Different Polyketide Folding Modes Converge to an Identical Molecular Architecture

Abstract. Metabolic diversity is being studied intensely by evolutionary biologists, but so far there has been no comparison of biosynthetic pathways leading to a particular secondary metabolite in both prokaryotes and eukaryotes. We have detected the bioactive anthraquinone chrysophanol, which serves as a chemical defence in diverse eukaryotic organisms, in a bacterial *Nocardia* strain, thereby permitting the first comparative biosynthetic study. Two basic modes of folding a polyketide chain to fused-ring aromatic structures have so far been described (Thomas 2001): modes F (referring to fungi) and mode S (from *Streptomyces*). We have demonstrated that in eukaryotes (fungi, higher plants and insects) chrysophanol is formed *via* the folding mode F. In actinomycetes, by contrast, the cyclisation follows mode S. Thus, chrysophanol is the first polyketide synthase product that is built up by more than one polyketide folding mode.

Polyketide synthases (PKSs) convert simple building blocks such as acetate and/ or malonate into a huge variety of diverse acetogenic natural products. For the regioselective cyclisation of the reactive intermediate polyketide chains (in this context referred to as "folding"), actinomycetes and fungi have developed two different synthetic "strategies": folding modes S and F, respectively. Initial reports show that plants can cyclise polyketides *via* the mode F strategy (Bringmann et al. 1998; Bringmann et al. 2000). There is evidence of these folding mechanisms for different polyketides in diverse groups of organisms. However, diverse folding modes might still lead to identical basic structures (for example, anthraquinones). It is not known whether the biosynthesis of a specific polyketide is restricted to a particular folding mode. One reason for this is that as yet, no known polyketide is produced in sufficient quantities by both eukaryotic organisms (such as fungi, plants and insects) and prokaryotic organisms (such as actinomycetes). The aim of the present study is to determine whether a polyketide present in very different organisms is biosynthesised *via* one common pathway or in an organism-specific manner.

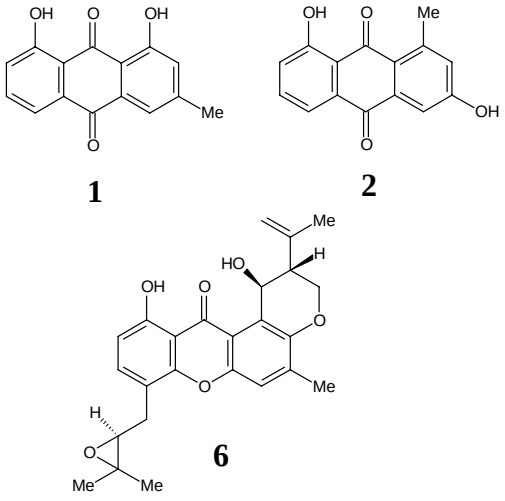

Figure 1. Chemical structures of the acetogenic metabolites chrysophanol (**1**), aloesaponarin II (**2**) and tajixanthone (**6**).

The widely distributed polyketidic anthraquinone chrysophanol **(1)** (Fig. 1) (Thomson 1987), has been found in several eukaryotic organisms: in fungi and plants (potential F-type organisms) and also in insects (Howard et al. 1982; Hilker and Schulz 1991; Kunze et al. 1996) and lichens (Mishchenko et al. 1980; Krivoshchekova et al. 1983). More recently, **1** has also been detected in small amounts in a *Streptomyces* strain (Fotso et al. 2003). Given its wide distribution in nature and its structure, **1** represents a prototype of a substance that might, in principle, be synthesised through different folding modes: folding

modes F and S are thought to be responsible for its formation in fungi and streptomycetes, respectively (Thomas 2001). However, the presence of only small amounts of **1** in streptomycetes did not allow detailed biosynthetic investigations by feeding labelled precursors, whereas the biosynthesis of other polyketides has been studied intensely in *Streptomyces* (Funa et al. 1999).

Besides chrysophanol (**1**), the isomeric anthraquinone aloesaponarin II (**2**) has also been assumed – but not proven – to be a potential candidate that might be synthesised from differing biosynthetic polyketidic origins, owing to its occurrence both, in plants (Yagi et al. 1974) and as a shunt product in recombinant strains of *Streptomyces galilaeus* (ATCC 31133 and ATCC 31671) transformed using DNA carrying the *actI*, *actIII*, *actIV* and *actVII* loci (Bartel et al. 1990). However, **2** has not been reported in fungi.

Our discovery that a *Nocardia* strain (that is, a prokaryote) produces **1** as a major metabolite allowed us to undertake the first comparative investigation of its biosynthetic formation in prokaryotes (actinomycetes) and eukaryotes (plants, fungi, insects).

For the biosynthetic origin of the tricyclic ring system of **1** from eight molecules of acetyl coenzyme A (**3**) (Fig. 2), four different folding modes of a hypothetical intermediate linear polyketide precursor **4** can be imagined: besides modes F (two intact C_2 units in the first ring of **1**) and S (three C_2 units), the additional, never-before-discussed modes S' and F' are conceivable.

As the molecule of **1** is always identical from a merely chemical point of view, regardless of whether it is derived from actinomycetes, fungi, plants or insects, different possible biogenetic origins cannot be determined directly from the structure of the molecule as such. This can, however, be analysed by feeding experiments with [1,2-^{13}C]acetate, as this can be expected to lead, *via* the different presumed intermediates (for example, *via* **5a**, 5**b**, 5**c** or 5**d**), to the production of different isotopomers.

The biosynthesis of chrysophanol (**1**) has been studied in higher plants (*Rumex alpinus*, *R. obtusifolius* and *Rhamnus frangula*) using radiolabelled ^{14}C-acetate (Leistner and Zenk 1969; Leistner 1971; Fairbairn and Muhtadi 1972) and indirectly in the fungus *Aspergillus variecolor* (Ahmed et al. 1987) during studies on the biogenesis of tajixanthone (**6**)

(Ahmed et al. 1987). In feeding experiments with [1,2-$^{13}C_2$]acetate, **6** gives an isotope pattern consistent with its origin *via* an F-type folding; [*methyl*-$^{2}H_3$]chrysophanol, in turn, is a specific precursor to **6**. However, direct experiments with doubly ^{13}C-labelled acetate have not been performed on **1**; hence, localisation of the intact acetate units has not been possible.

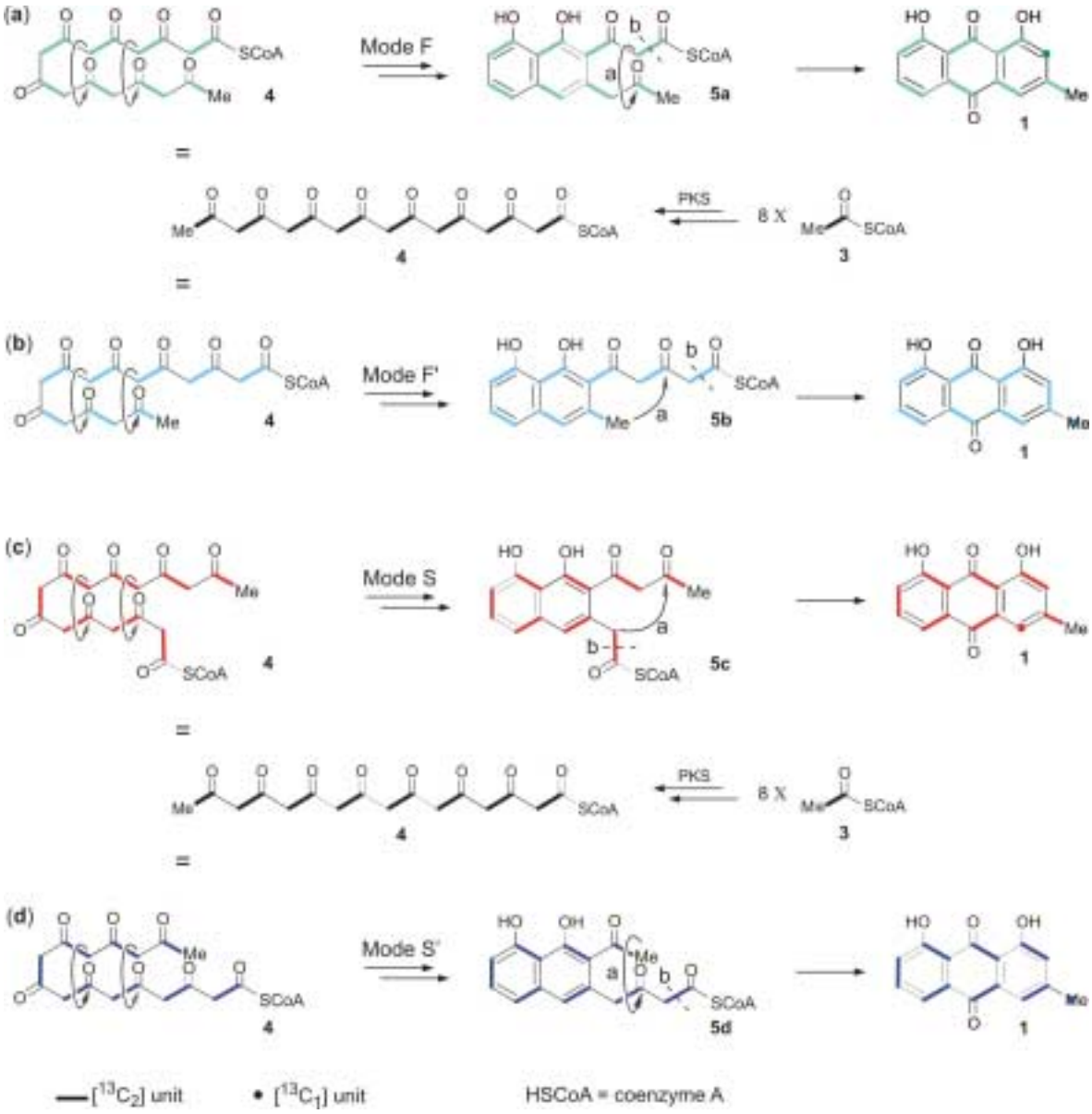

Figure 2. The four biosynthetic folding modes that might lead to chrysophanol (**1**) in nature, with their joint (**4**) and individually different (**5a**-**d**) hypothetical intermediates and with the expected characteristic ^{13}C labelling patterns for **1**. (**a**), mode F folding. (**b**), prospective mode F' folding. (**c**), mode S folding and (**d**), conceivable mode S' folding. The bold coloured lines indicate the positions of incorporated intact [1,2-$^{13}C_2$]acetate units. Aldol-type cyclisation reactions are illustrated by bent arrows, of which those leading to the third aromatic ring are denoted by "a", while the decarboxylation is indicated by "b". Note that –for reasons of clarity– the structure of **5** disregards the expected high degree of enolisation and, in particular, its presumable reduction at C9.

To determine the biosynthesis of **1** in a fungus, we treated a culture of the known chrysophanol producer *Drechslera catenaria* (also known as *Helminthosporium catenarium*) (Van Eijk 1974) with [1,2-$^{13}C_2$]acetate for four weeks. We then took 2D INADEQUATE NMR measurements on the isolated **1** (Bax et al. 1988). These showed interactions between C1a and C1, C3 and CH_3 at position 3, C4a and C4, C5a and C10, C5 and C6 and between C7 and C8 thereby clearly establishing, as expected, the mode F folding characteristic of a fungus (Fig. 3). We could not detect connectivity between C8a and C9, but we observed the C9 signal at the correct double quantum frequency for C9 and C8a correlation. The identical coupling constants of C9 and C8a (55 Hz) corroborated these assignments. We independently confirmed that C_2 fragment using a SELINQUATE (Berger 1988) experiment with irradiation on C9, clearly demonstrating this last missing connection. We did not identify the single C atom (that is, the residual methyl-C of acetate remaining after decarboxylation), C2, from a significant enhancement of the singlet in the ^{13}C NMR spectrum, but rather indirectly, from the complete pairwise labelling of all the other C atoms and from its appearance as a singlet in the ^{13}C NMR spectrum.

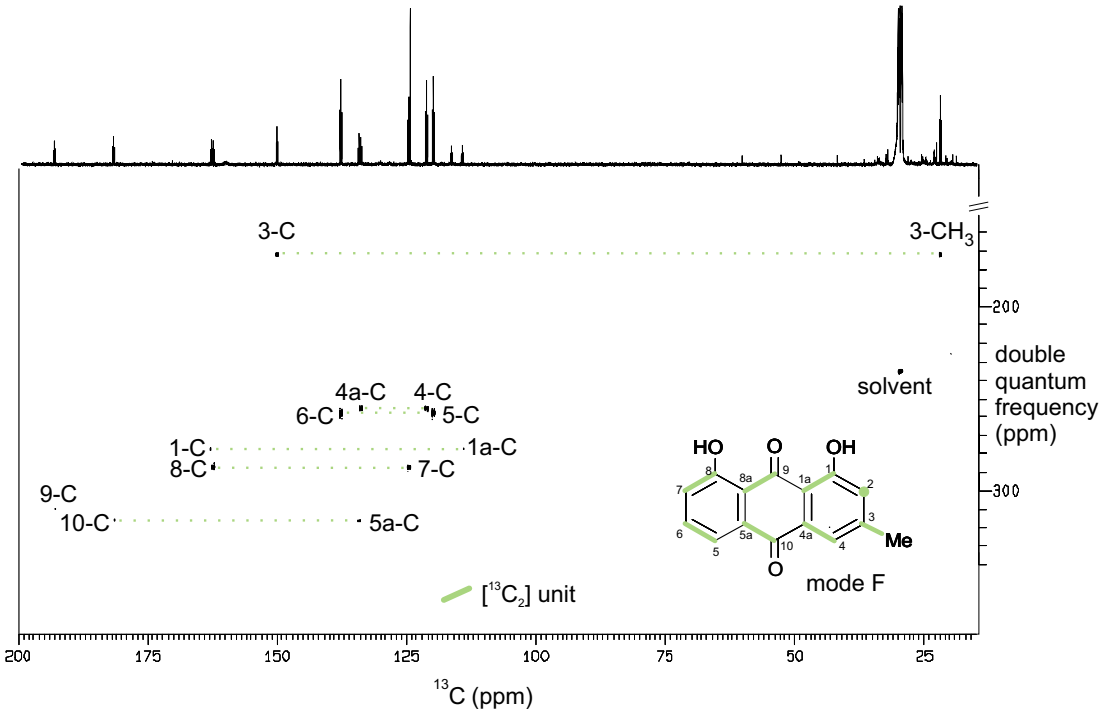

Figure 3. 2D INADEQUATE NMR spectrum of **1** isolated from the filamentous fungus *Drechslera catenaria* after feeding with sodium [1,2-$^{13}C_2$]acetate; the labelling pattern with its pairwise ^{13}C-^{13}C correlations of incorporated $^{13}C_2$ units (only two in the initially formed ring) indicates mode F folding; identical incorporation patterns were observed with liquid tissue cultures of the higher plant, *Kniphofia uvaria* and the leaf beetle *Galeruca tanaceti* (not shown).

We analysed the biosynthesis of **1** in a plant species by treating liquid callus cultures of the torch lily *Kniphofia uvaria*, cultivated as described earlier (Bringmann et al. 2002), with labelled acetate in a similar way. 2D INADEQUATE measurements gave results comparable to those for **1** derived from the fungal source described above, thereby proving the existence of F-mode folding in higher plants. Once again, we clearly identified this folding mode, although we detected fewer C-C connectivities owing to the significantly smaller amounts of the compound isolated from this biological system. NMR measurements using the more sensitive cryoprobe technique (Styles and Stoffe 1984) showed four of the seven expected connectivities; namely, the ones between C-methyl and C3, C4a and C4, C5 and C6 and between C7 and C8.

The study of the folding mode of chrysophanol (**1**) in a bacterial species was facilitated by the large amounts of the polyketide produced by the *Nocardia* strain Acta 1057, as discovered in the course of our HPLC diode array screening of actinomycetes isolated from soils collected from various sites in Northumberland, UK. The mycelium extract of strain Acta 1057 yielded a peak with the same retention time and an identical UV-visible spectrum to that of **1** (Fiedler 1993). We cultivated the strain in a 1-l stirred tank fermenter in a complex medium; we obtained maximal chrysophanol production of up to 30 mg/ l after 144 h. Feeding with [1,2-$^{13}C_2$]acetate during 85 and 105 h of fermentation led to the isolation of **1** that showed an isotopic pattern (Fig. 4) with a folding substantially different from the type F identified for the two organisms mentioned above.

A more detailed analysis of the compound uncovered the presence of a mode S folding (as postulated earlier (Thomas 2001); see above). Chemically this is the most probable, as it allows the formation of the "last" (that is, the methyl-bearing) ring by an aldol reaction of the doubly activated methylene followed by decarboxylation (Fig. 2). In addition to this dominant labelling pattern, there were also hints at the chemically less feasible mode S' as suggested by weak interactions between C3 and C4 (marked in blue), which, however, might also be due to interacetate couplings.

We studied chrysophanol biosynthesis in an insect species using the leaf beetle *Galeruca tanaceti* (Chrysomelidae), which is known to contain anthraquinones at different developmental stages (Hilker et al. 1992). This beetle does not sequester **1** from its host plant (Hilker et al. 1992); hence, the polyketide might be produced either by the insect or

by constituent endosymbiotic organisms. The production of **1** even upon treatment of the beetles with fungicides or antibiotics suggests that it is formed by the insect. After feeding *G. tanaceti* larvae with labelled sodium [1,2-$^{13}C_2$]acetate, NMR analysis of larval **1** uncovered the presence of mode F folding as evident from six out of the seven expected connections in the 2D INADEQUATE spectrum. Thus, only the coupling between C1 and C1a was undetectable; we observed the C1a signal at the expected double quantum frequency. These results clearly indicate that an eukaryotic PKS catalyses the formation of **1** in this species, which excludes an endosymbiotic prokaryotic producer of anthraquinones in the beetle.

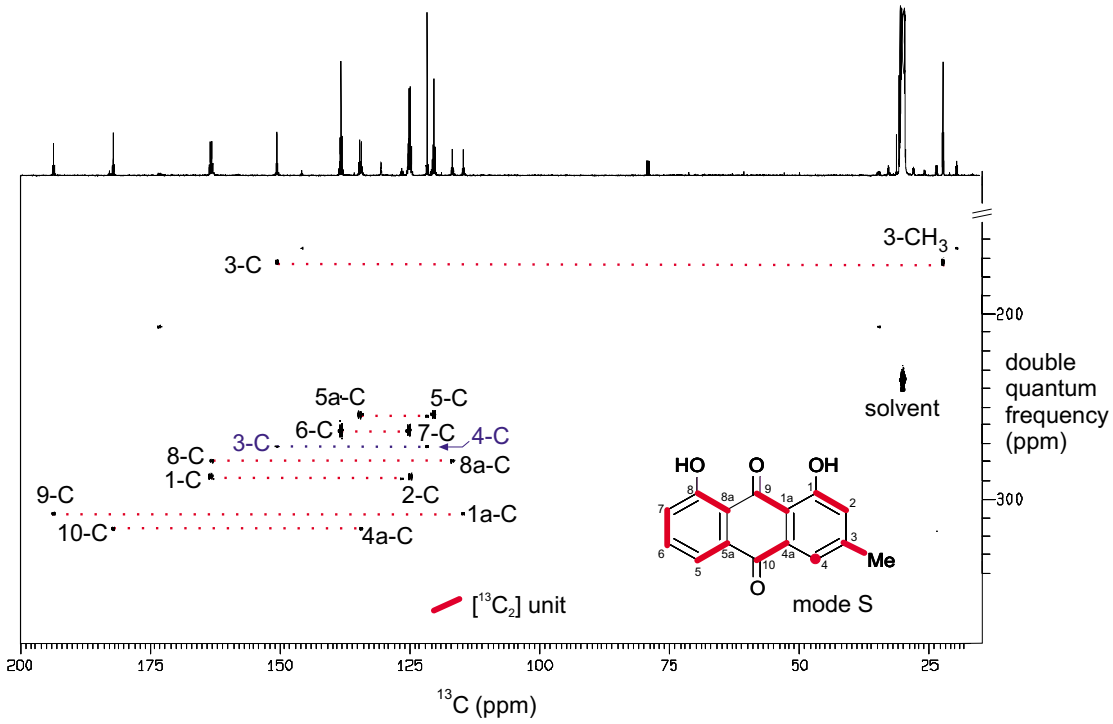

Figure 4. 2D INADEQUATE NMR spectrum of **1** from *Nocardia* strain Acta 1057 after feeding sodium [1,2-$^{13}C_2$]acetate; the labelling pattern with its pairwise ^{13}C-^{13}C correlations of incorporated intact [$^{13}C_2$] units indicates the presence of mode S folding. The blue dotted line shows an interaction between C3 and C4, indicating the possible existence of an additional mode S' folding.

Our comparative analysis of folding modes of chrysophanol (**1**) in very diverse organisms shows for the first time that this polyketide can be produced in nature in at least two of the four imaginable different ways (Fig. 2): the chemically most probable modes F and S. We did not detect the theoretically possible, but chemically less probable mode F', and the existence of mode S' can neither be proven unambiguously nor excluded. Thus, we have established the first example of a chemically convergent biosynthesis of a polyketide.

Hence, the biogenesis of chrysophanol (**1**) is simultaneously chemically divergent (one octaketide chain precursor (**4**) leading to differently cyclised regioisomeric intermediates of basic type **5**) and convergent: the biosynthetic intermediates, despite their different structures (such as **5a** and **5c**), converge, eventually leading to **1** with the very same chemical structure.

Our discoveries raise questions about the evolutionary origin and the ecological role of **1** in the organisms studied. Chrysophanol (**1**) shows antifungal (Agarwaj et al. 2000), antiviral (Semple et al. 2001) and antifeedant activities against insects (Hilker et al. 1992) and birds (Hilker and Köpf 1995) thereby giving the producers selective advantages in the fight for survival. The need for this defence compound in "chemical protection" thus may have driven the evolutionarily convergent development on the production of this anthraquinone through different "strategies". The cloning and characterisation of the involved PKSs may provide a highly rewarding tool to further elucidate the evolutionary success of this chemical defensive component.

Methods

Materials

[1,2-$^{13}C_2$]NaOAc (99% isotopic enrichment) was purchased from Eurisotop (Saarbrücken, Germany).

Biological material and sterile cultures

Cultures of *Drechslera catenaria* obtained from the CBS were cultivated in Czapek-Dox broth shake cultures in 250-ml Erlenmeyer flasks, using a procedure adapted from Van Eijk (Van Eijk 1974) (pH adjusted to 7.4 before autoclaving) on a rotary shaker (100 rpm) at 24°C in the dark. The ingredients of the media used for the fungi and plants were from Sigma.

Liquid callus cultures of the torch lily *Kniphofia uvaria* (Asphodelaceae) were established as previously reported (Bringmann et al. 2002).

Nocardia strain Acta 1057 was isolated from a sample of plot 6 soil collected from Cockle Park Experimental Farm near Morpeth, Northumberland. The strain was cultivated on

Gause's medium 2 (10 g glucose, 5 g peptone, 3 g tryptone, 5 g NaCl, 15 g agar per litre tap water; pH adjusted to 7.0 prior to sterilisation), supplemented with nalidixic acid (10 µg ml^{-1}), cycloheximide (50 µg ml^{-1}) and nystatin (50 µg ml^{-1}). The organism was shown to have chemotaxonomic and morphological properties consistent with its classification in the genus *Nocardia* (Goodfellow et al. 1999). This assignment was confirmed by 16S rRNA gene sequence data, which also showed that the organism formed a distinct phyletic line in the *Nocardia* gene tree. Fermentation of the strain was performed in a 1-l fermenter (Biostat S, B. Braun, Melsungen, Germany) at 27°C in a medium consisting of 15 g starch, 10 g glucose, 10 g glycerol, 2.5 g cornsteep powder (Marcor), 5 g Bacto peptone, 2 g yeast extract (Ohly Kat) and 1 g NaCl per litre tap water; the pH was adjusted to 7.3 prior to sterilisation. The fermenter was inoculated with 5 vol-% of a shake flask culture grown in the same medium for 72 h. The culture was incubated for 144 h at an aeration rate of 0.5 volume air/ volume medium per min (vvm) and agitation of 250 rpm.

For the biosynthetic investigations on the insects, approximately 800 *Galeruca tanaceti* larvae were kept on Chinese cabbage in a climate chamber (constant temperature: 25°C; with a photoperiod of 18/ 6 h (2000 Lux) and 60% relative humidity).

Feeding experiments

In the case of the fungi, feeding started after five days of incubation. A sterile-filtered solution of labelled acetate was fed in intervals over a period of 28 days to get a final concentration of 200 mg l^{-1}.

For the higher plants, a sterile-filtered solution (4 mg l^{-1}) of 0.05 M uniformly ^{13}C-labelled NaOAc in distilled water, adjusted to pH 5.8, was used.

Both, initially and after eight days, 1 ml per flask of this solution was administered to liquid callus cultures cultivated in 50 ml medium in 100-ml Erlenmeyer flasks; the cultures were harvested after 16 d.

In the case of the *Nocardia* strain, an aqueous sterile-filtered solution (8.3 mg ml^{-1}) of 0.06 M uniformly ^{13}C labelled NaOAc in distilled water (pH adjusted to 7.0) was fed at a continuous flow rate of 3 ml/ h in the period between 85 and 105 h of fermentation.

The feeding of the insects started immediately after hatching of the larvae. A sterile-filtered solution of ^{13}C labelled NaOAc (2 mg ml^{-1}) was spread with a conventional vaporiser onto cabbage leaves, which were fed daily to the insects over a period of 14 days.

*Chrysophanol (**1**)*

The material from the respective biological sources was lyophilised, ground and extracted with 95:5 acetone/ water. The solvent was removed by rotary evaporation and the extract purified by HPLC on a preparative Waters Symmetry Prep C18 column (7 µm, 19.0 x 300 mm) using the following gradient (flow rate 11 ml/ min): water (A), acetonitrile (B) plus 0.05% trifluoroacetic acid; 0 min 15% B, 30 min 55% B, 35 min 100% B, 40 min 100% B. All solvents were of HPLC grade.

The spectroscopic data were in accordance with an authentic sample purchased from Aldrich.

NMR spectroscopy

NMR spectra were recorded on a Bruker DMX 600 spectrometer. For calibration of ^{13}C and ^{1}H chemical shifts, the methyl-carbon signal and the residual proton signal of the solvent were used, respectively (acetone-d_6: $\delta_H = 2.05$ p.p.m. and $\delta_C = 29.8$ p.p.m.). Proton-detected, heteronuclear correlations were measured using HMQC (optimised for $^{1}J_{HC} = 145$ Hz) and HMBC (optimised for $^{n}J_{HC} = 8$ Hz or $^{n}J_{HC} = 4$ Hz) pulse sequences. To establish ^{13}C-^{13}C connectivities, 2D INADEQUATE (Bax et al. 1981) experiments were performed at 150.9 MHz using a 5 mm carbon-sensitive probe head; in the case of **1** derived from bacteria and insects a carbon-sensitive cryoprobe (Styles and Stoffe 1984) was used. The spectra of labelled **1** isolated from the fungal, plant, bacterial and insect-derived materials were measured for 162 h, 91 h, 113 h and 87 h, respectively, and optimised for $^{1}J(^{13}C, ^{13}C)$ coupling constants between 50 and 62 Hz. For the SELINQUATE (Berger 1988) spectrum (optimised for $^{1}J(^{13}C, ^{13}C) = 55$ Hz) of the fungal source, a selective 270° Gauss pulse with 5 ms pulse length at 193.7 p.p.m. was used for the selective coherence transfer.

An additional 2D INADEQUATE spectrum (51 h measurement time) of the plant-derived chrysophanol (**1**) was recorded on a 5 mm carbon-sensitive cryoprobe (Styles and Stoffe 1984) at 500 MHz on a Bruker Avance 500 spectrometer to improve the signal-to-noise ratio.

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