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Production of anticancer polyenes through precursor-directed biosynthesis

Benjamin R. Clark,[a] Stephen O'Connor,[a] Deirdre Fox,[a] Jacques Leroy[b] and Cormac D. Murphy*[a]

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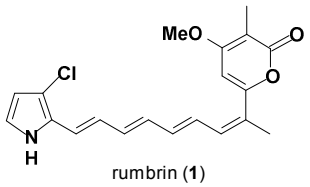
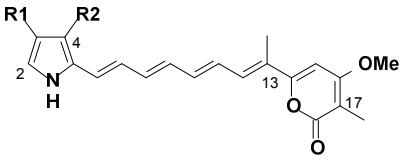
The biosynthesis of the pyrrolyl moiety of the fungal metabolite rumbrin originates from pyrrole-2-carboxylic acid. In an effort to produce novel derivatives with enhanced biological activity a series of substituted pyrrole-2-carboxylates were synthesised and incubated with the producing organism, *Auxarthron umbrinum*. Several 4-halo-pyrrole-2-carboxylic acids were incorporated into the metabolite yielding three new derivatives: 3-fluoro-, 3-chloro- and 3-bromo-isorumbrin, which were generated in milligram quantities enabling cytotoxicity assays to be conducted. The 3-chloro- and 3-bromo-isorumbrins had improved activity against HeLa cells compared with rumbrin; 3-bromoisorumbrin also showed dramatically improved activity towards a lung cancer cell line (A549).

Introduction

Rumbrin (**1**) is a polyenyl pyrrole produced by a number of fungi from the Onygenales family.[1,2] Naturally-occurring polyenyl-pyrroles are very rare, limited to a handful of examples, including the fungus-derived auxarconjugatins,[3] wallemias,[4] and malbranchpyrroles,[5] in addition to the keronopsins, brominated metabolites isolated from the marine ciliate *Pseudokeronopsis rubra*.[6]



rumbrin (**1**)



(12*E*)-isorumbrin (**1a**)	R_1 = H, R_2 = Cl
dechloroisorumbrin (**2**)	R_1 = H, R_2 = H
3-chloroisorumbrin (**3**)	R_1 = Cl, R_2 = H
3-bromoisorumbrin (**4**)	R_1 = Br, R_2 = H
3-fluoroisorumbrin (**5**)	R_1 = F, R_2 = H

The biological activity of **1** is of particular interest; it has been reported to possess cytoprotective activity,[1] and also acts as a calcium accumulation inhibitory agent and lipid peroxide production inhibitor.[7] An isomer of rumbrin, (12*E*)-isorumbrin (**1a**), displays nanomolar activity as a cytotoxic agent, showing particular activity against lung and ovarian cancer cell lines.[8]

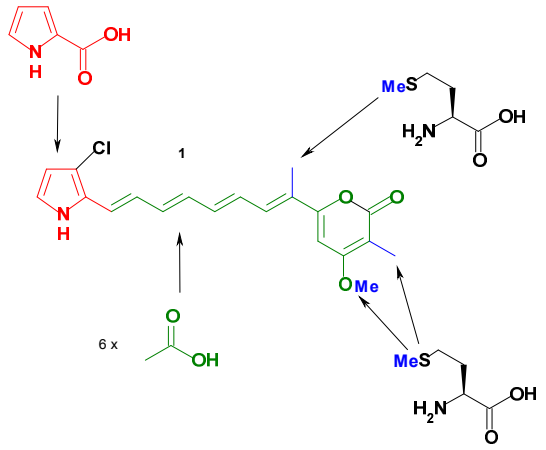
The related polyenes auxarconjugatins A and B, which contain a chloropyrrole-group, have also been reported to possess cytotoxic properties,[9] while in contrast, the furan-containing gymnoconjugatins possessed no significant activity.[8] Another group of pyrrolepolyenes, the malbrachpyrroles, were also found to possess cytotoxic properties against a range of cancer cell lines: it was suggested that they act by causing cell cycle arrest at the G0/G1 phase.[5]

Previous studies have produced a small number of unnatural rumbrin analogues. Supplementation of the growth medium of a rumbrin-producing strain *Gymnoascus reessii* with sodium bromide led to the production of a brominated analogue, in addition to the non-halogenated derivative dechlororumbrin (**2**), though both compounds possessed much reduced cytotoxicity.[10] In 2010, Fang and coworkers reported the synthetic production of rumbrin and a range of derivatives.[9] One of these analogues (17-demethyl, 17-butyl-rumbrin) possessed nanomolar activity against a lung cancer cell line (A549). Derivatives incorporating phenyl or thiophene rings in place of the terminal chloropyrrole group displayed no activity. Thus, the available evidence from both natural and modified pyrrolylpolyenes suggests a crucial role for the chloropyrrole moiety in their biological activity.

The biosynthesis of rumbrin has been investigated using feeding studies with isotopically labelled substrates. These studies revealed that rumbrin is biosynthesised from proline, methionine, and acetate (Scheme 1).[11] Intriguingly, it was also found that pyrrole-2-carboxylic acid is a direct precursor in the biosynthesis of rumbrin, and that the un-natural precursor 4-chloro-pyrrole-2-carboxylate can be successfully incorporated to form 3-chloroisorumbrin (**3**). Though the modified polyene was not isolated in this instance, this observation opened the possibility of producing additional rumbrin derivatives through precursor-directed biosynthesis.

In the current study we have followed up on these findings,

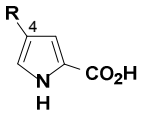
carrying out precursor-director biosynthesis with a range of substituted pyrrole-2-carboxylic acids. We report the production, characterisation and biological properties of three rumbrin derivatives: 3-chloroisorumbrin (**3**), 3-bromoisorumbrin (**4**) and 3-fluoroisorumbrin (**5**).



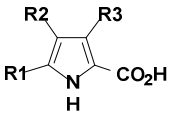
Scheme 1 The biosynthesis of rumbrin (**1**)

Results and Discussion

A small suite of substituted pyrrole-2-carboxylates (**6-17**) was used in the study. Acetyl-, chloro-, bromo-, and iodo-pyrrole carboxylic acids were synthesised from trichloroacetylpyrrole or pyrrole carboxylate methyl ester using electrophilic substitution strategies.[12] While 4-iodo-pyrrole carboxylic acid (**10**) was formed in quantitative yield, the other substitution reactions produced mixtures of 4-, 5-, and/or di-substituted pyrrole carboxylic acids. Attempts to produce fluorinated pyrrole-2-carboxylic acids by direct fluorination of trichloroacetylpyrrole using a range of fluorinating agents gave poor yields. Thus, alternative literature syntheses were used to produce pyrroles **9** and **16** instead.[13,14]



6 R = H
7 R = Cl
8 R = Br
9 R = F
10 R = I
11 R = $COCH_3$
12 R = NO_2



13 R_1 = Br, R_2 = H, R_3 = H
14 R_1 = $COCH_3$, R_2 = H, R_3 = H
15 R_1 = Cl, R_2 = Cl, R_3 = H
16 R_1 = H, R_2 = F, R_3 = F
17 R_1 = Me, R_2 = H, R_3 = Me

The commercially available strain *Auxarthron umbrinum* DSM-3193 was used in precursor-directed biosynthesis experiments, and grown using a rich cottonseed-flour based medium.[11] Precursors (3 mM) were introduced to the fungal culture after three days, immediately prior to the commencement of polyene biosynthesis. After seven days the cultures (25 mL) were filtered, the mycelium extracted with MeOH-CH_2Cl_2 (3:1), and the extracts analysed by electrospray mass spectrometry (ESI(+)MS) and HPLC to determine whether new polyenes had been produced. Altered HPLC profiles (Fig 1) were observed

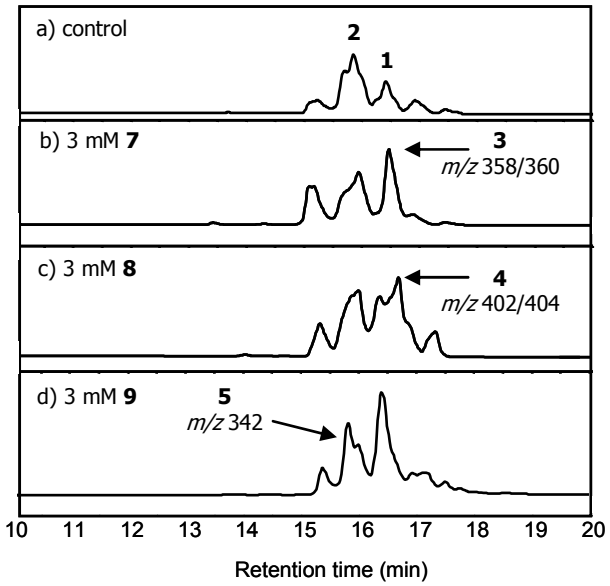


Fig. 1. HPLC analysis (DAD, 440 nm) of polyenes produced by *A. umbrinum* DSM-3193 cultures. (a) Unsupplemented medium, (b) medium plus 3 mM **7**, (c) plus 3 mM **8**, (d) plus 3 mM **9.** *M/z* values for selected peaks, corresponding to the new compounds **3-5**, are also given.

when the growth medium was supplemented with 4-chloro-, 4-bromo-, and 4-fluoro-pyrrole-2-carboxylates (**7-9**). In the latter two cases, new peaks were also observed in the mass spectrum, at *m/z* 402/404, and 342, respectively, suggesting successful production of the target polyenes. Pyrrole-carboxylates with larger substituents in the 4-position, **10-12**, were not accepted as the biosynthetic starter unit, nor were the 5-substituted (**13**, **14**) or di-substituted (**15-17**) acids. Supplementing the growth medium with pyrrole-2-carboxylic acid (**6**) led to an increased production of non-halogenated polyenes such as dechlororumbrin (**2**), as reported previously.[11]

Syntheses of the successfully incorporated precursor pyrroles **7-9** were conducted on a larger scale (100-200 mg), and the feeding studies repeated using 8 x 25 mL cultures for each experiment. The fungal mycelia were extracted as before and the extracts purified by solid-phase extraction and repeated HPLC to yield the new polyenes **3-5** in milligram quantities (1.6, 1.9, and 1.3 mg,).[†] The structures of the new polyenes were confirmed by spectroscopic analysis. High-resolution electrospray mass spectrometry revealed ions at *m/z* 358.1212 $(M+H)^+$, 402.0732 $(M+H)^+$, and 340.1353 $(M-H)^-$, respectively, consistent with the proposed molecular formulae.

For all three of the new compounds, ^1H and ^{13}C NMR resonances (Table 1) for the pyrone and polyene portions were very similar to those for isorumbrin (**1a**). However, significant changes were observed in the ^1H and ^{13}C NMR resonances for the pyrrole moiety. In **3**, the characteristic pyrrole resonances of **1a** at 6.90 (dd, J = 2.9, 3.0 Hz; H-2) and 6.13 (dd, J = 2.6, 3.0 Hz; H-3) had shifted to 6.94 (t, J = 1.8 Hz; H-2) and 6.29 (t, J = 1.8 Hz; H-4) ppm. The altered coupling constants, in particular, were characteristic of a 3-substituted pyrrole moeity. Similar pyrrole resonances at δ_H 6.95 (t, J = 1.8 Hz; H-2) and 6.33 (t, J = 1.9 Hz; H-4) were observed for **4**. Corresponding changes in the ^{13}C NMR spectra, in particular the observation of a relatively deshielded quaternary ^{13}C resonance (δ_C 96.2) indicative of the

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Table 1: ^{1}H and ^{13}C NMR data for rumbrin derivatives **3-5**

	3-chlororumbrin (**3**)		3-bromorumbrin (**4**)		3-fluororumbrin (**5**)	
	δ_C	δ_H [a]	δ_C	δ_H [a]	δ_C	δ_H [a]
N-1		11.39, br s		11.46, br s		10.92, s
2	117.5	6.94, t (1.8)	120.0	6.95, t (1.9)	103.4[b]	6.72, ovl
3	111.6	-	96.2	-	152.2[c]	-
4	108.2	6.29, t (1.8)	110.8	6.33, t (1.9)	95.9[d]	6.13, t (2.3)
5	130.7	-	131.5	-	136.9	-
6	123.8	6.42, d (15.3)	123.7	6.46, d (15.4)	124.5	6.42, d (15.0)
7	125.1	6.69, dd (15.3, 10.5)	125.1	6.71, dd (10.5, 15.6)	124.8	6.67, dd (11.0, 15.6)
8	135.9	6.52, dd (10.5, 14.4)	136.0	6.54, dd (10.9, 14.2)	136.1	6.53, dd (10.9, 14.5)
9	131.6	6.43, ovl	131.5	6.50, ovl	131.3	6.46, ovl
10	138.6	6.74, ovl	138.9	6.74, ovl	138.6	6.73, ovl
11	127.8	6.74, ovl	127.7	6.74, ovl	127.6	6.73, ovl
12	131.0	7.05, d (12.6)	131.2	7.05, d (10.0)	131.0	7.05, d (10.0)
13	125.8	-	125.8	-	125.7	-
14	158.8	-	159.0	-	158.8	-
15	93.7	6.57 (s)	93.6	6.57 (s)	93.6	6.56 (s)
16	166.0	-	165.9	-	166.0	-
17	100.3	-	100.4	-	100.3	-
18	163.3	-	163.4	-	163.3	-
13-Me	12.4	2.04, s	12.4	2.05, s	12.3	2.03, s
17-Me	8.7	1.82, s	8.7	1.82, s	8.7	1.82, s
OMe	56.7	3.98, s	56.7	3.96, s	56.7	3.97, s

[a] δ_H, mult. (coupling (Hz)). [b] doublet (J = 26.7 Hz). [c] doublet (J = 236 Hz). [d] doublet (J = 16.3 Hz). ovl = overlapping resonances.

presence of bromine substitution at C-3, were sufficient to unambiguously confirm the structure as shown. Compound **5** also possessed ^{1}H NMR resonances characteristic of a 3-substituted pyrrole moiety at 6.72 (brs; H-2) and 6.13 (t, J = 2.3 Hz; H-4) ppm. Confirmation of the presence of fluorine was given by the presence of a single fluorine resonance (δ_F -165.3, br s). In the ^{13}C NMR, one quaternary carbon resonance showed a very high chemical shift and large coupling constant, characteristic of fluorine attachment (δ_C 152.2, d, J = 236 Hz; C-3), while two other pyrrole carbons at δ_C 103.4 (d, J = 26.7 Hz; C-2) and 95.9 (d, J = 16.3 Hz, C-4) also displayed splitting due to proximity to fluorine. These data were all consistent with the proposed structure.

Compounds **1a, 3-5** were tested for cytotoxic activity against a range of cancer cell lines (Table 2) using an MTT colourimetric assay.[15] These showed promising results, with **1a**, **3**, and **4** showing nanomolar activity against a cervical cancer cell line (HeLa), with **4** displaying the highest activity (35 nM). This compound also displayed nanomolar activity against a lung cancer line (A549). Compounds **1a**, **3** and **4** displayed low levels of activity against a non-neoplastic control line (HDF). In contrast, the fluorinated derivative **5** exhibited much reduced activity against HeLa cells but moderate activity against the other three cell lines tested.

Conclusions

Precursor-directed biosynthesis is a convenient way to introduce new atoms or groups into known natural products, in particular halogens, which can impact on the biological activity of the compound in question[16] or provide a functionalizable handle for further modification.[17] These findings are of great interest as they show that the 3-chloropyrrole moiety is not absolutely essential for biological activity in this class of polyenes. In addition, the differences in selectivity displayed by the various derivatives, show that rumbrin derivatives may have potential as clinical anticancer agents, and that their potency and selectivity may be tuned by synthetic or biosynthetic manipulation of the pyrrole moiety.

Table 2 IC_{50} values of 12*E*-isorumbrin (**1a**) and derivatives **3-5** against selected cell lines.

	HeLa (cervical)	A549 (lung)	Jurkat (leukemia)	HDF[a] (fibroblast)
12*E*-isorumbrin (**1a**)	167 ± 8 nM	> 1 mM	~ 1 mM	> 1 mM
3-chloroisorumbrin (**3**)	64 ± 7 nM	> 1 mM	~ 1 mM	> 1 mM
3-bromoisorumbrin (**4**)	35 ± 5 nM	28 ± 1 nM	> 1 mM	> 1 mM
3-fluoroisorumbrin (**5**)	> 1 mM	25± 2 μM	27 ± 3μM	50± 5 μM

[a] Human dermal fibroblast.

Experimental

Auxarthron umbrinum DSM-3193 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). All media components were purchased from Sigma-Aldrich and Oxoid. 4-nitro-pyrrole-2-carboxylic acid (**12**) was purchased from Fluorochem. The fluorinated pyrroles **9** and **16** were synthesised according to literature procedures.[13, 14, 18] All other chemicals were purchased from Sigma-Aldrich and AGB, except as otherwise specified. All solvents were HPLC grade and used

without further purification.

HPLC was carried out using a Varian Prostar system consisting of two solvent delivery modules (210), diode array detector (335), autoinjector (410) and fraction collector (710). A Zorbax StableBond C-18 column, 9.4 x 250 mm, 5 μm particle size, was used in all preparative HPLC separations. NMR was conducted using Varian Inova 300, 400, and 500 MHz spectrometers, and referenced to residual signals in the solvent (for DMSO, δ_H 2.50 and δ_C 39.5 ppm), and $CFCl_3$ for ^{19}F. UV-vis spectra were recorded 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.

Cellular viability was assessed 48 h after addition of the test compound using the MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colourimetric assay. The assay is based on the reduction of the soluble yellow tetrazolium MTT salt, via the mitochondria of metabolically active cells, leading to the precipitation of purple water-insoluble formazan crystals. Relative numbers of viable cells are determined spectrophotometrically following solubilisation of the formazan crystals in DMSO. Prior to treatment, cells were seeded at a density of 10,000 cells per well (For HeLa and A549) in 96-well plates, in a volume of 150 μL of medium per well. After 24 h the medium was aspirated from cells and replaced with media containing the test compound at concentrations ranging from 1 x 10^{-4} M to 0.8 x 10^{-6} M. The plates were placed in the incubator for 48 h, after which 50 μL of a fresh sterile filtered solution of MTT (5 mg/mL) was added to each well of treated plates. The MTT solution was prepared using sterile PBS and filter sterilised using a 0.2 μm filter (Millipore). Plates were then returned to the incubator for 3 h. The media/MTT solution was aspirated from the well, with care taken not to dislodge the formazan crystals from the bottom of each well. The crystals were dissolved following addition of 200 μL DMSO to each well. Finally, absorbance of the resulting solution measured at 570 nm using a Wallac 1420 Multilabel HTS plate reader (Wallac, MD, USA). Absorbance values in treated plates were expressed as a percentage of untreated controls in order to obtain percentage viability values. The percentage viability values were plotted against the log of test compound concentration and a sigmoidal log dose curve was calculated by non-linear regression analysis using Graphpad Prism software version 5.0 for Windows (Graphpad Software, CA, USA). From these curves, IC_{50} values were obtained.

Chloropyrrole-2-carboxylic acids (7, 15)[12,19]

Trichloroacetylpyrrole (213 mg, 1 mmol) was dissolved in $CHCl_3$ (5 mL), and cooled on ice. SO_2Cl_2 (84 μL, 1 mmol) was added dropwise with stirring. Additional aliquots of SO_2Cl_2 were added until no starting material remained. 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 (30%, 0.05% HCOOH) to yield the two chlorinated products **7** (100 mg, 67%), and **15** (8.3 mg, 5%) as off-white solids.

4-chloro- (**7**) ^1H NMR (d_6-DMSO, 500 MHz) δ_H 12.57 (br s), 12.02 (br s), 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, 122.8, 120.9, 113.2, 111.3; ESI(–)MS *m/z* 144.2/146.2 (M–H), 100.1/102.1 (M–CO_2H).

4,5-dichloro- (**15**) ^1H NMR (d_6-DMSO, 300 MHz) δ_H 12.88 (br s), 6.80 (s); ^{13}C NMR (d_6-DMSO, 125 MHz) δ_C 160.1, 121.8, 116.4, 113.9, 108.8; ESI(–)MS *m/z* 178.3/180.3/182.3 (M–H), 134.2/136.2/138.2 (M–CO_2H).

Bromopyrrole-2-carboxylic acids (8, 13)[12, 20]

Trichloroacetylpyrrole (213 mg, 1 mmol) was dissolved in $CHCl_3$ (5 mL), and cooled on ice. Br_2 (60 μL, 1 mmol) was added dropwise with stirring. The solution was warmed to room temperature over an hour. Additional aliquots of Br_2 (60 μL) were added until MS revealed that no starting material remained. The reaction mixture was quenched by adding ice, extracted repeatedly 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 (30 min, rt). 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, 3 mL/min, isocratic MeCN/H_2O (35%, 0.05% HCOOH)) to yield **13** (8.2 mg, 4.4 %) and **8** (123 mg, 66%) as off-white solids.

4-bromo- (**8**). ^1H NMR (d_6-DMSO, 300 MHz) δ_H 12.58 (brs), 12.09 (brs), 7.09 (t, J = 1.5 Hz), 6.73 (br s); ^{13}C NMR (d_6-DMSO, 75 MHz) δ_C 161.4, 124.3, 123.7, 116.1, 96.0; ESI(–)MS *m/z* 188.1/190.1 (M–H), 144.0/146.0 (M–CO_2H).

5-bromo- (**13**). ^1H NMR (d_6-DMSO, 300 MHz) δ_H 12.43 (brs), 6.69 (d, J = 3.6 Hz), 6.18 (d, J = 3.6 Hz); ^{13}C NMR (d_6-DMSO, 75 MHz) δ_C 161.0, 125.3, 116.2, 111.6, 104.3; ESI(–)MS *m/z* 188.1/190.1 (M–H), 144.0/146.0 (M–CO_2H).

4-Iodopyrrole-2-carboxylic acid (10)[12, 21]

Iodine (120 mg, 0.47 mmol) was added in small portions to stirred solution of trichloroacetylpyrrole (110 mg, 0.52 mmol) and $AgNO_3$ (85 mg, 0.50 mmol) in $CHCl_3$ (5 mL) at 0 °C. The reaction mixture was allowed to warm to room termperature and stirred for a further 2 h. The suspension was then filtered, washed with Na_2SO_3 solution (5 mL, 5%) and water (5 mL, x 2), dried over $MgSO_4$, and concentrated under nitrogen. The residue was suspended in DMSO (0.5 mL) and K_2CO_3 (2 M, 1.5 mL) added dropwise. The mixture was stirred at room temperature for 3 h. This was then acidified with HCl, extracted with EtOAc (5 mL x 3), the organic layer dried over $MgSO_4$, and reduced to dryness under vacuum to yield **10** (123 mg, 100 %).

^1H NMR (d_6-DMSO, 300 MHz) δ_H 12.50 (br s), 12.01 (br s), 7.06 (t, J = 1.5 Hz), 6.76 (br s); ^{13}C NMR (d_6-DMSO, 75 MHz) δ_C 160.7, 128.1, 125.1, 120.6, 111.3, 61.5; ESI(–)MS *m/z* 236.0 (M–H), 192.0 (M–CO_2H).

Acetylpyrrole-2-carboxylic acids (11, 14)[12, 22]

A solution of methyl pyrrole-2-carboxylate (100 mg, 0.8 mmol) in $CHCl_3$ (2 mL) was added dropwise to an ice-cooled solution of acetic anhydride (160 μL, 1.6 mmol) and $AlCl_3$ (200 mg, 1.6 mmol) and stirred for 1 hour. The reaction was quenched by adding water, and the mixture extracted with CH_2Cl_2. The organic phase was washed with saturated $NaHCO_3$ (5 mL, x 2) and NaCl (5 mL), dried over $MgSO_4$ and reduced to dryness under vacuum to give 127 mg of crude product. This material was fractionated using HPLC (25% MeCN, 4 mL/min) to yield 4-acetyl- and 5-acetyl-pyrrole-2-carboxylate methyl esters. These were then hydrolysed separately by stirring in 2 M K_2CO_3 (1 mL, rt, 2 h), followed by a standard aqueous workup to yield the free acids **11** (26.5 mg, 22%) and **14** (19.4 mg, 16%).

4-acetyl (**11**). ^{1}H NMR (d_6-DMSO, 300 MHz) δ_H 12.67 (brs), 12.33 (brs), 7.66 (br s), 7.05 (br s) 2.35 (s, 3H); ^{13}C NMR (d_6-DMSO, 75 MHz) δ_C 192.5, 161.6, 127.8, 126.2, 124.6, 113.8, 27.1; ESI(–)MS *m/z* 152.1 (M–H).

5-acetyl (**14**). ^{1}H NMR (d_6-DMSO, 300 MHz) δ_H 12.85 (brs), 12.17 (brs), 6.94 (d, J = 3.4 Hz), 6.77 (d, J = 3.4 Hz) 2.43 (s, 3H); ^{13}C NMR (d_6-DMSO, 75 MHz) δ_C 188.3, 160.6, 134.8, 128.2, 116.0, 115.0, 26.4; ESI(–)MS *m/z* 152.1 (M–H).

3,5-Dimethylpyrrole-2-carboyxlic acid (17)

3,5-Dimethylpyrrole-2-carboxylate ethyl ester (50 mg, 0.3 mmol), was dissolved in ethanol-5 M NaOH (1:3, 4 mL). The mixture was stirred at 80 ˚C for 2 h. Ethanol was removed on a rotary evaporator, water added (5 mL), and the reaction mixture extracted with EtOAc (10 mL x 2). The aqueous phase was adjusted to pH 4.5 with HCl, re-extracted with EtOAc (10 mL x 2), and the resulting organic layer dried over $MgSO_4$ and reduced under vacuum to yield **17** as a pale pink solid (31 mg, 73 %). Control of the pH during workup was important, as over-acidification led to product decomposition and very low yields.

^{1}H NMR (d_6-DMSO, 300 MHz) δ_H 11.78 (br s), 11.02 (br s), 5.69 (d, J = 1.8 Hz), 2.18 (s, 3H), 2.12 (s, 3H); ^{13}C NMR (d_6-DMSO, 75 MHz) δ_C 162.3, 132.1, 127.1, 117.4, 110.4, 12.6, 12.5; ESI(–)MS *m/z* 138.1 (M–H).

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 d. 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).

3-Chloroisorumbrin (3)

Eight flasks were used in the feeding experiment, using conditions as detailed above. After three days, 4-chloro-pyrrole-2-carboxylic acid (**7**, 20 mg/flask, 5.5 mM) was added to each flask. The mycelium from all flasks was combined and extracted twice with MeOH–CH_2Cl_2 (1:1, 200 mL) for one hour. The organic extract was reduced under vacuum, and partitioned between water and CH_2Cl_2 (150 mL), and the organic fraction reduced under vacuum and re-partitioned between hexane and methanol. The methanolic fraction was reduced under vacuum and fractionated by solid-phase extraction (Thermo-Fisher Hypersep C_{18} 1 g cartridge, eluting with 70, 80, 90, 100% MeOH/H_2O). The 90% and 100% MeOH fractions (69.6 mg) contained a mixture of polyenes and were further purified by RP HPLC (75% MeOH/H_2O at 3 mL/min, followed by 55% MeCN/H_2O at 4 mL/min) to yield 3-chloroisorumbrin (**3**, 1.6 mg, 0.44%). UV λ_{max}(MeOH)/nm (ε) 270 (6300), 340 (7400), 439 (21500); ^{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* 358.1212 (M+H. $C_{20}H_{21}NO_3Cl$ requires 358.1210).

3-Bromoisorumbrin (4)

Eight flasks were used in the feeding experiment, using conditions as detailed above. After three days, 4-bromo-pyrrole-2-carboxylic acid (**8**, 15 mg/flask, 3.2 mM) was added to each flask. The mycelium from all flasks was combined and extracted twice with MeOH–CH_2Cl_2 (1:1) for one hour. The organic extract was reduced under vacuum, partitioned between water-CH_2Cl_2, and the organic fraction reduced under vacuum and re-partitioned between hexane and methanol. The methanolic fraction was reduced under vacuum and fractionated by solid-phase extraction (Thermo-Fisher Hypersep C_{18} 1 g cartridge, eluting with 70, 80, 90, 100% MeOH/H_2O). The 90% and 100% MeOH fractions (60.5 mg) contained a mixture of polyenes and were further purified by RP HPLC (75% MeOH/H_2O, 3 mL/min) to yield 3-bromoisorumbrin (**4**, 1.9 mg, 0.74%). UV λ_{max}(MeOH)/nm (ε) 270 (7800), 330 (8200), 340 (8500), 439 (26100); ^{1}H NMR (d_6-DMSO, 500 MHz) see Table 1; ^{13}C NMR (d_6-DMSO, 100 MHz) see Table 1; HRESI(+)MS *m/z* 402.0732 (M+H. $C_{20}H_{21}NO_3Br$ requires 402.0705).

3-Fluoroisorumbrin (5)

Eight flasks were used in the feeding experiment, using conditions as detailed above. After three days, 4-fluoro-pyrrole-2-carboxylic acid (**9**, 15 mg/flask, 4.7 mM) was added to each flask. The mycelium from all flasks was combined and extracted twice with MeOH–CH_2Cl_2 (1:1) for one hour. The organic extract was reduced under vacuum, and partitioned between water-CH_2Cl_2, and the organic fraction reduced under vacuum and re-partitioned between hexane and methanol. The methanolic fraction was reduced under vacuum and fractionated by solid-phase extraction (Thermo-Fisher Hypersep C_{18} 1 g cartridge, eluting with 70, 80, 90, 100% MeOH/H_2O). The 90% and 100% MeOH fractions (121 mg) contained a mixture of polyenes and were further purified by RP HPLC (53% MeCN/H_2O at 4 mL/min, followed by 72% MeOH/H_2O at 3.5 mL/min) to yield 3-fluoroisorumbrin (**5**, 1.3 mg, 0.41%). UV λ_{max}(MeCN)/nm (ε) 225 (3400), 267 (5800), 338 (3900), 438 (22000); ^{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* 340.1353 (M-H. $C_{20}H_{19}NO_3F$ requires 340.1349).

Notes and references

[a] *School of Biomolecular and Biomedical Sciences, Centre for Synthesis and Chemical Biology, Ardmore House Annexe, University College Dublin, Belfield, Dublin 4, Republic of Ireland. Fax: (+353 1) 716 1183; Tel: (+353 1) 716 1311; E-mail: cormac.d.murphy@ucd.ie*

[b] *Ecole Normale Supérieure, Département de Chimie, 24 rue Lhomond F-75231, Paris Cedex 05, France. Fax: (+33 1)4432 3325; E-mail: jacques.leroy@ens.fr*

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