were

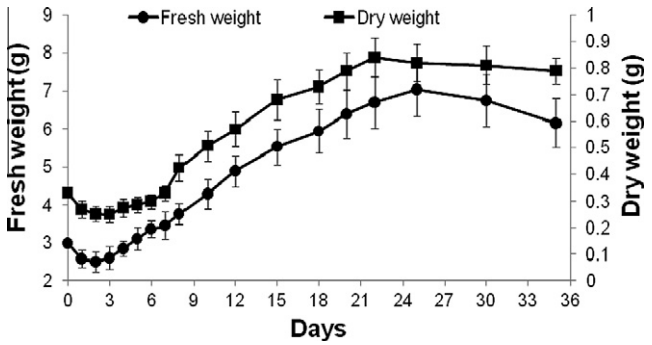

Fig. 3. Growth curves of *C. bicapsularis* cell cultures.

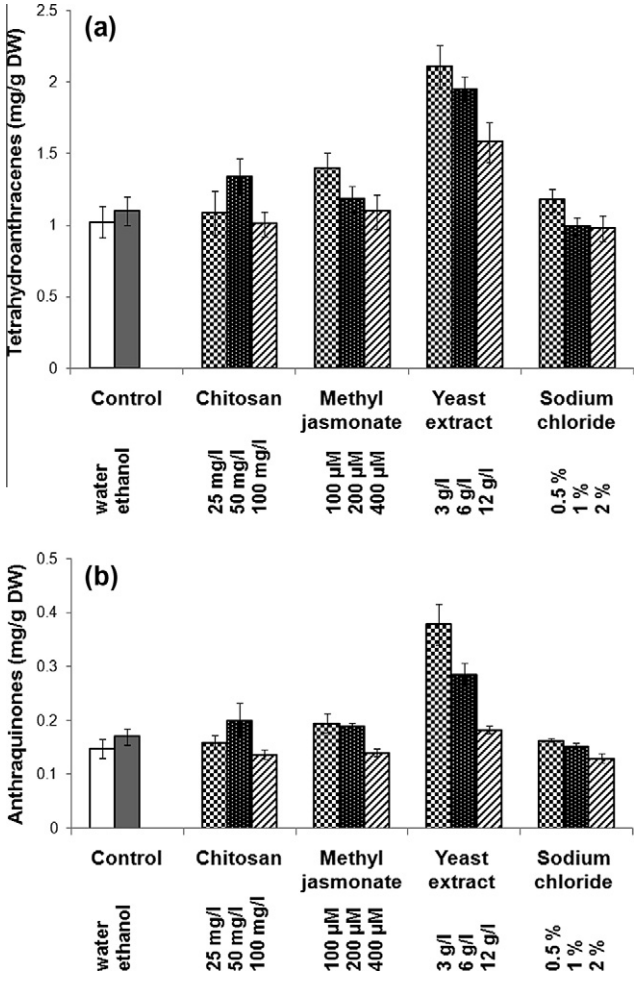

Fig. 4. Effect of elicitors and salt stress on tetrahydroanthracene (a) and anthraquinone accumulation (b) in *C. bicapsularis* cell cultures. Data are mean values ± SD of three independent experiments.

Table 1
Effect of increasing yeast extract concentrations on formation of germichrysone (**1**), torosachrysone (**2**), and 7-methyltorosachrysone (**4**) as well as the total tetrahydroanthracene (THA) and anthraquinone (AQ) contents in *C. bicapsularis* cell cultures. Data are mean values ± SD of three independent experiments.

Yeast extract (g/l)	**1**	**2**	**4**	Total THA	Total AQ
	(mg/g DW)			(mg/g DW)	
0	0.89 ± 0.071	0.11 ± 0.009	0.025 ± 0.001	1.02 ± 0.081	0.14 ± 0.012
1	1.13 ± 0.107	0.12 ± 0.020	0.028 ± 0.003	1.27 ± 0.130	0.16 ± 0.014
2	1.19 ± 0.110	0.14 ± 0.012	0.030 ± 0.004	1.36 ± 0.126	0.24 ± 0.019
3	1.90 ± 0.127	0.18 ± 0.020	0.039 ± 0.003	2.11 ± 0.150	0.37 ± 0.032
4	2.32 ± 0.191	0.21 ± 0.029	0.042 ± 0.005	2.57 ± 0.225	0.39 ± 0.034
6	1.86 ± 0.144	0.17 ± 0.016	0.033 ± 0.002	1.94 ± 0.162	0.28 ± 0.025
8	1.66 ± 0.172	0.16 ± 0.019	0.031 ± 0.002	1.85 ± 0.193	0.26 ± 0.022
10	1.60 ± 0.159	0.15 ± 0.021	0.028 ± 0.003	1.77 ± 0.183	0.25 ± 0.025
12	1.41 ± 0.122	0.14 ± 0.023	0.025 ± 0.004	1.57 ± 0.149	0.18 ± 0.020

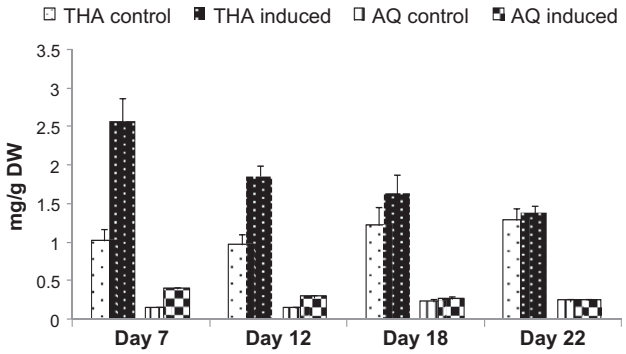

Fig. 5. Tetrahydroanthracene (THA) and anthraquinone (AQ) levels in *C. bicapsularis* cell cultures that were treated with yeast extract at various times after transfer to new medium. Control cultures received only water. Data are mean values ± SD of three independent experiments.

studied in more detail after addition of YE on day 7. The continuous increase in dry wt observed with control cell cultures was delayed by three days in YE-treated cultures (Fig. 6a). Growth retardation after elicitor treatment was previously reported for some other cell cultures (Monir and Proksch, 1989; Chen and Chen, 2000). The content of THAs strongly increased between day 2 and day 4 (Fig. 6b). The AQ level also peaked at day 4 after the addition of YE (Fig. 6c). In control cell cultures, the contents of both classes of anthracene derivatives decreased continuously.

2.4. Detection of anthranoid formation in enzyme assays

Since OKS activity was expected to precede THA and AQ accumulation, cell-free extracts were prepared from cell cultures harvested at various times up to 54 h after the addition of YE (Fig. 7). OKS activity strongly increased after 18 h, peaked at 27 h and decreased thereafter slowly. Compared to the time point of YE addition (0 h), OKS activity increased approx. 35-fold. In view of this profile, enzyme assays were carried out using cell-free extract prepared from cells 27 h after the onset of elicitation. Incubation for 1 h of acetyl-CoA as a starter substrate and [2-^{14}C]malonyl-CoA as an extender substrate resulted in formation of four enzymatic products (R_t = 11.5, 13.4, 15.9, and 19.6), as demonstrated by radiodetector-coupled HPLC analysis (Fig. 8a). Enzyme assays containing heat-denatured cell-free extract failed to form these products (Fig. 8b). Nor were the products present in assays that lacked acetyl-CoA. Based on the retention times of the authentic reference compounds, the enzymatic products R_t 15.9 and 19.6 appeared to be torosachrysone (**2**) and emodin anthrone (**7**). Emodin anthrone as a reference compound was chemically synthesized from the anthraquinone emodin (**10**, Fig. S2). For authentication,

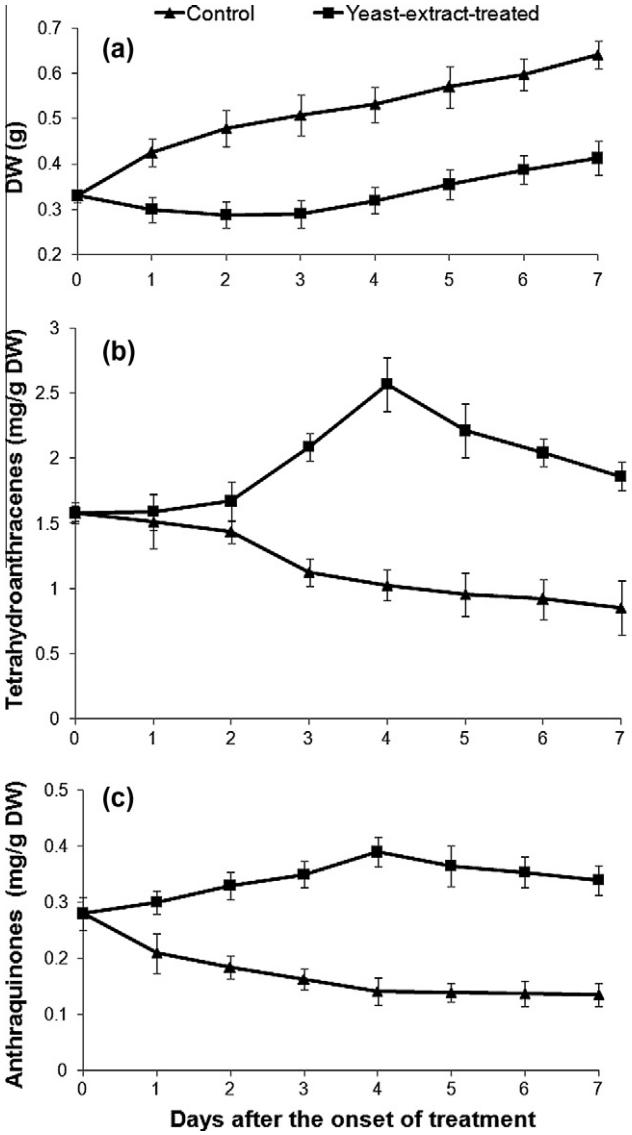

Fig. 6. Changes in dry weight (a) and the tetrahydroanthracene (b) and anthraquinone levels (c) in *C. bicapsularis* cell cultures after treatment with either yeast extract or water only (control). Data are mean values ± SD of three independent experiments.

the enzymatic products needed to be isolated from large-scale incubations, however, cell-free extracts used for the enzyme assays already contained, although buffer-based, traces of the respective compounds due to biosynthesis in the cultivated cells. Thus,

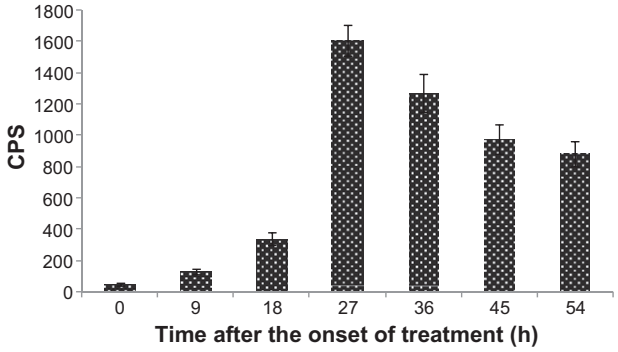

Fig. 7. Changes in OKS activity in *C. bicapsularis* cell cultures after the addition of yeast extract. Data are mean values ± SD of two independent experiments.

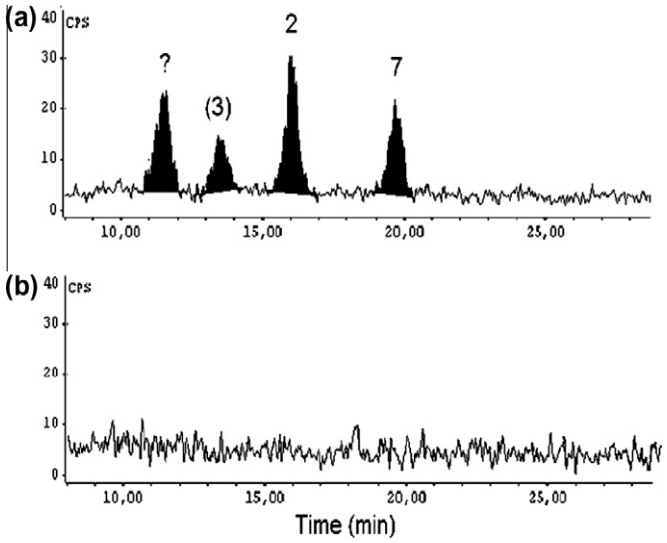

Fig. 8. Radiodetector-coupled HPLC analysis of OKS assays containing cell-free extract and [2-^{14}C]malonyl-CoA (a). Heat-denaturation led to enzyme inactivation (b).

isolated compounds would have been mixtures of products formed in intact cells and cell-free extracts. All efforts to completely remove the constituents from cell-free extracts while retaining OKS activity failed. For example, crude extracts were repeatedly passed through gel chromatography columns and adsorbent matrices.

2.5. Incubation of cell-free extracts with [U-$^{13}C_3$]malonyl-CoA

As an alternative approach to confirm *in vitro* anthranoid production, cell-free extracts were incubated with [U-$^{13}C_3$]malonyl-CoA in the presence of acetyl-CoA. Subsequently, the enzymatic products R_t 15.9 and 19.6 were isolated from large-scale incubations and analyzed for ^{13}C incorporation by ESI-MS/MS in comparison to the corresponding unlabeled compounds. The mass spectra of the two compounds from the ^{13}C experiment contained additional signals at higher *m/z* values. The *m/z* values of the pseudo molecular ions, $[M+H]^+$ for torosachrysone (**2**) and $[M-H]^-$ for emodin anthrone (**7**), were increased by 13 u each, which was due to the incorporation of 7 C_2 units from [U-$^{13}C_3$]malonyl-CoA followed by a decarboxylative loss of one ^{13}C atom (Fig. 1). The proposed ESI-MS/MS fragmentations of unlabeled torosachrysone (**2**; $[M+H]^+$ in ESI positive mode) and emodin anthrone (**7**; $[M-H]^-$ in ESI negative mode) are summarized in Fig. 9a and b, respectively. The proposed structures of the fragment ions include marks (black dots), which highlight labeled carbon atoms. For both enzymatic products, all major fragment ions were confirmed by incubation of cell-free extract with [U-$^{13}C_3$]malonyl-CoA, yielding shifts by the indicated number of ^{13}C atoms. For example, the fragment 247 u of torosachrysone (**2**; Fig. 9a, base peak) showed a peak at 258 u in the ^{13}C experiment. This shift by 11 u is indicated by the same number of black dots in the proposed structure of the fragment ion 247 u.

2.6. Sequential formation of enzymatic products

Both torosachrysone (**2**) and emodin anthrone (**7**) are likely to arise biosynthetically from atrochrysone (**3**) via *O*-methylation and loss of water, respectively (Fig. 1). Thus, atrochrysone (**3**) was supposed to be one of the other two enzymatic products found in radiodetector-coupled HPLC analysis (Fig. 8a; R_t 11.5 and 13.4). However, atrochrysone (**3**) was not available as an authentic reference compound and efforts to isolate the two yet unidentified enzymatic products in sufficient quantities for structural analysis failed due to the low amounts present. Since atrochrysone (**3**) is formed in the postulated pathway (Fig. 1) prior to biosynthesis of torosachrysone (**2**) and emodin anthrone (**7**), the standard incubation time (1 h) for enzyme assays containing [2-^{14}C]malonyl-CoA was stepwise reduced. A 5-min-incubation primarily yielded the peak 2 compound, suggesting that this enzymatic product may be atrochrysone (**3**, Fig. 10a). After 15 and 30 min, formation of the peak 1 compound and emodin anthrone (**7**) increased (Fig. 10b and c). After 1 h incubation (Fig. 10d), torosachrysone (**2**) appeared as the fourth enzymatic product at the expense of atrochrysone (**3**), indicating that *O*-methylation of atrochrysone (**3**) is a delayed reaction.

3. Discussion

Cell-free extracts from yeast-extract-treated *C. bicapsularis* cell cultures formed THAs from acetyl- and malonyl-CoAs as starter and extender substrates, respectively. In contrast, recombinant OKSs from *A. arborescens* and *H. perforatum* failed to produce anthranoids but released aromatic octaketides called SEK4 and SEK4b (Abe et al., 2005a; Karppinen et al., 2008). Thus, although catalyzing the correct number of decarboxylative condensations with malonyl-CoA to give a linear octaketide, both OKSs were incapable of subjecting the intermediate to appropriate folding and cyclization. Formation of the shunt products SEK4 and SEK4b by the affinity-purified OKSs may be attributed to the lack of a yet unknown factor or tailoring enzyme that may interact with OKS (Abe et al., 2005a; Karppinen et al., 2008). Intact *A. arborescens* and *H. perforatum* plants do not contain detectable amounts of SEK4 and SEK4b or derivatives thereof. Recently, two additional OKS cDNAs have been isolated from *A. arborescens* (Mizuuchi et al., 2009). The encoded enzymes functionally resembled the above OKSs, i.e. they released SEK4 and SEK4b only. All three *A. arborescens* OKSs were active with malonyl-CoA as sole substrate by generating the starter unit via decarboxylation of malonyl-CoA, whereas *H. perforatum* OKS and the *C. bicapsularis* enzyme relied on acetyl-CoA. SEK4 and SEK4b were also observed as shunt products of the minimal type II PKS of the *Streptomyces coelicolor* actinorhodin biosynthetic gene cluster, when incubated in the absence of downstream tailoring enzymes (Fu et al., 1994a,b). The catalytic specificity can be influenced by aromatases and cyclases, which interact with the minimal type II PKS and stabilize the highly reactive poly-β-keto chain. The finding that cell-free extracts from yeast-extract-treated *C. bicapsularis* cell cultures were capable of forming anthranoids indicated that a potentially required tailoring enzyme or factor retained its activity upon extraction and appropriately

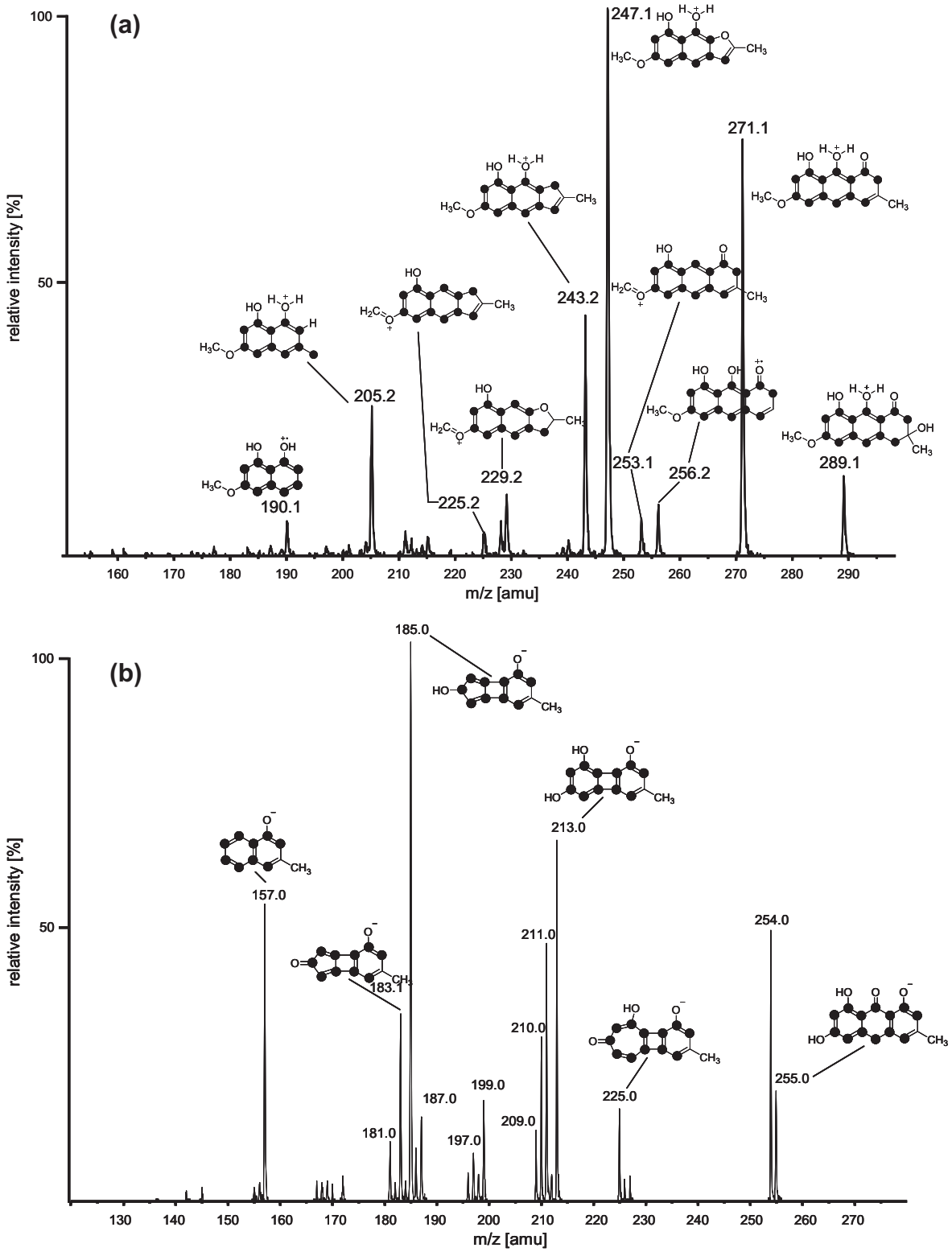

Fig. 9. Proposed fragmentation patterns of (a) torosachrysone (**2**) and (b) emodin anthrone (**7**). Black dots highlight incorporated ^{13}C atoms verified by incubation with [U-$^{13}C_3$]malonyl-CoA.

interacted with OKS in the enzyme assays. Very recently, a polyketide cyclase which functions in concert with a tetraketide synthase has been identified in *C. sativa* (Gagne et al., 2012).

Aloe arborescens OKS shared 91% amino acid sequence identity with *A. arborescens* pentaketide chromone synthase (PCS) producing 5,7-dihydroxy-2-methylchromone from five malonyl-CoAs, and the two enzymes were functionally interconvertible by a single amino acid substitution (Abe et al., 2005a,b; Morita et al., 2007a). The PCS M207G mutant produced a 1:4 mixture of SEK4 and SEK4b rather than 5,7-dihydroxy-2-methylchromone, whereas

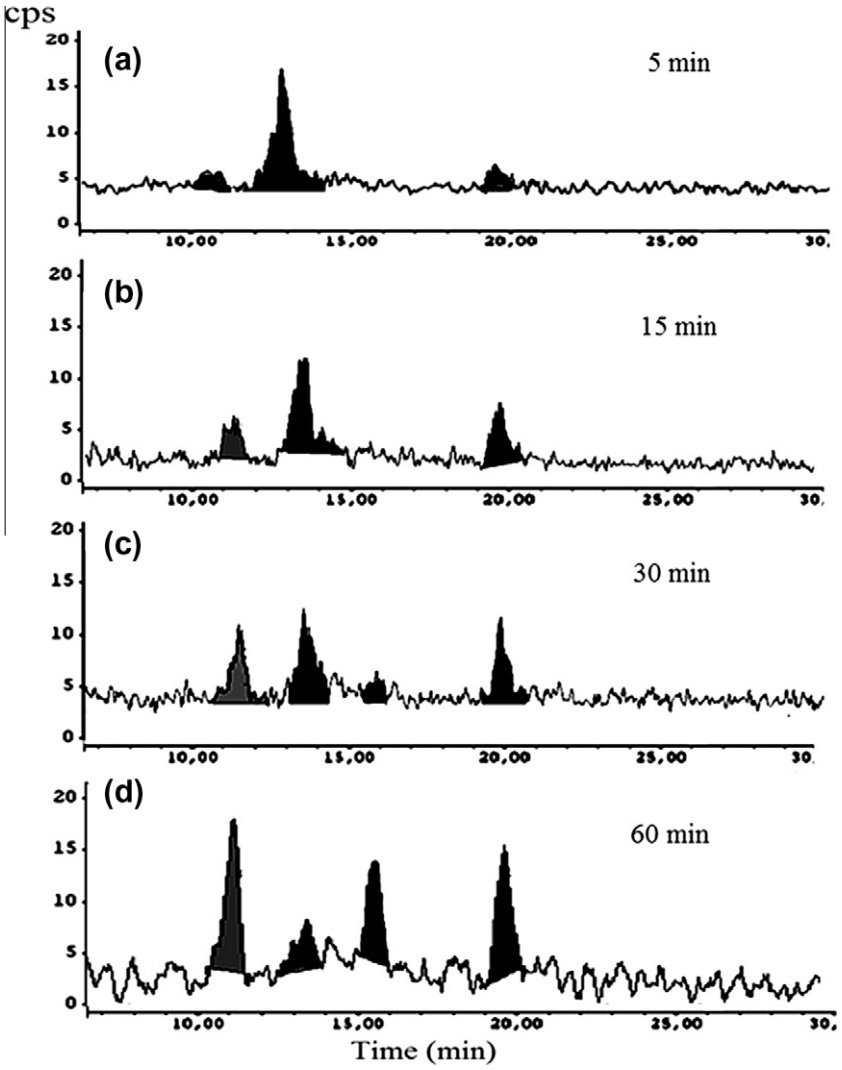

Fig. 10. Radiodetecter-coupled HPLC analysis of OKS assays incubated between 5 and 60 min.

the OKS G207M mutant produced an isomeric pentaketide, 2,7-dihydroxy-5-methylchromone, instead of SEK4 and SEK4b. The crystal structure of *A. arborescens* OKS was resolved and the structure-based N222G mutant, whose polyketide chain elongation tunnel was expanded at the bottom by a large-to-small substitution, formed a benzophenone derivative as a single product (Morita et al., 2007b; Shi et al., 2009). This C_{20} compound, which resulted from the iterative condensation of ten molecules of malonyl-CoA, is the longest polyketide chain produced by a structurally simple type III PKS.

Incorrect folding and cyclization of polyketide intermediates also occurred with recombinant hexaketide synthases from *Plumbago indica* and *Drosophyllum lusitanicum*, which formed hexaketide pyrones rather than the expected hexaketide naphthoquinones (Springob et al., 2007; Jindaprasert et al., 2008). Intact plants exclusively contained the naphthoquinones, such as plumbagin. Both recombinant enzymes correctly catalyzed extension of the acetyl starter unit with five malonyl-CoAs to produce a linear hexaketide intermediate which, however, underwent false folding and cyclization into a α-pyrone as derailment product. Again, the absence of an additional cofactor, such as a cyclase-like cofactor, was made responsible for the unexpected product profile (Springob et al., 2007; Jindaprasert et al., 2008). The failure of producing a naphthoquinone might also be due to the lack of a ketoreductase, which interacts in the polyketide elongation process, thereby preventing nonspecific cyclization. Notably, in contrast to *C. bicapsularis* extracts, cell-free preparations from *D. lusitanicum* cultures did not catalyze formation of the expected products, namely naphthalenes, in *in vitro* PKS assays (Jindaprasert et al., 2008).

Cassia bicapsularis cell cultures primarily accumulated THAs, which were undetectable in field-grown plants and *in vitro*-grown shoot cultures (Abdel-Rahman, 2006). Conversely, intact plants and shoot cultures contained AQs, which were minor constituents of cell cultures. Dehydration of THAs leads to formation of anthrones, which then are oxidized to AQs (Takahashi et al., 1976; Kitanaka and Takido, 1984; Delle Monache et al., 1991). Obviously, these reactions proceed to only low extents in cell cultures, resulting in accumulation of THAs. Formation of THAs was previously observed with hairy root and cell suspension cultures of various *Cassia* species (Noguchi and Sankawa, 1982; Kitanaka et al., 1985; Delle Monache et al., 1991; Ko et al., 1995). The major THA that was accumulated in yeast-extract-treated *C. bicapsularis* cell cultures was germichrysone, formation of which requires a reduction step involving NADPH (Fig. 1). A similar reaction proceeds in Fabaceae in the biosynthesis of 6′-deoxychalcones, which result from the coupled catalytic action of chalcone synthase (CHS) and NADPH-dependent chalcone reductase (CHR; Bomati et al.,

2005). CHR activity was first demonstrated in crude extracts from *Glycyrrhiza echinata* and a CHR cDNA was cloned from *Glycine max* (Ayabe et al., 1988; Welle et al., 1991). Finally, the crystal structure of *Medicago sativa* CHR was resolved and provided evidence for that the cyclic and prochiral coumaryl-trione is the most likely substrate for CHR (Bomati et al., 2005). Reduction of a carbonyl of the fully extended and CHS-cyclized trione by CHR needs only a single CHS/CHR diffusion event, followed by spontaneous aromatization in solution. In contrast, CHR reduction of a linear polyketide-CoA intermediate, e.g. at the tetraketide stage (Oguro et al., 2004), would require passive diffusion between the CHS and CHR active sites.

A minor THA present in *C. bicapsularis* cell cultures was 7-methyltorosachrysone, which may arise from *C*-methylation of torosachrysone. Genes for *C*-methyltransferases, which transfer the alkyl group from *S*-adenosyl-methionine (SAM) to a ring system, were isolated from *Streptomyces resistomycificus*, *Streptomyces spheroides*, and *Streptomyces rishiriensis* (Pacholec et al., 2005; Ishida et al., 2007). Alternatively, the alkyl side group of 7-methyltorosachrysone may originate from the use of methylmalonyl-CoA as extender substrate in octaketide formation. *Pinus strobus* contains *C*-methylated flavonoids and two PKSs appear to contribute to their biosynthesis by exchanging polyketide intermediates (Schröder et al., 1998). While one PKS uses malonyl-CoA for carrying out the first and third condensation reactions, the other PKS prefers methylmalonyl-CoA for catalyzing only the second condensation reaction, thereby converting the diketide intermediate into a methylated triketide intermediate. Some PKSs from bacteria were even reported to use ethylmalonyl-CoA as extender substrate (Stassi et al., 1998; Song et al., 2006).

4. Conclusions

Biosynthesis of medicinally valuable anthranoids *via* the polyketide pathway is poorly understood. Unlike recombinant OKSs, cell-free extracts from yeast-extract-treated *C. bicapsularis* cell cultures were capable of forming the anthranoid scaffold *in vitro*. Thus, the cultivated cells provide an interesting system to gain deeper insight into anthranoid biosynthesis. Co-incubation of cell-free extracts with either heterologous OKSs or the homologous enzyme, once a cDNA has been cloned, may lead to detection and subsequent identification of a component that interacts with OKS for correct folding and cyclization of the linear octaketide intermediate.

5. Experimental

5.1. Chemicals

Chemicals were of analytical grade and purchased from Fluka (Buchs, Switzerland) and Sigma–Aldrich (Taufkirchen, Germany). Solvents were of HPLC grade. [2-^{14}C]Malonyl-CoA (55.2 mCi/mmol) was obtained from PerkinElmer NEN (Rodgau, Germany). [U-$^{13}C_3$]Malonyl-CoA (lithium salt) was purchased from Sigma–Aldrich. Emodin anthrone was synthesized from emodin, as described by Falk et al. (1993).

5.2. Establishment of C. bicapsularis cell cultures

Cassia bicapsularis L. (Fabaceae, subfamily Caesalpinioideae) was cultivated in the experimental station of the Pharmacognosy Department of the Faculty of Pharmacy, Assiut University (Assiut, Egypt) and kindly identified by Dr. Trease Demian, Orman Botanical Garden, Giza, Egypt. Seeds of the plant were collected in Mai 2009 and aseptically germinated in petri dishes containing solidified basic MS medium (Murashige and Skoog, 1962). Resulting plantlets were cultivated in a growth chamber at 25 ± 2 °C under a 16 h photoperiod and leaves of 8-week-old plantlets were used as explants for the induction of callus cultures on solidified MS medium either under a 16 h photoperiod or in the dark. Finally, callus material was transferred to 250 ml Erlenmeyer flasks containing 50 ml liquid MS medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l kinetin. The resulting cell cultures were grown on a rotary shaker at 120 rpm at 25 °C in the dark. Cultivated cells (3 g) were transferred to fresh medium every 3 weeks.

5.3. Preparation and use of elicitors

Yeast extract (3 g) was dissolved in 10 ml distilled water, filter-sterilized, and stored at 4 °C. A stock solution of chitosan prepared by dissolving 125 mg in 100 ml acetic acid (1%, v/v) and adjusting the pH to 5.5 was autoclaved and kept at 4 °C (Pitta-Alvarez and Giulietti, 1999). Methyl jasmonate was dissolved in ethanol (95%, v/v) to give a 50 mM solution (freshly prepared). An aqueous stock solution of sodium chloride (25%, w/v) was autoclaved and stored at 4 °C. Elicitor solutions were commonly added to 7-day-old cell cultures and the resulting effects were studied after additional 4 days. A single stock culture grown in a 500 ml Erlenmeyer flask in the dark at 25 °C and 120 rpm was used as an inoculum for all experimental flasks. Control cell cultures were only treated with either distilled water (2 ml) or ethanol (400 µl).

5.4. Extraction and isolation of constituents

Cultivated cells (500 g) were dried at 50 °C in an oven, giving approx. 46 g dry wt. The constituents were exhaustively extracted with methanol, the combined extract was evaporated to dryness at 40 °C, and the residue was dissolved in the smallest possible volume of water and exhaustively extracted with dichloromethane. After solvent evaporation, the residue of the dichloromethane phase was subjected to silica gel (Merck, Darmstadt, Germany) column chromatography (20 × 2.5 cm; 25 g) using an *n*-hexane–ethyl acetate gradient for elution of three anthraquinones (2–3 mg each). Identification of these compounds was based on their UV, mass, and NMR spectra in comparison with authentic samples. The aqueous phase was passed through a column of an adsorbent resin (Amberlite XAD-16, 30 × 2.5 cm, 30 g; Serva, Heidelberg, Germany). Phenolic compounds were bound to the column matrix, whereas sugars were eluted with water (Ferreres et al., 1994). The column was washed with acidified water (pH 2) and distilled water and the total phenolics fraction was eluted with methanol. The residue after concentration to dryness was dissolved in methanol and separated by preparative HPLC (Elite LaChrom equipped with an L-2455 photodiode array detector) on a Hyperclone C_{18} column (250 × 10 mm i.d., 5 µm; Phenomenex, Aschaffenburg, Germany; sample injection volume 100 µl). The mobile phase consisted of 0.1% formic acid (A) and methanol (B). The gradient was 55% B for 2 min, 55–90% B in 15 min, and 90–100% B in 3 min at a flow rate of 5.0 ml/min. Corresponding peaks of multiple runs were collected, combined, and concentrated. Three tetrahydroanthracenes (**1**, **2**, **4**) were obtained and identified according to their UV, MS, and NMR data in comparison to the literature. Data analysis was carried out using the EZChrom Elite software on Windows XP.

5.5. Quantitative HPLC analysis of tetrahydroanthracenes

Cultivated cells were harvested by suction filtration, dried at 50 °C in an oven and powdered. An aliquot of the powder (0.2 g) was exhaustively macerated with methanol (2 ml each). The com-

bined extract was filtered and the solvent was evaporated under reduced pressure at 40 °C. The residue was dissolved in 5 ml methanol and analyzed by HPLC (Elite LaChrom equipped with an L-2455 photodiode array detector) on a Hyperclone C_{18} column (150 × 3.2 mm i.d., 3 μm; Phenomenex). The mobile phase consisted of 0.1% formic acid (A) and methanol (B) and the gradient was 55% B for 2 min, 55–90% B in 15 min, and 90–100% B in 3 min. The flow rate was 0.3 ml/min, the sample injection volume 20 μl, and the detection wavelength 270 nm. A calibration curve was established using germichrysone (**1**).

5.6. Quantitative spectrophotometric analysis of total anthraquinones

The total AQ content was measured, as described by Sakulpanich and Gritsanapan (2008). Cultivated cells were dried and homogenized. An aliquot of the powder (0.2 g) was exhaustively macerated with ether (2 ml each). The combined extract was filtered and the solvent was evaporated under reduced pressure at 40 °C. The residue was dissolved in 2 ml magnesium acetate in methanol (0.5%, w/v), yielding a red solution. Absorbance was measured at 515 nm using an Ultrospect 1000 spectrophotometer. A calibration curve was prepared using chrysophanol (**8**).

5.7. Conversion of tetrahydroanthracenes to anthraquinones

THAs **1** and **2** were dissolved in 5% NaOH and kept at room temperature for 3 h. The solution was neutralized with 1% HCl, diluted with water and extracted with ethyl acetate. After solvent evaporation, the residue was dissolved in methanol and subjected to HPLC analysis using the conditions described under 5.5. The products were identified as physcion and chrysophanol (Kitanaka and Takido, 1984; Kitanaka et al., 1985).

5.8. Synthesis of emodin anthrone

Emodin anthrone was prepared, as described by Falk et al. (1993). Emodin (40 mg) was dissolved in 2.8 ml acetic acid and a solution consisting of 0.112 g $SnCl_2{\cdot}2H_2O$ in 0.32 ml HCl_{conc}. After refluxing for 5 h and cooling to room temperature, the precipitate was filtered off, dried under vacuum, and purified by silica gel column chromatography using petroleum ether:ethyl acetate 7:3 (v/v) as solvent.

5.9. NMR

NMR spectra (600 MHz ^1H and 151 MHz ^{13}C) of $CDCl_3$ solutions of compounds **1**, **2**, and **4** were obtained on a Bruker Avance II 600 spectrometer with a 5 mm TCI CryoProbe at a sample temperature of 20 °C. Internal chemical shift references were tetramethylsilane (δ_H = 0.00 ppm) and the solvent (δ_H = 7.25 ppm and δ_C = 77.01 ppm). The ^1H and ^{13}C chemical shifts of **2** were assigned by 1D (DEPT-135) and 2D (COSY, NOESY, HSQC, HMBC) NMR techniques, those of **4** by comparison with **2**. The ^{13}C assignments of the aromatic carbon nuclei of **1** were derived from **2** by subtracting the corresponding substituent chemical shifts (SCS) of the OMe group in 2-methoxynaphthalene (Ernst, 1975) and adding the SCS of the Me group in 2-methylnaphthalene (Ernst and Schulz, 1992).

5.10. Electrospray ionization-mass spectrometry (ESI-MS/MS)

THAs **1**, **2**, **4** and **7** were analyzed via direct infusion. Samples were introduced to an electrospray ion source (ESI; Turbo V, ABSciex, Ontario, Canada) using a Hamilton syringe pump and a flow rate of 5–10 μl/min coupled to a 3200 QTRAP mass spectrometer (ABSciex). Tuning and ionization optimization of the instrument were achieved using the corresponding reference compounds. MS/MS-experiments for **1**, **2** and **4** were carried out in the EPI^+ mode (enhanced product ion scan, positive mode) of the instrument. Analysis of emodin anthrone (**7**) was carried out in the EPI^- mode of the instrument. Data acquisition and processing were performed by the Analyst Software version 1.4.2 (ABSciex, Ontario, Canada).

5.11. Preparation of cell-free extracts

Yeast-extract-treated cell cultures were extracted, as described by Klingauf et al. (2005). Cells (5 g) were collected by suction filtration and mixed with 10% (w/w) Polyclar AT (Serva) and a spatula of seasand. After homogenization at 0–4 °C in 3 ml 100 mM HEPES buffer pH 7.0 containing 1 mM dithiothreitol (DTT), the homogenate was centrifuged at 9000 rpm for 15 min at 4 °C. The supernatant was used for carrying out enzyme assays.

5.12. Octaketide synthase (OKS) assay

The standard assay (150 μl) contained 0.1 M HEPES buffer pH 7.0, 16 μM acetyl-CoA as a starter substrate, 60 μM malonyl-CoA as an extender substrate, 16 μM [2-^{14}C]malonyl-CoA (55.2 mCi/mmol), and approx. 100 μg protein. The mixture was incubated at 37 °C for 60 min. The reaction was stopped by addition of 15 μl HCl (10%). The assay was extracted twice with 150 μl ethyl acetate and centrifuged at 13,000 rpm for 10 min. The combined organic phase was dried under vacuum and the residue was dissolved in 50 μl methanol. Analysis of enzymatic products was performed by HPLC (Agilent 1200 series) coupled with a radiodetector (RAMONA star 2; Raytest, Straubenhardt, Germany). Data analysis was performed using Gina star 4.06 software on Windows XP. Separation was achieved at 25 °C on a Lichrospher C_{18} column (250 × 4.6 mm i.d., 5 μm; WICOM, Heppenheim, Germany). The mobile phase consisted of 0.1% formic acid (A) and methanol (B) using the following gradient: 55% B for 2 min, 55–90% B for 15 min, and 90–100% B for 3 min at a flow rate of 1 ml/min. The sample injection volume was 30 μl. Large-scale assays (5 ml) containing [U-$^{13}C_3$]malonyl-CoA (2 mM) consisted of 3 mg protein, 0.5 mM acetyl-CoA, and 0.1 M HEPES buffer pH 7.0. After incubation at 37 °C for 60 min, the reaction was stopped by addition of 500 μl HCl (10%) and extracted three times with 5 ml ethyl acetate each. After the combined organic phase had been dried under vacuum, the residue was dissolved in methanol and analyzed by HPLC. The peaks with R_t 15.9 and 19.6 min were collected and analyzed by ESI-MS/MS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.01.001.

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