Biosynthesis of pyrrolylpolyenes in *Auxarthron umbrinum*

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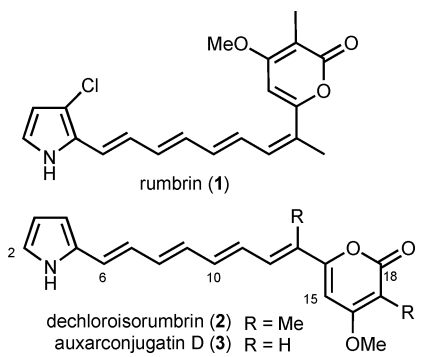
The biosynthesis of the pyrrolylpolyene rumbrin (**1**) in the fungus *Auxarthron umbrinum* was elucidated using feeding studies with labelled precursors. Incorporation of stable isotopes from [^{15}N]-proline, [^{13}C]-methionine and [^{13}C]-acetate confirmed that these were the precursors of the pyrrole moiety, methyl groups, and backbone of rumbrin, respectively. Label-dilution experiments with pyrrole-2-carboxylate confirmed it was a direct precursor in the biosynthesis of rumbrin. Both 3- and 4-chloropyrrolecarboxylates were also accepted as precursors in polyene production.

Introduction

Rumbrin (**1**) is an unusual pyrrolylpolyene produced by a small number of fungi from the order Onygenales. First isolated in 1993 from an *Auxarthron umbrinum* strain,[1,2] it has since been isolated a number of times along with several isomers, in addition to closely related polyenes including the auxarconjugatins[3] and gymnoconjugatins.[4] Rumbrin possesses several interesting biological properties, including anticancer[4] and cytoprotective activities.[1] Despite its intriguing structure, very little is known about the biosynthesis of rumbrin. A single feeding study has been carried out using [1,2-$^{13}C_2$]-acetate, confirming the polyketide nature of the polyene; however the study was conducted for structural elucidation purposes, and the biosynthetic implications were not addressed.[2] More recently, molecular techniques have been used to probe the biosynthesis of other fungal polyenes, showing them to be biosynthesised by iterative polyketide synthase (PKS) systems.[5,6] However, these polyenes lack the highly unusual 3-chloropyrrole moiety and potent biological activities of rumbrin. In this study we describe investigations into the biosynthesis of rumbrin using feeding studies with both isotopically labelled and unlabelled precursors.



Results and discussion

The commercially available strain *A. umbrinum* DSM-3193 was used for the studies. Polyene production was assessed by HPLC and MS analysis under a range of culture conditions and was found to be best using the rich medium employed by Alvi *et. al.*[7] with production beginning after approximately four days growth. Polyene production can also be visually monitored by the development of a deep red colour in the fungal mycelium. While some strains used in previous studies have produced almost exclusively chlorinated polyenes,[8] the strain used in the current investigation produced both non-chlorinated and chlorinated products, in a ratio of ~3:1, as determined by HPLC and MS analysis. The major product was dechloroisorumbrin (**2**), though rumbrin (**1**) and a range of isomers and related metabolites such as the auxarconjugatins were also detectable (Fig. 1a). Monitoring the production in *A. umbrinum* cultures over a one month period showed no significant change in the composition of the polyenes over time. Given the relatively high production of **2**, most of the biosynthetic studies focused on this as a model for production of all polyenes.

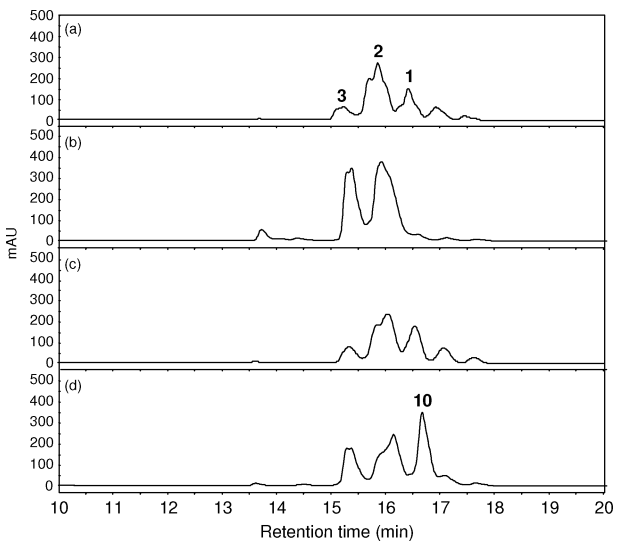


Fig. 1 HPLC analysis (DAD, 440 nm) of polyenes produced by *A. umbrinum* DSM-3193 cultures. (a) Unsupplemented medium, (b) medium plus 2 mM pyrrolecarboxylate (**4**), (c) plus 2 mM **5**, (d) plus 2 mM **6**.

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Stable isotope labelling studies

It was thought that the polyene backbone of rumbrin was likely to be derived from acetate, with the pyrrole moiety originating from proline and the methyl groups from methionine. Thus, [1-^{13}C]-acetate, [^{15}N]-L-proline, and [methyl-^{13}C]-L-methionine were added to growing cultures of *A. umbrinum* DSM-3193 in order to determine whether they were incorporated into the polyene. Precursors were introduced to the fungal culture after three days, immediately prior to the commencement of polyene biosynthesis. After seven days the cultures were filtered, the mycelium extracted with MeOH-CH_2Cl_2 (3:1), and the extracts analysed by electrospray mass spectrometry (ESI(+)MS) and HPLC. Incorporation of precursors was assessed by comparison of peak heights at *m/z* 324 and 325 (dechloroisorumbrin, M+1 and M+2) in the mass spectrum.

When [^{15}N]-L-proline was added to the culture medium at concentrations of 2 and 5 mM, isotope incorporation of 14% and 43% was observed, confirming proline as a precursor. In comparison, feeding with a biosynthetically unrelated amino acid such as [^{15}N]-glycine gave a much lower incorporation at the same concentations (5.1% and 8.1%, respectively). This incorporation presumably arose from enrichment of the cellular nitrogen pool by deamination of glycine, for example *via* the actions of serine hydroxymethyltransferase, which converts two molecules of glycine to serine with the liberation of NH_4^+, and serine dehydratase, which converts serine to pyruvate and NH_4^+.

Mass spectral evidence suggested that [methyl-^{13}C]-methionine (3 mM) was also incorporated into dechlororumbrin (**2**), since both single and double isotope incorporation of 13.7% and 5.5%, respectively, were detected. In order to determine the location of the ^{13}C labels, the crude fungal extract was purified by solvent partitioning and reversed phase SPE to yield a mixture of polyenes. ^{13}C NMR of this crude polyene mixture revealed enhancements in clusters of ^{13}C resonances at 8.7, 12.8, 55.5 ppm, corresponding to the 13-methyl, 17-methyl, and 16-*O*-methyl resonances of the polyenes, respectively. For dechloroisorumbrin (**2**), percentage incorporation at each position was 8.5%, 6.1%, and 10.7%. A cluster of peaks at 21.7 ppm, corresponding to the 13-methyl group of a 12*Z*-polyene,[2] was also enhanced (8.3%).

[1-^{13}C]-Acetate (25 mM) was also incorporated into dechloroisorumbrin, presumably as malonate, with multiple ^{13}C-labeled acetates incorporated into a single molecule. In the mass spectrum, species containing a single label (17.9%), double label (12.3%), triple label (11.9%), four labels (8.9%) and >4 labels (4.8%) were all detected. ^{13}C NMR of the partially purified polyenes (as above) gave a complex mixture of peaks that prevented a rigorous analysis. Thus, further purification was carried out using reversed-phase HPLC to yield several purified polyene metabolites. Due to difficulties in the purification of dechloroisorumbrin (**2**), the purified polyene selected for further ^{13}C analysis was the didemethyl polyene auxarconjugatin D (**3**).† Enhancement of the ^{13}C NMR signals at 137.4, 139.2, 135.3, 158.5, 170.7, and 162.6 ppm revealed the incorporation of the C-1 of acetate at C-8, C-10, C-12, C-14, C-16, and C-18, as expected (Table 1).

Table 1 ^{1}H and ^{13}C NMR data (500 MHz, d_6-DMSO) and $\%^{13}$C incorporation for auxarconjugatin D (**3**) from [1-^{13}C]-acetate.

	δ_H (m, *J* (Hz))	δ_C	$\%^{13}C^{a,b}$
N-1	11.16 (br s)		
2	6.85 (dd, 3.8, 2.6)	120.7	**9.4**
3	6.07 (dd, 3.0, 2.6)	109.4	1.1
4	6.24 (br s)	110.1	0.8
5		130.5	1.0
6	6.54 (d, 15.6)	125.4	**5.2**
7	6.65 (dd, 15.6, 11.2)	123.2	1.2
8	6.55 (dd, 14.1, 11.2)	137.4	**6.2**
9	6.35 (dd, 14.1, 11.4)	129.8	1.1
10	6.73 (dd, 14.5, 11.4)	139.2	**6.9**
11	6.43 (dd, 14.5, 11.3)	129.7	1.2
12	7.04 (dd, 15.2, 11.3)	135.3	**5.6**
13	6.27 (d, 15.2)	121.1	1.6
14		158.5	**5.1**
15	6.22 (d, 2.0)	100.5	1.1
16		170.8	**4.9**
17	5.58 (d, 2.0)	88.3	1.1
18		162.6	**5.6**
OMe	3.81 (s)	56.2	1.1

[a] Calculated by comparison of the ^{13}C peak heights relative to a ^{13}C spectrum of unlabelled **3**. The OMe peak was used as an internal standard and defined as 1.1%. [b] Numbers in bold are the enriched positions.

However, two further signals at 120.7 and 125.4 ppm, C-2 and C-6, were also enhanced. This was initially perplexing, as this portion of the molecule was believed to be proline-derived. On further consideration the origin of these labels became clear: proline is biosynthesised from glutamate, which is in turn derived from α-ketoglutarate, an intermediate in the acetate-fuelled TCA cycle. Thus, C-1 of acetate is incorporated into proline at the C-1 and C-5 positions, and subsequently into the polyene as C-6 and C-2 (Scheme 1).

The incorporation of these precursors is consistent with what is already known about production of fungal polyketides. Given the polyketide nature of the polyene, incorporation of acetate was expected, though the high retention of label in the proline-derived portion of the molecule was surprising, and was probably a consequence of the high concentration of labelled acetate used in the experiment.

The incorporation of [methyl-^{13}C]-L-methionine is consistent with previous studies. Pendant methyl groups on fungal polyketides are invariably derived from the *S*-methyl group of methionine, *via* *S*-adenosyl methionine.[9,10] The production of polyenes with a variable degree of methylation by *A. umbrinum* DSM-3193 suggests that methylation by the hypothetical PKS cluster is unpredictable, and that a methyltransferase module may be "skipped" during the biosynthetic process.[11]

The biosynthesis of pyrrole-containing natural products has not previously been investigated in fungi, and this represents the first investigation of the biosynthesis of a pyrrole-containing metabolite. Our observations that proline is the biosynthetic origin of the pyrrole moiety has also been recognized for several bacterial pyrrole-containing natural products.[12]

Incorporation of pyrrole-2-carboxylates

With the origins of the atoms established, the next step was to see if any more advanced intermediates could be incorporated. Biosynthesis of bacterial pyrrole-containing antibiotics, such

† The structure of **3**, a new metabolite, was determined by comparison of ^{1}H and ^{13}C NMR shifts (Table 1) with those of related metabolites, in particular auxarconjugatins B and C.[3]

Table 2 Relative proportions of chlorinated and non-chlorinated polyenes produced upon addition of various pyrrole-2-carboxylic acids to the culture medium

Pyrrole-2-carboxylic acid(s) (2 mM)	Total polyenes[a] (mg/L)	Non-chlorinated polyene (%)	Chlorinated polyene (%)
None	106	77	23
4	174	88	12
5	111	61	39
5+4	123	76	24
6	135	48	52
6+4	194	80	20

[a] Determined by integration of peak areas at 440 nm.

as pyoluteorin and coumermycin A1 proceeds with pyrrolyl-2-carboxyl (originally proline) tethered to a peptidyl carrier protein.[13,14] Nevertheless, experiments were conducted to examine to possibility of free pyrrole-2-carboxylate (**4**) being incorporated into the polyenes. Thus, a label dilution experiment, where both [^{15}N]-L-proline and pyrrole-2-carboxylate (2 mM each) were added to an *A. umbrinum* culture, was conducted. Control experiments with no substrate, [^{15}N]-L-proline only, and **4** only were carried out in parallel. Cultures were worked up and analysed as before. The results were striking: while the proline-only extract showed excellent incorporation (22%), as before, the label-dilution extract showed a greatly reduced incorporation of [^{15}N]-proline (2.6%), indicating that **4** is a direct precursor in the biosynthesis of the polyenes. Furthermore, in both cultures to which pyrrole-2-caboxylate (**4**) had been added, drastic changes were observed in the HPLC profile of the polyenes, with a noticeable increase in the production of **2** and other non-halogenated polyenes, while production of chlorinated polyenes was not enhanced (Fig. 1b, Table 2). This effect was further investigated by culturing *A. umbrinum* in medium containing varying amounts of pyrrole-2-carboxylate (0, 1, 3, and 10 mM), which showed that polyene production was enhanced further with higher concentrations of pyrrolecarboxylate (Fig. 2) – a five-fold increase in polyene production was observed with the addition of 10 mM of **4**.

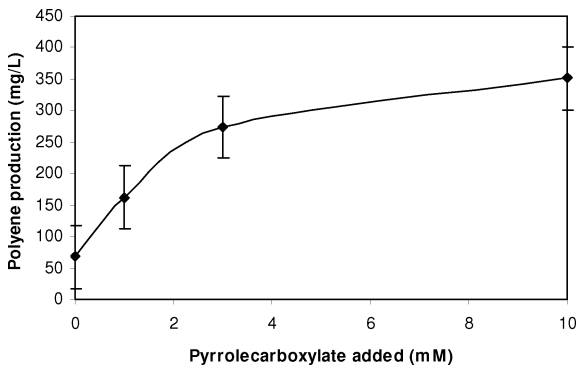
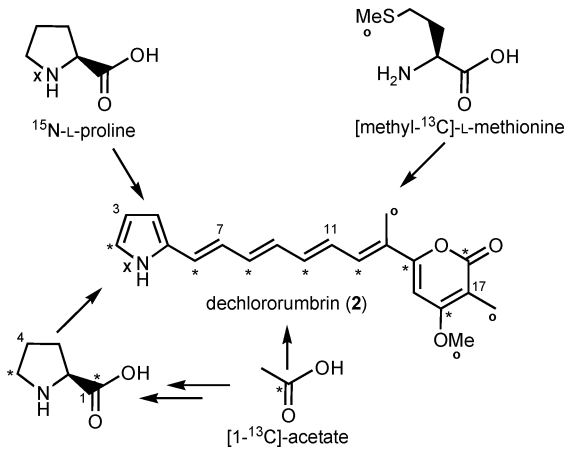
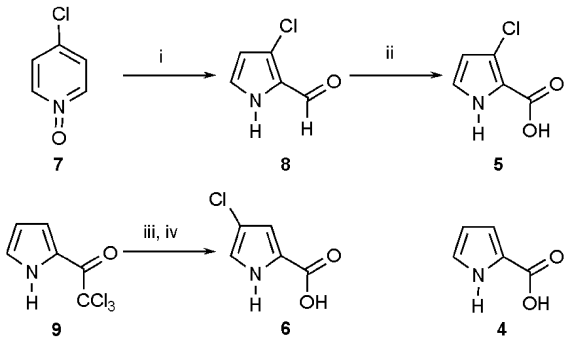


Fig. 2 Increased polyene production by *A. umbrinum* DSM-3193 with addition of pyrrole-2-carboyxlate (**4**). Average of duplicates.

The confirmation of pyrrole-2-carboxylate as a precursor of dechloroisorumbrin prompted investigation of the possible incorporation of chlorinated pyrroles into the polyenes. Both 3- and 4-chloropyrrole-2-carboxylate (**5**, **6**) were synthesized for use in feeding studies (Scheme 2). **5** was synthesized in two steps from 4-chloro-pyridine-*N*-oxide (**7**), which was subjected to photolysis



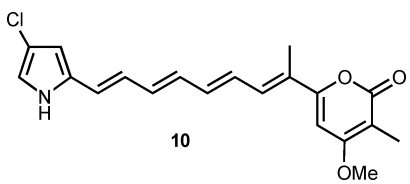
Scheme 1 Biosynthetic origin of atoms in dechlororumbrin (**2**).



Scheme 2 Synthesis of chloropyrroles. *Reagents and conditions*: (i) $CuSO_4$, H_2O, hν, rt, 18 h, 33%; (ii) Ag_2O, H_2O/MeOH, rt, 18 h, 85%; (iii) SO_2Cl_2, $CHCl_3$, 0–25°C, 1 h; (iv) NaOH, rt, 30 min, 35% over 2 steps.

in the presence of $CuSO_4$[15,16] to yield 3-chloro-2-formyl pyrrole (**8**) in 35% yield. **8** was subsequently oxidised with Ag_2O to give the final product.[17] 4-Chloropyrrole-2-carboxylate (**6**) was synthesised using literature methods from 2-trichloroacetylpyrrole (**9**).[14,18]

An analogous feeding study was conducted as before, revealing that addition of **5** slightly enhanced the proportion of chlorinated polyenes (Table 2), although the overall amount of polyenes did not noticeably increase. In contrast, the addition of **6** significantly boosted production of chlorinated metabolites, and led to the appearance of a new peak (16.7 min, Fig. 1). While this compound was not isolated, based on its mass spectrum (*m*/*z* 358/360, M+H$^+$), characteristic pyrrolylpolyene UV-vis spectrum, and on biosynthetic arguments, we propose the structure **10**. Previous studies have shown that the substitution of the pyrrole ring has a pronounced effect on the cytotoxic and anticancer activity of these polyenes,[8] and this discovery opens the way for further SAR studies.

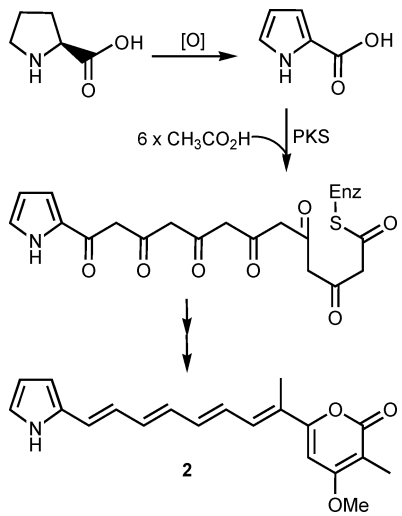


Label-dilution studies using chloropyrroles and [^{15}N]-L-proline were conducted as before. In an experiment in which 2 mM of both **5** and [^{15}N]-L-proline were added to a growing culture of

A. umbrinum, ^{15}N incorporation in dechloroisorumbrin (**2**) remained high (15.3%) while incorporation into rumbrin (**1**) was greatly reduced (3.1%). Similar results were observed for **6**, with moderate incorporation into **2** detected (9.1%), but almost none into the corresponding chlorinated polyene (2.1%).

Given the low incorporation of **5** relative to **4** and **6**, we suggest that while **5** is accepted as a substrate, it is not the natural precursor for rumbrin (**1**), and thus chlorination does not occur on the free pyrrole-2-carboxylate itself but at a later stage in the biosynthetic pathway.

In the biosynthesis of bacterial pyrrole-containing metabolites such as pyoluteorin and clorobiocin, the prolyl group is oxidised to a pyrrolecarboxyl moiety as a peptidyl carrier protein-bound species.[13,19] In clorobiocin biosynthesis, this is then transferred *via* several protein-bound intermediates to the growing metabolite.[20] In contrast, the facile incorporation of pyrrole-2-carboxylate (**4**) into rumbrin suggests that free **4**, rather than an enzyme-bound derivative, is taken up directly by the PKS as the starter unit for rumbrin biosynthesis. Similarly, we propose that the oxidation of proline to pyrrolecarboxylate occurs on the free amino acid and not a PCP-tethered derivative. The extension of the polyketide *via* the incorporation of acetate units is presumably catalyzed in the classical PKS fashion (Scheme 3), with subsequent reduction, dehydration, and methylation yielding the mature polyene.



Scheme 3 Proposed biosynthesis of **2**.

Biochemical chlorination assays

Halogenation of the pyrrole-containing bacterial metabolite pyoluteorin occurs *via* the actions of a $FADH_2$-dependent halogenase, PltA.[14] Thus the chlorination of rumbrin was further probed by incubating cell extracts of *A. umbrinum* DSM-3193 with NADH, NaCl plus either dechlororumbrin or pyrrole-2-carboxylate. However, no chlorinated product was detected by HPLC. However, it should be noted that the solubility of **2** in aqueous environments is very poor. In many halogenated pyrrole-containing natural products, C-4 of the pyrrole ring is the primary halogenation location. In many cases, this can be attributed to the presence of an electron withdrawing carbonyl group at C-2, which deactivates the C-3 and C-5 positions to electrophilic substitution. In contrast, the chlorine atom in rumbrin (**1**) occurs at C-3 of the pyrrole unit (C-4 of rumbrin), suggesting that in this instance halogenation occurs *after* polyketide extension and formation of the polyene chain. Whether this occurs on the finished polyene or on an ACP-bound intermediate species is unknown. For completeness the cell-free extract was also assayed for haloperoxidase activity, but none was detected.

Conclusions

Precursor-directed feeding studies have illustrated the origin of the atoms in the cytotoxic fungal polyene rumbrin (**1**), which is biosynthesed from proline, methionine, and acetate, probably by an iterative polyketide synthase enzyme complex. This represents the first biosynthetic study of a fungal pyrrolyl polyene. Label dilution experiments also confirmed pyrrole-2-carboxylate (**4**) as a direct precursor. This suggests that the incorporation of the pyrrole proceeds *via* a different mechanism to that which that occurs in the biosynthesis of bacterial pyrrole-containing metabolites. The chlorinated pyrrole **5** was also accepted as a substrate, though its low incorporation leads us to believe that it is not a natural precursor. Remarkably, the fungus was also able to incorporate the "un-natural" chloropyrrole **6** into the polyenes in high amounts. Further studies on the incorporation of other substituted pyrrolecarboxylates are in progress, and may allow the production of polyenes with improved biological properties.

Experimental

Auxarthron umbrinum DSM-3193 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). All chemicals were purchased from Sigma-Aldrich, except for labelled substrates, purchased from Cambridge Isotope Laboratories. All solvents were HPLC grade and used without further purification. Analytical TLC was performed on pre-coated plates (0.2 mm silica gel 60 on aluminium, Machery-Nagel). Compounds were visualised by staining with anisaldehyde dip and heating on a hot plate. Flash chromatography was performed on silica gel 60 (230–400 mesh, Merck). UV irradiation was conducted using a Phillips TUV 30W/G30 T8 Longlife Hg UV lamp.

HPLC was carried out using a Varian Prostar system consisting of two solvent delivery modules (210), DAD detector (335), autoinjector (410) and fraction collector (710). NMR was conducted using Varian Inova 300, 400, and 500 MHz spectrometers, and referenced to residual proton signals in the solvent (for DMSO, δ_H 2.50 and δ_C 39.5 ppm). IR spectra were recorded on a Varian 3100 Excalibur Series FT-IR spectrometer, and UV-vis spectra on a Thermo-Spectronic Helios-β spectrophotometer. Low resolution mass spectra were obtained using a Micromass Quattro mass spectrometer, and high resolution spectra on a Micromass LCT time-of-flight mass spectrometer, both coupled to a Waters Alliance 2695 solvent delivery system.

General procedure for feeding studies

A. umbrinum DSM-3193 was grown in 250 mL flasks containing 25 mL of a medium consisting of 2% glucose, 1.5% Pharmamedia (cottonseed flour), 0.5% yeast extract, 0.4% $CaCO_3$, 0.3% $(NH_4)_2SO_4$, and 0.003% $ZnSO_4.7H_2O$, at 28 °C on a rotary

shaker shaken at 200 rpm. Substrates for feeding studies were added after 3 days. After 7 days, the culture was filtered under vacuum and the mycelium extracted twice in 20 mL MeOH-CH_2Cl_2 (3:1) for one hour. Solvent extracts were analysed by HPLC and MS. Analytical HPLC was carried out using a standard gradient (Thermo Hypersil C18 4.6 × 150 mm 5 µm column, 0.8 mL/min, 10% MeCN/H_2O to 100% MeCN over 20 minutes), while amounts of individual polyene components were estimated by integration of peaks at 440 nm and comparison with standards. For determination of isotope incorporation, heights of the MS peaks at M+1 and M+2 (and M+3, M+4, as appropriate) were compared for the relevant metabolite(s), and corrected with reference to the unlabelled compounds.

[Methyl-^{13}C]-L-methionine feeding study

One culture flask was prepared as described above, with 12 mg (3 mM) [methyl-^{13}C]-L-methionine added after 3 days. The culture was worked up as described, and the polyene mixture partially purified as follows. The crude extract (34 mg) was dissolved in CH_2Cl_2 (5 mL), washed with water (2 × 5 mL), and the organic extract reduced to dryness under vacuum. This was resuspended in MeOH, washed with hexanes (3 × 5 mL), and the methanol layer reduced to dryness. The residue was futher purified by RP SPE (Thermo-Fisher Hypersep C_{18} 1 g cartridge, eluting with 60, 85, 100% MeOH/H_2O). The 85% MeOH fraction (1.4 mg) contained a mixture of polyenes and was analysed by ^{13}C NMR.

Production and isolation of labelled auxarconjugatin D (3)

Eight culture flasks were prepared as described above, with 40 mg (25 mM) [1–^{13}C]-acetate added to each flask after 3 days growth. The cultures were combined, extracted, and the crude extract (196 mg) partially purified by solvent partitioning and SPE as described above, to yield material enriched in polyenes (50.6 mg). This was further purified by HPLC (Zorbax StableBond 4.6 × 250 mm 5 µm column, 3 mL/min, isocratic 80% MeOH/H_2O) to yield **3** as a red solid (2.3 mg). UV λ_{max}(MeOH)/nm (ε) 262 (5300), 333 (5200), 444 (16600); IR ν_{max}(neat)/cm^{-1} 2917, 2849, 1688, 1628, 1539, 1449, 1404; ^{1}H NMR (d_6-DMSO, 500 MHz) see Table 1; ^{13}C NMR (d_6-DMSO, 125 MHz) see Table 1; HRESI(+)MS *m/z* 318.1098 (M+Na. $C_{18}H_{17}NO_3Na$ requires 318.1106).

2-Formyl-3-chloro-pyrrole (8)

4-Chloro-pyridine-*N*-oxide (**7**, 260 mg, 2 mmol) and $CuSO_4$.$5H_2O$ (4.85 g, 20 mmol) were dissolved in 100 mL dH_2O and irradiated under UV light for 18 h. The resulting brownish solution was extracted twice with $CHCl_3$, and the resulting organic extract washed with water, dried with $MgSO_4$, and reduced to dryness under vacuum. The crude product was purified by flash chromatography using EtOAc–hexane (1:4) to give **8** (83.5 mg, 33%) as a white solid. UV λ_{max}(MeOH)/nm (ε) 290 (8300); IR ν_{max}(neat)/cm^{-1} 3253, 1637; ^{1}H NMR (d_6-DMSO, 500 MHz) δ_H 12.35 (brs), 9.59 (d, $J = 0.9$), 7.24 (ddd, $J = 3.6, 2.8, 1.0$), 6.34 (dd, $J = 2.8, 2.5$); ^{13}C NMR (d_6-DMSO, 125 MHz) δ_C 176.8 (d), 127.1 (s), 126.5 (d), 121.8 (s), 110.6 (d); HRESI(–)MS *m/z* 127.9905 (M–H. C_5H_3NOCl requires 127.9903).

3-Chloropyrrole-2-carboxylate (5)

$AgNO_3$ (330 mg, 3 mmol) was dissolved in dH_2O (5 mL), and NaOH (200 mg, 5 mmol) added slowly to give a brown suspension of Ag_2O. To the stirred suspension was added a solution of 2-formyl-3-chloropyrrole (**8**, 50 mg, 0.38 mmol) in aqueous methanol (1:1). The resulting solution was stirred for 18 h at room temperature. TLC (hexane-EtOAc-HCOOH, 1:1:0.1) indicated no starting material was left, so the suspension was filtered and the solid residue washed with hot H_2O (2 × 10 mL). The aqueous filtrate was acidified and extracted with Et_2O (3 × 20 mL), and the combined ether layers dried over $MgSO_4$ and reduced to dryness under vacuum to yield **5** as an off-white solid (48 mg, 85%). UV λ_{max}(MeOH)/nm (ε) 259 (8500); IR ν_{max}(neat)/cm^{-1} 3335, 1667; ^{1}H NMR (d_6-DMSO, 500 MHz) δ_H 12.63 (brs), 11.91 (brs), 6.95 (dd, $J = 3.2, 2.6$), 6.20 (dd, $J = 2.6, 2.6$); ^{13}C NMR (d_6-DMSO, 125 MHz) δ_C 161.1 (s), 122.4 (d), 118.1 (s), 116.8 (s), 110.8 (d); HRESI(–)MS *m/z* 143.9854 (M–H. $C_5H_3NO_2Cl$ requires 143.9852).

4-Chloropyrrole-2-carboxylate (6)

Trichloroacetylpyrrole (213 mg, 1 mmol) was dissolved in $CHCl_3$ (5 mL), and cooled on ice. SO_2Cl_2 (168 µL, 2 mmol) was added dropwise with stirring. The solution was warmed to room temperature over an hour. The reaction mixture was quenched by adding ice, extracted with 50 mM Na_2HPO_4 (pH 7.0) until neutral, and the organic phase reduced to dryness under vacuum. The solid residue was dissolved in 2 M NaOH (10 mL) and stirred for 30 min at room temperature. The aqueous solution was acidified to pH 1, extracted with Et_2O (2 × 20 mL), and the organic extracts reduced to dryness. The crude product was purified by HPLC (Zorbax StableBond 4.6 × 250 mm 5 µm column, 5 mL/min, isocratic MeCN/H_2O (0.05% HCOOH)) to yield **6** as an off-white solid (38 mg, 35%). UV λ_{max}(EtOH)/nm (ε) 232 (5070), 265 (8150); IR ν_{max}(neat)/cm^{-1} 3363, 1695; ^{1}H NMR (d_6-DMSO, 500 MHz) δ_H 12.57 (brs), 12.02 (brs), 7.07 (dd, $J = 2.9, 1.7$), 6.68 (dd, $J = 2.3, 1.7$); ^{13}C NMR (d_6-DMSO, 125 MHz) δ_C 161.1 (s), 122.8 (s), 120.9 (d), 113.2 (d), 111.3 (s); HRESI(–)MS *m/z* 143.9848 (M–H. $C_5H_3NO_2Cl$ requires 143.9852).

Halogenase assays

Three-day cultures of *A. umbrinum* DSM-3193 were filtered, the mycelium resuspended in 50 mM PO_4^{3-} buffer (pH 7.0), and the cells ruptured using a French press at 1000 psi. The resulting lysate was centrifuged at 20,000 rpm to remove cell debris, and the supernatant decanted for use in assays.

Chloroperoxidase assay: cell-free extract (50 µL), 1 M NaCl (20 µL), 100 mM H_2O_2 (20 µL), and 10 mM monochlorodimedone (20 µL) were added to PO_4^{3-} buffer (pH 3.0, 900 µL) and incubated for 18 h at room temperature. Reaction progress was monitored by UV at 278 nm. As a positive control, chloroperoxidase enzyme (from *Caldariomyces fumago*, diluted × 100,000) was used instead of cell-free extract.

$FADH_2$-dependent halogenase assay: cell-free extract (50 µL), 1 M NaCl (20 µL), 10 mM NADH (50 µL), and substrate (5 µL) were added to PO_4^{3-} buffer (pH 7.0, 900 µL) and incubated for 18 h at room temperature. Reaction progress was monitored by HPLC.

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