The Effect of Halide Salts on the Production of *Gymnoascus reessii* Polyenylpyrroles

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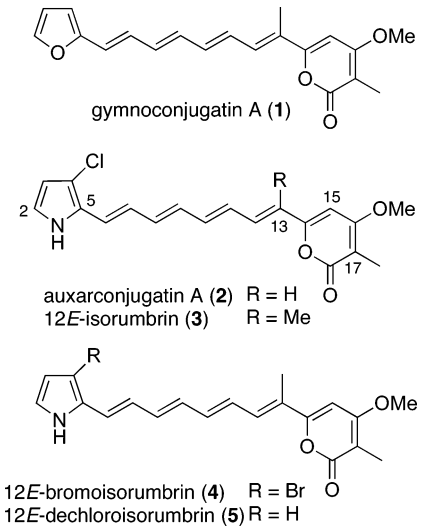
Addition of NaCl to the solid-phase fermentation of an Australian isolate of *Gymnoascus reessii* resulted in enhanced production of chloropolyenylpyrroles, while the addition of NaBr suppressed chloropolyenylpyrrole production in favor of bromo and dechloro analogues. Access to a wider selection of polyenylpyrroles provided scope for SAR comparisons on this rare class of cytotoxic natural products, with the bromo- and dechloropolyenylpyrroles displaying significantly reduced cytotoxicity. These results suggest that the chloro substituent is a critical element in the pharmacophore for this rare class of natural product.

During recent investigations into the chemistry of an Australian isolate of *Gymnoascus reessii*, MST-F9977, we reported novel compounds across a range of structural classes, including new butenolides (e.g., gymnoascolide A),[1] a new diketopiperazine (i.e., roquefortine E),[2] unprecedented polyenylfurans (e.g., gymnoconjugatin A (**1**)),[3] and chloropolyenylpyrroles [i.e., auxarconjugatin A (**2**) and 12*E*-isorumbrin (**3**)].[3] This latter class of compound was of particular interest both at a biological activity level, because of its potent cytotoxic properties, and at a biosynthetic level because of a compelling structural similarity to a rare family of marine ciliate bromopolyenylpyrroles, the keronopsins.[4] Halogenated polyenylpyrroles are a particularly uncommon class of natural product that are not available from commercial sources, nor are they readily available from microbial fermentation or chemical synthesis. Prior to our studies on *G. reessii*, chloropolyenylpyrroles were only known from two genera of terrestrial fungi, *Auxarthron*[5−7] and *Wallemia*,[8] while reports of bromopolyenylpyrroles were limited to a single investigation on the marine ciliate *Pseudokeronopsis rubra*.[4] Following from our earlier study we were keen to explore structure−activity relationships (SAR) within the *G. reessii* polyenylpyrroles. Encouraged by the precedent of brominated marine analogues, we manipulated the fermentation of MST-F9977 through the addition of halide salts in the hope of gaining access to a wider cross section of halopolyenylpyrroles. This report describes the production, isolation, and identification of 12*E*-bromoisorumbrin (**4**) and 12*E*-dechloroisorumbrin (**5**). These new analogues were used to advance SAR investigations into the cytotoxic properties of polyenylpyrrole natural products.

Preliminary small-scale (single Petri dish) solid-phase fermentations of MST-F9977, with and without the addition of NaCl or NaBr, provided promising results. HPLC-DAD-ESIMS analysis (Figure 1, Table 1) of MeOH extracts derived from 21-day fermentations confirmed that NaCl stimulated polyenylpyrrole production (× 2.5) relative to a control culture, while the addition of NaBr did not alter overall production levels. On the other hand, while chloro analogues were the dominant polyenylpyrroles in the HPLC-DAD-ESIMS analysis of control and NaCl-augmented extracts, these metabolites were almost entirely replaced in the NaBr-augmented extract by a suite of new metabolites *tentatively* identified as bromopolyenylpyrroles (i.e., **4** and isomers). These new bromo metabolites as listed in Table 1 displayed ESIMS pseudomolecular ions with characteristic bromine isotope patterns. The addition of NaBr to the fermentation media also stimulated the production of a dehalopolyenylpyrrole, **5**, as evidenced by an HPLC peak displaying a characteristic polyenylpyrrole UV−vis spectrum (DAD) but with ESIMS pseudomolecular ions lacking halogen isotope patterns. Given these initial promising results, further small-scale fermentations were carried out with the addition of KI and KSCN to the culture medium. However, HPLC-DAD-ESIMS analyses of MeOH extracts derived from these fermentations were identical to those of the control culture.



In an attempt to isolate and identify new polyenylpyrroles we undertook a larger scale, solid-phase fermentation of MST-F9977 on malt extract agar (MEA) containing 0.5% (w/v) NaBr (100 × 15 g petri dishes, incubated for 21 days at 24 °C). The resulting fermentation product was extracted with MeOH (2 × 2 L) and fractionated by solvent partition, silica column chromatography, and reversed-phase HPLC, to yield the new polyenylpyrroles **4** and **5**.

High-resolution ESI(+)MS analysis of **4** revealed a pseudomolecular ion (M + Na) consistent with a molecular formula ($C_{20}H_{20}O_3N^{79}Br$, Δmmu −1.3) indicative of a bromopolyenylpyrrole. The ^1H and ^{13}C NMR data for the pyrone ring and most of the polyenyl chain in **4** were virtually identical to those for 12*E*-isorumbrin (**3**), while analysis of ^1H NMR coupling constants confirmed an all-*E* geometry along the polyene chain (Table 2).

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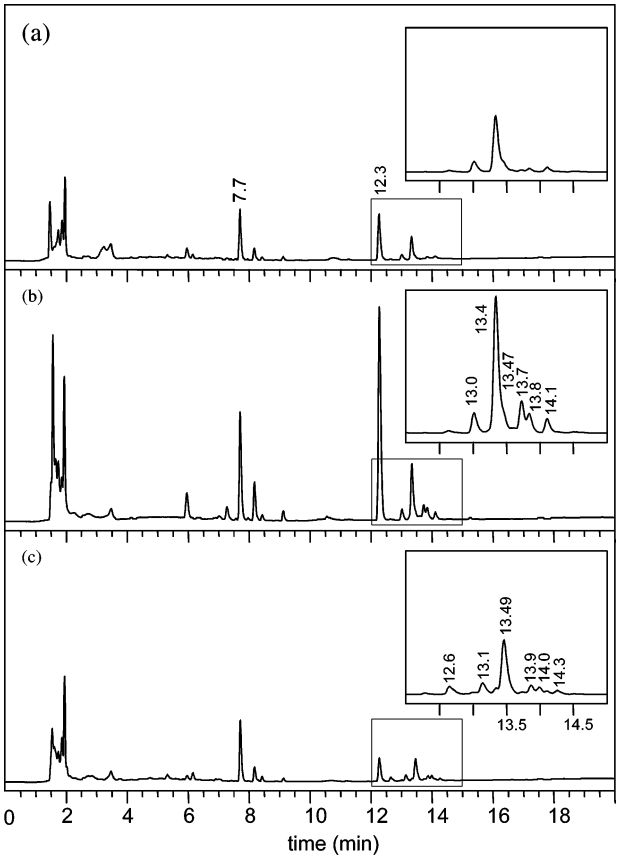

Figure 1. HPLC-DAD-ESIMS analysis of *G. reessii* cultures after 21 days, with (a) no added halide salts; (b) added NaCl; (c) added NaBr. Main traces are 254 nm DAD extracts, while expanded traces targeting polyenylpyrroles are 400 nm DAD extracts. Selected components are listed and tentatively identified in Table 1.

Table 1. Analysis of Selected Metabolites Produced by *G. reessii* Refermentations

retention time (min)	compound	ESI(+)MS $[M + H]^+$
	Non-polyenes	
7.7	roquefortine E	458
12.3	gymnoascolide A	251
	Non-halogenated Polyenes	
12.6	**5**	324
13.47	**1**	325
	Chlorinated Polyenes	
13.0	**2**	344/346[a]
13.40	**3**	358/360[a]
13.7−14.1	**3** isomers	358/360[a]
	Brominated Polyenes	
13.1	unknown	388/390[b]
13.49	**4**	402/404[b]
13.9−14.3	**4** isomers	402/404[b]

[a] ~3:1 ratio, consistent with the presence of Cl. [b] ~1:1 ratio, consistent with the presence of Br.

The most notable difference in the NMR data between **4** and **3** was centered around the pyrrole moiety, with a significant upfield shift of C-4 in **4** (δ_C 98.3) relative to **3** (δ_C 111.8), consistent with a C-4 bromo versus chloro substituent (Table 2). This analysis permitted the structure elucidation of **4** as 12*E*-bromoisorumbrin.

High-resolution ESI(+)MS analysis of **5** revealed a pseudomolecular ion (M + Na) consistent with a molecular formula ($C_{20}H_{21}O_3N$, Δmmu −0.4) indicative of a dehalopolyenylpyrrole. As with **4**, the ^{1}H and ^{13}C NMR data for the pyrone ring and most of the polyenyl chain in **5** were virtually identical to those for **3** (Table 2), while the appearance of an additional pyrrolomethine proton resonance (δ_H 6.23) confirmed the absence of a C-4 halo substituent. Analysis of the ^{1}H NMR coupling constants along the polyenyl chain was supportive of an all-*E* geometry; however, the multiplicity of some resonances appeared complicated, possibly by long-range and/or second-order effects. In light of this potential ambiguity the all-*E* geometry was further confirmed by the observation of a strong correlation between the ^{1}H and ^{13}C NMR polyenyl resonances in **5** with those for the all-*E* metabolites 12*E*-isorumbrin (**3**) and auxarconjugatin C.[5] Hence **5** could be identified as 12*E*-dechloroisorumbrin.

Cytotoxicity screening against an NS-1 cell line confirmed that 12*E*-dechloroisorumbrin (**5**) was noncytotoxic at concentrations up to 15 μM, while 12*E*-bromoisorumbrin (**4**) was considerably less cytotoxic (LD_{99} = 1.9 μM) than 12*E*-isorumbrin (**3**) (LD_{99} = 0.034 μM). This study suggests that the C-4 halo substituent on *G. reessii* polyenylpyrroles is a key structural characteristic in the cytotoxicity pharmacophore, with chlorine exhibiting a greater cytotoxic influence than bromine. Of note, *G. reessii* halopolyenylpyrroles appear to exhibit selective cytotoxicity insofar as none of these compounds displayed antibacterial, nematocidal, or antiprotozoan activity at concentrations up to 30 μM, nor did they display antifungal activity at concentrations up to 70 μM for **3** and **4**, and 30 μM for **5**.

Experimental Section

General Experimental Procedures. The procedures are as previously reported.[9] Silica column chromatography was carried out under nitrogen pressure with a flow rate of 5 mL/min.

Biological Material. The fungal strain (MST-F9977) was isolated from a roadside soil sample collected in July 1995 near Sussex Inlet on the southern coast of New South Wales, Australia, in an area regenerating from a recent bushfire. The isolate was identified as an ascomycete, *Gymnoascus reessii* Baranetzki, on morphological grounds. On malt extract agar (MEA) it is characterized by orange hyphae and a pinkish reverse.

Analysis. Analytical HPLC-DAD-MS traces were acquired using the following conditions: 1 mL/min gradient elution from 90% H_2O/MeCN (0.05% HCOOH) to MeCN (0.05% HCOOH) over 15 min, followed by 5 min flush with 100% MeCN (0.05% HCOOH). A Phenomemex LUNA $C_{8(2)}$ column (4.6 mm × 15 cm, 5 μm particle size) was used for the analyses.

Bioassays. Cytotoxicity, antibacterial, antifungal, nematocidal, and antiprotozoan assays were carried out as described previously.[3]

Preliminary Fermentation Study. MST-F9977 was cultured on MEA (malt extract (2%), peptone (0.1%), glucose (2%), agar (2%)) in individual Petri dishes for 21 days at 24 °C in the presence and absence of 0.5% (w/v) NaCl or NaBr or KI or KSCN. The cultures were excised from the Petri dishes and extracted with MeOH (20 mL).

Fermentation, Extraction, and Isolation. A solid-phase fermentation of MST-F9977 on MEA supplemented with NaBr (0.5% w/v) (100 × 15 g Petri dishes, 21 days, 24 °C) was extracted with MeOH (2 × 2 L). The extract was concentrated *in vacuo* to an aqueous residue (~800 mL) and then eluted with MeOH (3 × 40 mL) through two parallel 10 g Varian HF C_{18} SPE cartridges. The combined MeOH fractions were concentrated *in vacuo* to yield a residue (1.51 g), a portion of which (735 mg) was subjected to solvent partitioning between *n*-BuOH and H_2O. The *n*-BuOH-soluble material (562 mg) was concentrated *in vacuo* and then partitioned between petroleum ether (bp 60−80 °C) and 20% H_2O/MeOH, with the aqueous MeOH phase further partitioned with CH_2Cl_2. The CH_2Cl_2-soluble material (329 mg) was then subjected to silica column chromatography (20 mm diameter × 12 cm, Merck Keiselgel 60), eluting with 2% MeOH/CH_2Cl_2 to yield a fraction highly enriched in polyenes (46 mg). Finally, repeated preparative reversed-phase HPLC of 40 mg of this polyene fraction (20 mL/min isocratic elution with 25% H_2O/MeOH through a Zorbax StableBond C_{18} 5 μm 20 × 150 mm column, followed by 3.2 mL/min isocratic elution with 30% H_2O/MeCN, through a Zorbax StableBond C_{18} 5 μm 10 × 250 mm column) yielded the two purified polyenes 12*E*-bromoisorumbrin (**4**, 2.5 mg, 0.5%) and 12*E*-dechloroisorumbrin (**5**, 0.4 mg, 0.08%). Significant additional amounts of the polyenes in the form of isomers were also detected analytically, but were not

Table 2. NMR Data (d_6-DMSO, 600 MHz)[a]

	12*E*-bromoisorumbrin (**4**)			12*E*-dechloroisorumbrin (**5**)	
position	δ_C	δ_H (*J* in Hz)	HMBC (^{1}H to ^{13}C)	δ_C	δ_H (*J* in Hz)
N-1		11.57, s	C-3, C-4, C-5		11.16, brs
C-2	121.4	6.93, brs	C-3, C-4, C-5	120.3	6.84, dd (2.4, 3.7)
C-3	111.6	6.19, brs	C-2, C-5	109.0	6.06, dd (2.7, 5.5)
C-4	98.3			109.8	6.23, brs
C-5	127.7			130.6	
C-6	121.2	6.48, d (15.8)	C-4, C-8	124.9	6.53, d (15.6)
C-7	125.1	6.80, dd (11.3, 15.8)	C-5, C-8, C-9	123.3	6.65, dd (10.4, 15.6)
C-8	136.2	6.62, dd (11.3, 15.0)	C-6, C-9, C-10	136.8	6.55, dd (10.4, 14.8)
C-9	131.7	6.47, dd (10.8, 15.0)	C-7, C-11	130.2	6.42, dd (10.0, 14.8)[b]
C-10	138.6	6.72, dd (10.8, 14.4)	C-12	138.7	6.71, m
C-11	127.9	6.79, dd (11.6, 14.4)	C-9	126.9	6.71, m
C-12	131.1	7.05, d (11.6)	C-10, C-14, 13-Me	131.0	7.05, d (9.4)[b]
C-13	125.9			125.1	
C-14	158.8			158.9	
C-15	93.7	6.58, s	C-13, C-14, C-16, C-17	93.3	6.56, s
C-16	166.0			166.0	
C-17	100.3			100.1	
C-18	163.4			163.4	
13-Me	12.4	2.05, s	C-12, C-13, C-14	12.1	2.05, s
16-OMe	56.7	3.95, s	C-16	56.4	3.95, s
17-Me	8.7	1.81, s	C-16, C-17, C-18	8.5	1.81, s

[a] All assignments made with the assistance of gHSQC and gHMBC correlations and by comparison to known compounds. [b] Displays an irregular potentially second-order coupling pattern.

isolated. The percent yield is calculated against the *n*-BuOH-soluble mass (562 mg).

12*E*-Bromoisorumbrin (4): red solid; UV−vis (MeOH) λ_{max} (ϵ) 441 (58 000), 341 (17 000), 327 (sh) (15 000), 268 (17 000), 225 (12 000) nm; ^{1}H NMR (d_6-DMSO, 600 MHz) see Table 2; ^{13}C NMR (d_6-DMSO, 150 MHz) see Table 2; ESI(+)MS *m*/*z* 825/827/829 [2M + Na]$^+$, 424/426 [M + Na]$^+$, 402/404 [M + H]$^+$; HRESI(+)MS *m*/*z* 424.0511 ([M + Na]$^+$, $C_{20}H_{20}O_3N^{79}BrNa$ requires 424.0524).

12*E*-Dechloroisorumbrin (5): red solid; UV−vis (MeOH) λ_{max} (ϵ) 446 (28 000), 340 (8000), 327 (sh) (7200), 268 (9000), 225 (6900) nm; ^{1}H NMR (d_6-DMSO, 600 MHz) see Table 2; ^{13}C NMR (d_6-DMSO, 150 MHz) see Table 2; ESI(+)MS *m*/*z* 669 [2M + Na]$^+$, 346 [M + Na]$^+$, 324 [M + H]$^+$; HRESI(+)MS *m*/*z* 346.1415 ([M + Na]$^+$, $C_{20}H_{21}O_3NNa$ requires 346.1419).

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References and Notes

(1) Clark, B.; Capon, R. J.; Lacey, E.; Tennant, S.; Gill, J. H.; Bringmann, G.; Bulheller, B. *J. Nat. Prod.* **2005**, *68*, 1226−1230.
(2) Clark, B.; Capon, R. J.; Lacey, E.; Tennant, S.; Gill, J. H. *J. Nat. Prod.* **2005**, *68*, 1661−1663.
(3) Clark, B. R.; Capon, R. J.; Lacey, E.; Tennant, S.; Gill, J. H. *Org. Lett.* **2006**, *8*, 701−704.
(4) Hoefle, G.; Pohlan, S.; Uhlig, G.; Kabbe, K.; Schumacher, D. *Angew. Chem.* **1994**, *106*, 1561−1563.
(5) Hoseo, T.; Fukushima, K.; Takizawa, K.; Miyaji, M.; Kawai, K.-I. *Phytochemistry* **1999**, *52*, 459−463.
(6) Yamagishi, Y.; Shindo, K.; Kawai, H. *J. Antibiot.* **1993**, *46*, 888−891.
(7) Alvi, K. A.; Rabenstein, J. *J. Ind. Microbiol. Biotechnol.* **2004**, *31*, 11−15.
(8) Badar, Y.; Lockley, W. J. S.; Toube, T. P.; Weedon, B. C. L.; Valadon, L. R. G. *J. Chem. Soc., Perkin Trans. 1* **1973**, 1416−1424.
(9) Clark, B.; Capon, R. J.; Lacey, E.; Tennant, S.; Gill, J. H. *Org. Biomol. Chem.* **2006**, *4*, 1512−1519.

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