

Different polyketide folding modes converge to an identical molecular architecture

Gerhard Bringmann¹, Torsten F Noll¹, Tobias A M Gulder¹, Matthias Grüne¹, Michael Dreyer¹, Christopher Wilde², Florian Pankewitz³, Monika Hilker³, Gail D Payne⁴, Amanda L Jones⁴, Michael Goodfellow⁴ & Hans-Peter Fiedler²

Metabolic diversity is being studied intensively 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 defense 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 described¹: mode F (referring to fungi) and mode S (from *Streptomyces*). We have demonstrated that in eukaryotes (fungi, higher plants and insects), chrysophanol is formed via folding mode F. In actinomycetes, by contrast, the cyclization 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 cyclization 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 cyclize polyketides via the mode F strategy^{2,3}. 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 biosynthesized via one common pathway or in an organism-specific manner.

The widely distributed polyketidic anthraquinone chrysophanol⁴ (1) (Fig. 1), has been found in several eukaryotic organisms: in

fungi and plants (potential F-type organisms) and also in insects^{5–8} and lichens^{9,10}. More recently, 1 has also been detected in small amounts in a *Streptomyces* strain¹¹. Given its wide distribution in nature and its structure, 1 represents a prototype of a substance that might, in principle, be synthesized through different folding modes: folding modes F and S are thought to be responsible for its formation in fungi and streptomycetes, respectively¹. However, the presence of only small amounts of 1 in streptomycetes did not allow detailed biosynthetic investigations by feeding labeled precursors, whereas the biosynthesis of other polyketides has been studied intensively in *Streptomyces*¹².

Besides chrysophanol (1), the isomeric anthraquinone aloesapinarin II (2) has also been assumed—but not proven—to be a potential candidate that might be synthesized from differing biosynthetic polyketidic origins, owing to its occurrence both in plants¹³ 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* loci¹⁴. However, 2 has not been reported in fungi.

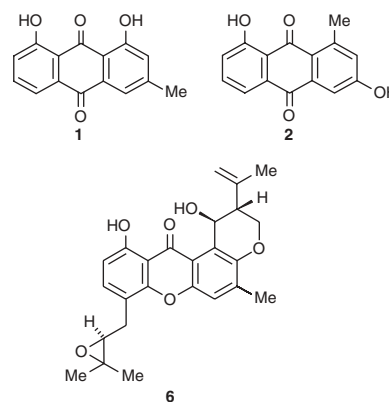


Figure 1 Chemical structures of the acetogenic metabolites chrysophanol (1), aloesapinarin II (2) and tajixanthone (6).

¹Institute of Organic Chemistry, University of Würzburg, Am Hubland, D-97074 Würzburg, Germany. ²Institute of Microbiology, University of Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany. ³Institute of Biology, Free University Berlin, Haderslebener Strasse 9, D-12163 Berlin, Germany. ⁴Division of Biology, University of Newcastle, Newcastle upon Tyne, NE1 7RU, UK. Correspondence should be addressed to G.B. (bringman@chemie.uni-wuerzburg.de).

Received 12 May; accepted 13 June; published online 9 July 2006; doi:10.1038/nchembio805

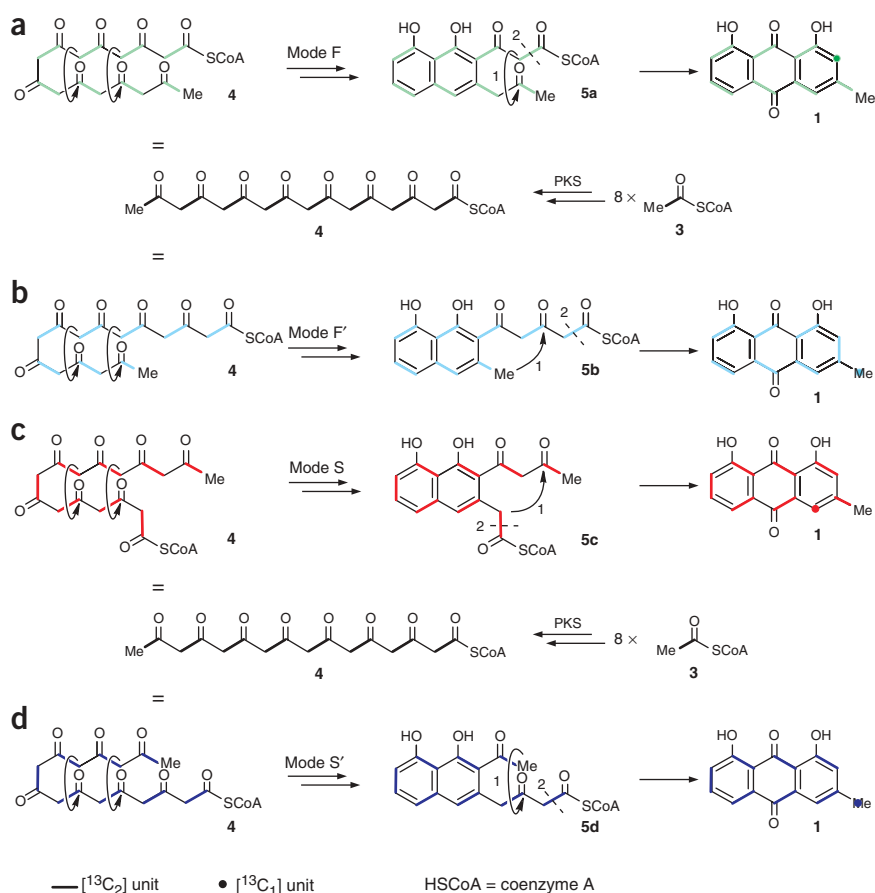


Figure 2 The four biosynthetic folding modes that might lead from acetyl coenzyme A to chrysophanol (1) in nature, with their joint (4) and individually different (5a–5d) hypothetical intermediates, with the expected characteristic ¹³C labeling patterns for 1. (a) Mode F folding. (b) Prospective mode F' folding. (c) Mode S folding. (d) Conceivable mode S' folding. The bold colored lines indicate the positions of incorporated intact [1,2-¹³C₂]acetate units. Aldol-type cyclization reactions are illustrated by curved arrows; those leading to the third aromatic ring are denoted by '1', and decarboxylation is indicated by '2'. Note that for reasons of clarity, the structure of 4 disregards the expected high degree of enolization and, in particular, its presumable reduction at C-9.

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*)¹⁹ with [1,2-¹³C₂]acetate for four weeks. We then took 2D INADEQUATE NMR measurements on the isolated 1 (ref. 20). These showed interactions between C1a and C1, C3 and CH₃ 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 C₂ fragment using a SELINQUATE²¹ 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 that singlet in the ¹³C NMR spectrum, but rather indirectly from the complete pairwise labeling of all the other C atoms and from its appearance as a singlet in the ¹³C NMR spectrum.

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 C₂ units in the first ring of 1) and S (three C₂ units), the additional, never-before-discussed modes S' and F' are conceivable.

We analyzed the biosynthesis of 1 in a plant species by treating liquid callus cultures of the torch lily *Kniphofia uvaria*, cultivated as described earlier²², with labeled 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 technique²³ 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.

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 analyzed by feeding experiments with [1,2-¹³C]acetate, as this can be expected to lead, via the different presumed intermediates (for example, via 5a, 5b, 5c or 5d), 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 radiolabeled [¹⁴C]acetate^{15–17} and indirectly in the fungus *Aspergillus varicolor*¹⁸ during studies on the biogenesis of tajixanthone (6) (ref. 18). In feeding experiments with [1,2-¹³C₂]acetate, 6 gives an isotope pattern consistent with its origin via an F-type folding; [methyl-²H₃]chrysophanol, in turn, is a specific precursor to 6. However, direct experiments with doubly ¹³C-labeled acetate have not been performed on 1; hence, localization of the intact acetate units has not been possible.

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 (ref. 24). We cultivated the strain in a 1-l stirred tank fermenter in

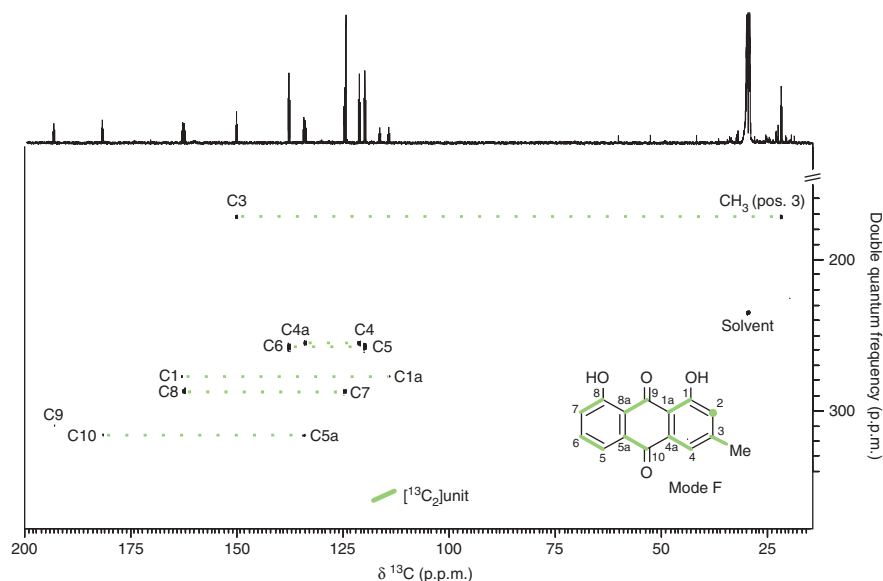


Figure 3 2D INADEQUATE NMR spectrum of **1** isolated from the filamentous fungus *Drechslera catenaria* after feeding with sodium $[1,2-^{13}\text{C}_2]$ acetate; the labeling pattern with its pairwise ^{13}C - ^{13}C correlations of incorporated $^{13}\text{C}_2$ units (only two in the initially formed 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 tanacetii* (not shown).

a complex medium; we obtained maximal chrysophanol production of up to 30 mg l^{-1} after 144 h. Feeding with $[1,2-^{13}\text{C}_2]$ 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 mode S folding (as postulated earlier¹; see above). Chemically this

and C1a was undetectable; we observed the C1a signal at the expected double quantum frequency. These results clearly indicate that a eukaryotic PKS catalyzes the formation of **1** in this species, which excludes an endosymbiotic prokaryotic producer of anthraquinones in the beetle.

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 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.

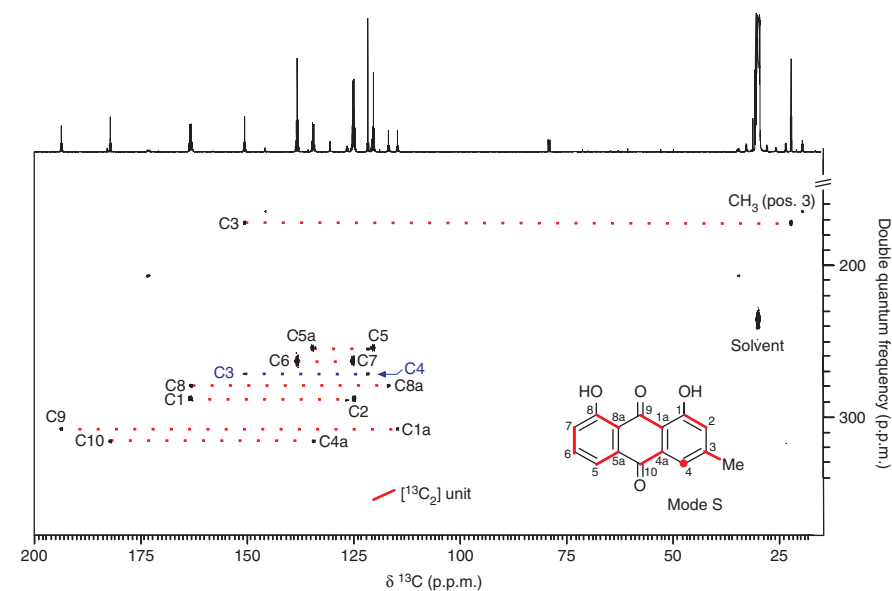


Figure 4 2D INADEQUATE NMR spectrum of **1** from *Nocardia* strain Acta 1057 after feeding sodium $[1,2-^{13}\text{C}_2]$ acetate; the labeling pattern with its pairwise ^{13}C - ^{13}C correlations of incorporated intact $^{13}\text{C}_2$ units indicates the presence of mode S folding. The blue dotted line shows an interaction between C3 and C4, indicating the possible existence of an additional mode S' folding.

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 labeling 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 tanacetii* (Chrysomelidae), which is known to contain anthraquinones at different developmental stages⁶. This beetle does not sequester **1** from its host plant⁶; 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. tanacetii* larvae with labeled sodium $[1,2-^{13}\text{C}_2]$ 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

convergent development on the production of this anthraquinone through different 'strategies'. The cloning and characterization 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- $^{13}\text{C}_2$]NaOAc (99% isotopic enrichment) was purchased from Eurisotop.

Biological material and sterile cultures. Cultures of *Drechslera catenaria* obtained from the CBS were cultivated in Czapek-Dox broth in 250-ml Erlenmeyer flasks, using a procedure adapted from van Eijk¹⁹ (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 reported²².

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 liter tap water; pH adjusted to 7.0 before sterilization), supplemented with nalidixic acid (10 $\mu\text{g ml}^{-1}$), cycloheximide (50 $\mu\text{g ml}^{-1}$) and nystatin (50 $\mu\text{g ml}^{-1}$). The organism was shown to have chemotaxonomic and morphological properties consistent with its classification in the genus *Nocardia*²⁸. 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, Braun) at 27 °C in a medium consisting of 15 g starch, 10 g glucose, 10 g glycerol, 2.5 g corn steep powder (Marcor), 5 g Bacto peptone, 2 g yeast extract (Ohly Kat) and 1 g NaCl per liter tap water; the pH was adjusted to 7.3 before sterilization. The fermenter was inoculated with 5 vol-% of a 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 tanacetii* larvae were kept on Chinese cabbage in a climate-controlled chamber (constant temperature of 25 °C, with a photoperiod of 18 h light (2,000 lx)/6 h dark and 60% relative humidity).

Feeding experiments. In the case of the fungi, feeding started after 5 d of incubation. A sterile-filtered solution of labeled acetate was fed in intervals over a period of 28 d to reach 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 -labeled NaOAc in distilled water (adjusted to pH 5.8) was used.

Both initially and after 8 d, 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 -labeled NaOAc in distilled water (pH adjusted to 7.0) was fed at a continuous flow rate of 3 ml h^{-1} 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 -labeled NaOAc (2 mg ml^{-1}) was spread with a conventional vaporizer onto cabbage leaves, which were fed daily to the insects over a period of 14 d.

Chrysophanol (1). The material from the respective biological sources was lyophilized, ground and extracted with 95:5 acetone/water (vol/vol). 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 \times 300 mm) using the following gradient (flow rate 11 ml min^{-1}): 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 ^1H chemical shifts, the methyl-carbon

signal and the residual proton signal of the solvent were used, respectively (acetone- d_6 : $\delta_{\text{H}} = 2.05$ parts per million (p.p.m.) and $\delta_{\text{C}} = 29.8$ p.p.m.). Proton-detected, heteronuclear correlations were measured using heteronuclear multiple quantum coherence (HMQC) (optimized for $^1\text{J}_{\text{HC}} = 145$ Hz) and heteronuclear multiple bond correlation (HMBC) (optimized for $^1\text{J}_{\text{HC}} = 8$ Hz or $^n\text{J}_{\text{HC}} = 4$ Hz) pulse sequences. To establish ^{13}C - ^{13}C connectivities, 2D INADEQUATE²⁰ 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 cryoprobe²³ was used. The spectra of labeled **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 optimized for $^1\text{J}(^{13}\text{C}, ^{13}\text{C})$ coupling constants between 50 and 62 Hz. For the SELINQUATE²¹ spectrum (optimized for $^1\text{J}(^{13}\text{C}, ^{13}\text{C}) = 55$ 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 cryoprobe²³ at 500 MHz on a Bruker Avance 500 spectrometer to improve the signal-to-noise ratio.

ACKNOWLEDGMENTS

This work is dedicated to Burchard Franck on the occasion of his 80th birthday. Financial support from the Fonds der Chemischen Industrie, the European Commission (grant QLK3-CT-2001-01783; project ACTAPHARM), and the German Research Foundation (DFG Hi 416/16-2) are gratefully acknowledged. We thank D. Moskau (Bruker Biospin AG, Fällanden, Switzerland) for the acquisition of a 2D INADEQUATE spectrum with a cryoprobe and F. Meyer for technical support.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Published online at <http://www.nature.com/naturechemicalbiology>

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions/>

1. Thomas, R. A biosynthetic classification of fungal and streptomycete fused-ring aromatic polyketides. *ChemBioChem* **2**, 612–627 (2001).
2. Bringmann, G., Wohlfarth, M., Rischer, H., Rückert, M. & Schlauer, J. The polyketide folding in the biogenesis of isoshinanolone and plumbagin from *Ancistrocladus heyneanus* (Ancistrocladaceae). *Tetrahed. Lett.* **39**, 8445 (1998).
3. Bringmann, G., Wohlfarth, M., Rischer, H., Grüne, M. & Schlauer, J. A new biosynthetic pathway to alkaloids in plants: acetogenic isoquinolines. *Angew. Chem. Int. Edn. Engl.* **39**, 1464–1466 (2000).
4. Thomson, R.H. Naturally occurring quinones III 383 (Chapman and Hall, New York, 1987).
5. Howard, D.F., Phillips, D.W., Jones, T.H. & Blum, M.S. Anthraquinones and anthrones: occurrence and defensive function in a chrysomelid beetle. *Naturwissenschaften* **69**, 91–92 (1982).
6. Hilker, M. & Schulz, S. Anthraquinones in different development stages of *Galeruca tanacetii* (Coleoptera: Chrysomelidae). *J. Chem. Ecol.* **17**, 2323 (1991).
7. Hilker, M., Eschbach, U. & Dettner, K. Occurrence of anthraquinones in eggs and larvae of several Galerucinae (Coleoptera: Chrysomelidae). *Naturwissenschaften* **79**, 271 (1992).
8. Kunze, A., Witte, L., Aregullin, M., Rodriguez, E. & Proksch, P. Anthraquinones in the leaf beetle *Triurhabda geminata* (Chrysomelidae). *Z. Naturforsch. [C]* **51**, 249–252 (1996).
9. Mishchenko, N.P., Stepanenko, L.S., Krivoshechkova, O.E. & Maksimov, O.B. Anthraquinones of the lichen *Asahinea chrysantha*. *Him. Prir. Soedin.* **2**, 160–165 (1980).
10. Krivoshechkova, O.E., Stepanenko, L.S., Mishchenko, N.P., Denisenko, V.A. & Maksimov, O.B. Study of lichens from the Parmeliaceae family. II. Pigments. *Him. Prir. Soedin.* **3**, 283–289 (1983).
11. Fotso, S. *et al.* Bhimamycin A ~ E and bhimanone: isolation, structure elucidation and biological activity of novel quinone antibiotics from a terrestrial streptomycete. *J. Antibiot.* **56**, 931–941 (2003).
12. Funa, N. *et al.* A new pathway for polyketide synthesis in microorganisms. *Nature* **400**, 897–899 (1999).
13. Yagi, A., Makino, K. & Nishioka, I. Constituents of *Aloe saponaria*. I. Structures of tetrahydroanthracene derivatives and the related anthraquinones. *Chem. Pharm. Bull. (Tokyo)* **22**, 1159–1166 (1974).
14. Bartel, P.L. *et al.* Biosynthesis of anthraquinones by interspecies cloning of actinorhodin genes in streptomycetes: clarification of actinorhodin gene functions. *J. Bacteriol.* **172**, 4816–4826 (1990).
15. Leistner, E. & Zenk, M. Chrysophanol (1,8-dihydroxy-3-methylanthraquinone) biosynthesis in higher plants. *J. Chem. Soc. Chem. Commun.* 210–211 (1969).
16. Fairbairn, J.W. & Muhtadi, F.J. Biosynthesis and metabolism of anthraquinones in *Rumex obtusifolius*. *Phytochemistry* **11**, 215–219 (1972).

17. Leistner, E. Second pathway leading to anthraquinones in higher plants. *Phytochemistry* **10**, 3015–3020 (1971).
18. Ahmed, S.A., Bardshiri, E. & Simpson, T.J. A convenient synthesis of isotopically labeled anthraquinones, chrysophanol, islandicin, and emodin. Incorporation of [methyl- $^2\text{H}_3$]chrysophanol into tajixanthone in *Aspergillus varicolor*. *J. Chem. Soc. Chem. Commun.* 883–884 (1987).
19. Van Eijk, G.W. Chrysophanol and emodin from *Drechslera catenaria*. *Phytochemistry* **13**, 650 (1974).
20. Bax, A., Freeman, R. & Frenkiel, T.A. An NMR technique for tracing out the carbon skeleton of an organic molecule. *J. Am. Chem. Soc.* **103**, 2102–2104 (1981).
21. Berger, S. Selective INADEQUATE. A farewell to 2D-NMR? *Angew. Chem. Int. Edn. Engl.* **27**, 1196–1197 (1988).
22. Bringmann, G., Noll, T. & Rischer, H. In vitro germination and establishment of tissue cultures of *Bulbine caulescens* and of two *Kniphofia* species (Asphodelaceae). *Plant Cell Rep.* **21**, 125–129 (2002).
23. Styles, P. & Stoffe, N.F. A high-resolution NMR probe in which the coil and preamplifier are cooled with liquid helium. *J. Magn. Reson.* **60**, 397–404 (1984).
24. Fiedler, H-P. Biosynthetic capacities of actinomycetes. 1. Screening for new secondary metabolites using UV-visible absorbance spectral libraries. *Nat. Prod. Lett.* **2**, 119–128 (1993).
25. Agarwaj, S.K., Singh, S.S., Verma, S. & Kumar, S. Antifungal activity of anthraquinone derivatives from *Rheum emodi*. *J. Ethnopharmacol.* **72**, 43–46 (2000).
26. Semple, S.J., Pyke, S.M., Reynolds, G.D. & Flower, R.L.P. In vitro antiviral activity of the anthraquinone chrysophanic acid against poliovirus. *Antiviral Res.* **49**, 169–178 (2001).
27. Hilker, M. & Köpf, A. Evaluation of the palatability of chrysoamelid larvae containing anthraquinones to birds. *Oecologia* **100**, 421–429 (1995).
28. Goodfellow, M., Isik, K. & Yates, E. Actinomycete systematics: an unfinished synthesis. *Nova Acta Leopold.* **312**, 47–82 (1999).