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ORIGINAL PAPER

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Auxarthrol A and auxarthrol B: two new tetrahydoanthraquinones from *Auxarthron umbrinum*

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Abstract Two new 1,2,3,4-tetrahydroanthraquinones, auxarthrol A (compound 1) and auxarthrol B (2), along with three known pyrrolyloctatetraenyl-α-pyrone pigments, auxarconjugatin A (3), auxarconjugatin B (4) and rumbrin (5), were isolated from the fungus *Auxarthron umbrinum*. Structure elucidation of new compounds 1 and 2 was accomplished by spectroscopic data analysis while identification of the known pigments (3–5) was achieved by LC-MS-photodiode array detection.

Keywords *Auxarthron umbrinum* · 1,2,3,4-Tetrahydroanthraquinone · Auxarthrol A · Auxarthrol B · Pyrrolyloctatetraenyl-α-pyrone pigments

Introduction

In the course of investigating the tetrahydroanthraquinones produced by the fungus *Auxarthron umbrinum*, we isolated two new 1,2,3,4-tetrahydroanthraquinones, auxarthrol A (**1**) and auxarthrol B (**2**), along with three known pigments; auxarconjugatin A (**3**), auxarconjugatin B (**4**) [6] and rumbrin (**5**) [13]. The presence of 1,2,3,4-tetrahydroanthraquinones in *A. umbrinum* extract was unusual, since these octaketide derivatives were identified mainly from the fungal genera *Alternaria*, *Phomopsis*, *Juniperus*, *Dactylaria* and *Bostryconema* [3, 7, 8, 9, 11, 12]. This is the first report of 1,2,3,4-tetrahydroanthraquinones from the fungal genus *Auxarthron*.

All five compounds were isolated from countercurrent chromatography fractions; and their structures were determined by analyzing spectroscopic data. This paper describes the structure elucidation of the new compounds and the strategy used for the identification of the known compounds.

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Materials and methods

General

UV spectra were run on a Perkin–Elmer Lambda 6 spectrometer. For NMR experiments (in $CDCl_3$), a Bruker DRX-500 spectrometer was used, operated at 500 MHz for ^1H and 125 MHz for ^{13}C NMR. Mass spectra were recorded on an API 150 MCA mass spectrometer (PE Sciex Perkin–Elmer, Toronto, Ontario, Canada) interfaced with a PE Sciex ion-spray probe. Liquid chromatography was performed with a Hewlett Packard 1100 series binary pump on a C_8 column (5 μm, 30×4.6 mm; Monitor) and a Waters model 996 photodiode array spectrophotometric detector (Millipore Corp., Marlborough, Mass., USA). Optical rotation was measured on a AutoPol IV polarimeter (Rudolpu Research Analytical Corp., Flanders N.J., USA). Countercurrent partition chromatography (CPC) was performed on a high-speed countercurrent chromatograph (P.C. Inc., Potomac, Md., USA), equipped with a 320-ml coil (1.68 mm ID). Also included were a 10-ml sample loop (with six-way valve) and a valve to permit rapid switching of solvent between the head and tail ends of the coil. The instrument was connected to a Waters model 996 photodiode array spectrophotometric detector and a NEC Power Mate 486/33I computer.

The HPLC chromatography system consisted of a Waters HPLC equipped with a Waters 600 system controller and a Waters 996 photodiode array detector. Separation was achieved on a semi-preparative C_{18} column (250×10 mm; E. Merck) with a H_2O/CH_3CN linear gradient.

Microorganism

Fungal strain 45866 (*A. umbrinum*) was isolated from a soil sample collected in England, UK. The culture was isolated by suspending serially diluted soil in molten tap-water/agar (50 °C) with the composition (per liter): 15 g agar, 6 g lithium chloride, 200 mg tetracycline and 10 mg cyclosporin A. This was incubated in the dark at 25 °C for 5–14 days. Hyphae were immediately transferred to yeast/malt extract (YME) agar plates with the composition (per liter): 10 g malt extract, 4 g yeast extract, 4 g glucose, 15 g agar and 5 ml of a trace elements solution containing (per liter): 1 g $FeSO_4 \cdot 7H_2O$, 1 g $MnSO_4 \cdot H_2O$, 0.025 g $CuCl_2 \cdot 2H_2O$, 0.132 g $CaCl_2 \cdot 2H_2O$, 0.056 g H_3BO_3, 0.019 g $(NH_4)_6Mo_7O_{24} \cdot 2H_2O$ and 0.2 g $ZnSO_4 \cdot 7H_2O$ in 0.6 N HCl.

Taxonomic characterization of the producing strain

For morphological characterization, strain 45866 was grown on YME agar at 25 °C.

Colonies reached 1.9 cm after 10 days of growth. No soluble pigment was produced. The strain formed a dark-orange vegetative mycelium and a flocculose white aerial mycelium. Numerous orange-brown ascomata with loosely woven peridia were formed after several weeks of incubation, dispersed within the aerial mycelium. The peridial network consisted of thick-walled, orange-brown, smooth, anastomosing hyphae, 2.5–3.0 μm wide, slightly wider at the septa, with some short spines and some long, straight or distally bent appendages (Fig. 1B). Mature asci contained eight ascospores (Fig. 1D). Ascospores were globose, 2.5–3.6 μm diameter, hyaline to orange-brown and echinulate (Fig. 1A). Long, straight or distally curved peridial appendages were sparsely present (Fig. 1C). The presence of ornamented, globose ascospores justified the classification of this strain as a member of the genus *Auxarthron*, rather than *Gymnoascus*. In addition, the orange vegetative mycelium and presence of long, distally curved peridial appendages distinguished this strain as *A. umbrinum* (Boudier) Orr and Plunkett 1963 [4].

Fermentation

The fermentation procedure utilized was a two-step process, in which a suspension of spores and mycelia was inoculated into 250-ml flasks containing 25 ml of a nutrient seed medium having the composition (per liter): 20 g glucose, 15 g Pharmamedia (Trader's Protein), 5 g yeast extract, 3 g $(NH_4)_2SO_4$, 0.03 g $ZnSO_4$ and 4 g $CaCO_3$. After inoculation, the flasks were incubated on a rotary shaker at 250 rpm and 28 °C for 2 days. Then, 1-ml aliquots of the seed culture were used to inoculate 100 flasks (250 ml) containing 30 ml of a production medium with the composition (per liter): 10 g glucose, 30 g soluble starch (Sigma S-9765), 5 g casamino acids, 3 g beef extract (Difco 0126-17), 2 g yeast extract, 3 g NZ amine, 0.5 g dibasic K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.3 g KCl and 3 g $CaCO_3$. Following inoculation, the production flasks were incubated on a rotary shaker at 250 rpm and 28 °C for 7 days. The fermentation flasks were then harvested and the fermentation mixture from each flask was pooled (ca. 3 l) into a single extraction vessel for extraction.

Extraction and isolation

The pooled fermentation mixture (broth and mycelium) was homogenized and then extracted with three equal volumes of EtOAc. The combined extracts were evaporated under reduced pressure to dryness, to yield an oily residue (1.286 g). Initially, an aliquot (400 mg) of the crude extract was fractionated by dual-mode high-speed countercurrent chromatography. The solvent system and full chromatographic conditions were used as previously described [2].

Final purification was achieved using semi-preparative HPLC. Auxarthrol A (**1**, 7 mg) and auxarthrol B (**2**, 12 mg) were isolated from CPC fractions 69–74, while the known pyrrolyloctatetraenyl-α-pyrone pigments; auxarconjugatin A (**3**), auxarconjugatin B (**4**) and rumbrin (**5**) were isolated from pooled CPC fractions 14–17.

Auxarthrol A

Pale yellow powder; $[\alpha]_D^{25}$ +234.28 (c 0.007, MeOH); UV-vis λ_{max} = 200.0, 250.9, 312.5 and 362.5 nm; ^{1}H NMR see Table 1; ^{13}C NMR see Table 2; LRAPIMS (positive) m/z = 337 $[M+H]^+$; HRFAB (positive) = 337.1022 $[M+H]^+$, calculated for $C_{16}H_{17}O_8$ = 337.1030.

Auxarthrol B

Pale yellow powder; $[\alpha]_D^{25}$ +179.16 (c 0.012, MeOH); UV-vis λ_{max} = 190, 245, 291 and 346 nm; ^{1}H NMR see Table 1; ^{13}C NMR see Table 2; LRAPIMS (positive) m/z = 355 $[M+H]^+$; HRFAB (positive) = 355.1047 $[M+H]^+$, calculated for $C_{16}H_{19}O_9$ = 355.1040.

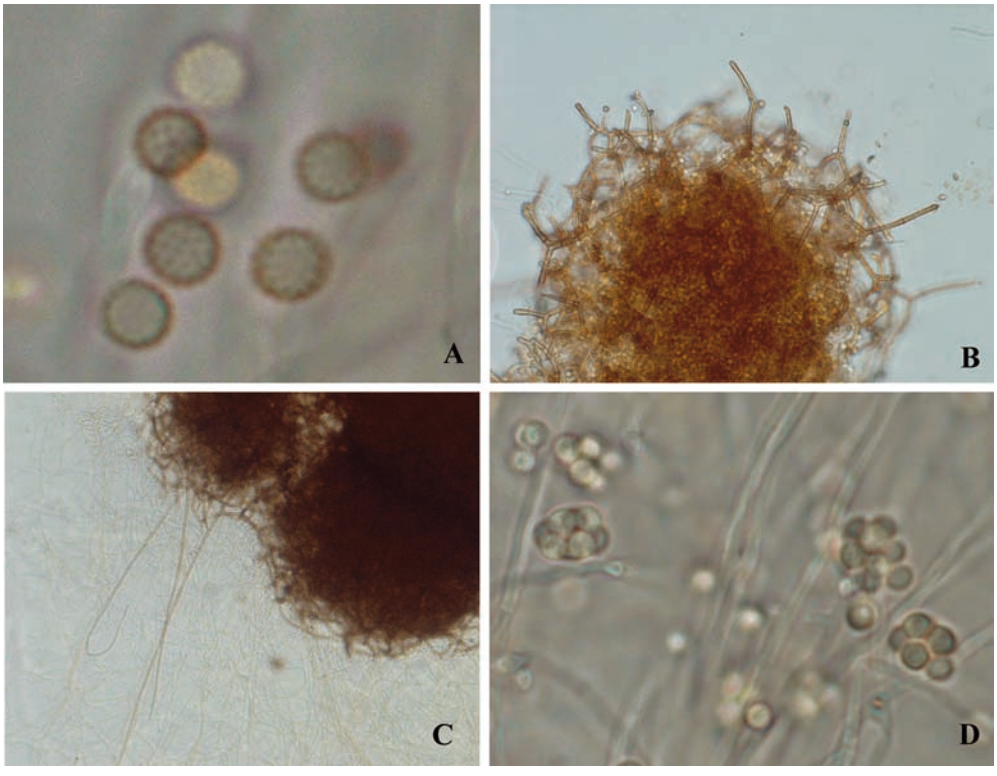
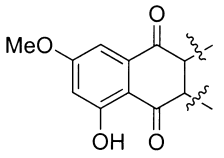
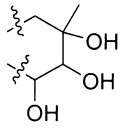


Fig. 1A–D Physical characteristics of *Auxarthron umbrinum* (Boudier) Orr and Plunkett 1963. **A** Ascospores are globose, 2.5–3.6 μm diameter, hyaline to orange-brown and echinulate. **B** The peridial network consists of thick-walled, orange-brown, smooth, anastomosing hyphae, 2.5–3.0 μm wide, slightly wider at the septa, with some short spines and some long, straight or distally bent appendages. **C** Long, straight or distally curved peridial appendages are sparsely present. **D** Mature asci contain eight ascospores



A



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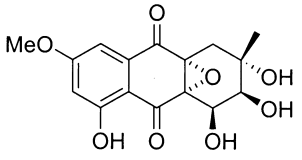

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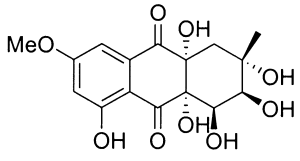

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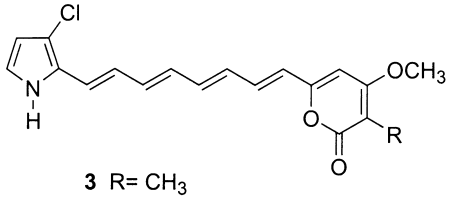
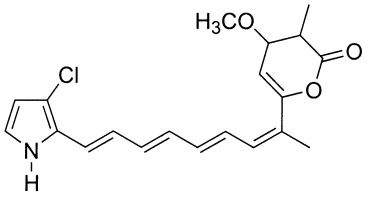


3 R= CH_3
4 R= H



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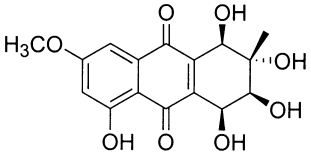

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Table 1 ^{1}H NMR data for Auxarthrols A-B and their derivatives. *d* Doublet, *dd* double doublet, *s*, *bs* singlet

Carbon number	Auxarthrol A (**1**)	Auxarthrol A acetonide	Auxarthrol B (**2**)	Auxarthrol B acetonide
1	2.11 (d, $J=15.9$ Hz)	2.30 (d, $J=15.7$ Hz)	1.67 (d, $J=14.9$ Hz)	1.87 (d, $J=14.9$ Hz)
1′	2.73 (d, $J=15.9$ Hz)	2.41 (d, $J=15.7$ Hz)	2.22 (d, $J=14.9$ Hz)	2.05 (d, $J=14.9$ Hz)
3	3.46 (d, $J=4.7$ Hz)	4.11 (d, $J=6.3$ Hz)	3.60 (d, $J=2.9$ Hz)	4.17 (d, $J=5.9$ Hz)
3-OH	4.51 (bs)		4.02 (bs)	
4	4.66 (dd, $J=4.7$, 5.4 Hz)	5.03 (d, $J=6.3$ Hz)	4.54 (d, $J=2.9$ Hz)	4.83 (d, $J=5.9$ Hz)
4-OH	5.39 (d, $J=5.4$ Hz)		4.70 (bs)	
5-OH	11.83 (bs)	11.93 (s)	11.79 (s)	11.59 (s)
6	6.94 (d, $J=2.1$ Hz)	6.90 (d, $J=2.1$ Hz)	6.79 (d, $J=2.1$ Hz)	6.80 (d, $J=2.1$ Hz)
8	6.98 (d, $J=2.1$ Hz)	6.99 (d, $J=2.1$ Hz)	6.92 (d, $J=2.1$ Hz)	6.92 (d, $J=2.1$ Hz)
Me	1.20 (s)	1.22 (s)	1.26 (s)	1.30 (s)
OMe	3.87 (s)	3.89 (s)	3.90 (s)	3.88 (s)
Me_2C		1.22 (s)		1.34 (s)
		1.31 (s)		1.37 (s)

Acetonides of auxarthrol A and auxarthrol B

One drop of sulfuric acid was added to a solution of auxarthrol A (4 mg) and auxarthrol B (4 mg) in dry acetone (1 ml). After stirring at room temperature for 2 h, the solution was poured into a dilute solution of sodium bicarbonate and the solution was extracted with ethyl acetate. The solvent was removed under vacuum.

Table 2 ^{13}C NMR data for auxarthrols A and B and their derivatives

Carbon number	Auxarthrol A (**1**)	Auxarthrol A acetonide	Auxarthrol B (**2**)	Auxarthrol B acetonide
1	35.9	29.7	31.4	31.1
2	69.4	67.3	72.8	70.9
3	63.9	70.4	62.8	70.2
4	72.3	79.2	76.3	76.2
5	164.0	165.3	163.3	163.3
6	106.6	107.9	105.1	105.4
7	166.0	167.0	165.1	165.3
8	107.3	108.3	106.2	105.9
9	189.6	190.1	193.2	192.4
10	193.3	193.2	200.0	200.5
11	108.5	109.5	110.7	110.2
12	133.0	133.4	135.4	135.1
13	64.6	61.8	79.8	79.6
14	65.6	63.9	79.9	79.4
Me	25.7	25.7	27.2	27.4
OMe	56.4	57.3	56.1	56.2
Me_2C		28.2		26.1
		29.3		25.9
Me_2C		108.9		108.7

Auxarthrol A acetonides: pale yellow powder; LRAPIMS (positive) m/z = 377 $[M+H]^+$.

Auxarthrol B acetonides: pale yellow powder; LRAPIMS (positive) m/z = 395 $[M+H]^+$.

Auxarconjugatin A

Dark red solid; UV λ_{max} = 218, 267, 330 and 440 nm; LRAPIMS (positive) m/z = 344 $[M+H]^+$.

Auxarconjugatin B

Dark red solid; UV λ_{max} = 221, 265, 331 and 432 nm; LRAPIMS (positive) m/z = 330 $[M+H]^+$.

Rumbrin

Dark red solid; UV λ_{max} = 225, 267, 338 and 441 nm; LRAPIMS (positive) m/z = 358 $[M+H]^+$.

Results and discussion

Auxarthrol A is a pale yellow compound. The ion-spray mass spectrum showed the molecular weight to be 336, or 337 $[M+H]^+$. The molecular formula of auxarthrol A was established to be $C_{16}H_{16}O_8$, $[M+H]^+$ 337.1022. The UV spectrum showed maxima at 200.0, 250.9, 312.5 and 362.5 nm. The UV spectrum of auxarthrol A is reminiscent of a dihydronaphthoquinonoid chromophore [7, 9]. Supporting evidence for this portion of the carbon skeleton was obtained by the ^{13}C NMR spectrum (Table 2), which suggested the presence of two conjugated carbonyl groups (δ 189.6, 193.3), and a teterasubstituted benzene (δ 106.6–166.0). Furthermore, the ^1H NMR spectrum (Table 1) showed a methyoxy singlet at δ 3.87, a phenolic proton resonance at δ 11.83 and two *m*-coupled aromatic proton doublets at δ 6.94 (J = 2.1 Hz) and δ 6.98 (J = 2.1 Hz). These data suggested the presence of a partial structure (system A), which was confirmed by a HMBC experiment, showing long-range coupling of H-8 to C-6 (δ 106.6), C-7 (δ 166.0), C-12 (δ 133.0), C-9 (δ 189.6) and C-11 (δ 108.5), similarly H-6 to C-5 (δ 164.0), C-7 and C-8 (δ 107.3). Furthermore, the methoxy protons showed two bonds coupling to C-7, while the phenolic proton showed two bonds coupling to C-5, confirming their attachment location. Thus, the existence of system A was confirmed.

The ^1H NMR spectrum also showed an AB doublet at δ 2.73 (J = 15.9 Hz) and δ 2.11 (J = 15.9 Hz), one doublet at δ 3.46 (J = 4.7 Hz), a double doublet at δ 4.66 (dd, J = 4.7, 5.4 Hz) and a singlet at δ 1.20. The carbon and proton connectivities were established through a HMQC experiment. Chemical shift considerations allowed us to place the oxygen atoms at C-2, C-3 and C-4. The ^1H-^1H connectivities determined from the COSY spectrum and the HMBC experiment allowed us to assemble a second partial structure (system B). Taking into consideration the number of oxygen atoms contained in auxarthrol A, it remained to account for the residual ethereal oxygen atom. The only positions available for attachment were at C-13 and C-14, which was further supported by the chemical shift of C-13 and C-14 and by comparison of the ^{13}C NMR chemical shift with that of other known naphthoquinone oxide systems [7, 9]. Subsequently, the overall structure was assembled by analysis of a few key long-range ^1H-^{13}C connectivities gleaned from a HMBC experiment: a two-bond ^1H-^{13}C correlation was observed between H-1 and C-13 and between H-4 and C-14. The gross structure of auxarthrol A appeared to be an oxidative derivative of 1,2,3,4-tetrahydroanthraquinone [10]. The overall framework again was confirmed by analysis of a ^1H-^1H COSY spectrum and HMQC and HMBC spectra.

The alicyclic ring of auxarthrol A is closely related to that of altersolanol E (**6** [10]), except for the absence of one methine proton attached to the carbon bearing a hydroxyl group. Instead, two upfield shifted methylene proton signals appeared at δ 2.73 (J = 15.9 Hz) and δ 2.11(J = 15.9 Hz). The chemical shifts and magnitude of vicinal coupling constants between C-3 and C-4 of auxarthrol A are almost identical to those reported for altersolanol E [10], suggesting the same stereochemistry at these centers. Corroboration for this conclusion was obtained from some other observations. Auxarthrol A formed an acetonide, which confirmed the presence of the C-3/C-4 *cis*-diol. The ^1H NMR spectrum of the acetonide showed the gem-dimethyl groups at δ 1.22 (s) and δ 1.31 (s), together with diequatorial proton signals of C-3 and C-4 at δ 4.11 (d, J = 6.3 Hz) and δ 5.03 (d, J = 6.3 Hz). These ^1H NMR data are very similar to those reported for altersolanol E acetonide, which further confirmed that both compounds share the same stereochemistry and conformation in the alicyclic ring. The ^1H NMR spectrum of auxarthrol A acetonide (Table 1) also provided some information about the relative stereochemistry of the epoxide ring. In the ^1H NMR spectrum of auxarthrol

A acetonide, one of the gem-dimethyl groups of the isopropylidene residue experiences considerable shielding (Table 1), which is clearly attributable to its position above the quinine double bond. It can be seen from Dreiding models that only when the oxiran ring and the acetonide are *trans* to each other in the acetonide would one of the gem-dimethyl groups of the acetonide be positioned above the quinine double bond. The proposed stereochemistry for the epoxide ring is further confirmed by comparing the C-13 and C-14 chemical shifts with those reported for nanaomycin E [7]. While this evidence is by no means unequivocal, it allows us to make a tentative assignment of the relative stereochemistry of the epoxide ring as shown in **1**.

The second compound, auxarthrol B, is also a pale yellow amorphous powder. The UV spectrum showed maxima at 190, 245, 291 and 346 nm. The UV spectrum suggested that compound **2** has a similar chromophore to that observed in **1**. This is further established by its ^{1}H NMR spectrum, which showed a methyoxy singlet at δ 3.90, a phenolic proton resonance at δ 11.79 and two *m*-coupled aromatic proton doublets at δ 6.79 ($J = 2.1$ Hz) and δ 6.92 ($J = 2.1$ Hz), a characteristic of system A. The ion-spray mass spectrum showed the molecular weight to be 354, or 355 $[(M+H]^{+}$. The molecular formula of auxarthrol B was established to be $C_{16}H_{18}O_9$, 355.1047 $[M+H]^{+}$, indicating that **2** may be a hydrated derivative of **1**. Most of the spectroscopic properties of **2** are very similar to **1** (Tables 1, 2). However, there were significant shift differences between carbon atoms C-13 and C-14, which were shifted by approximately 15 ppm and 14 ppm downfield from their positions in **1** and appeared at δ 79.6 and δ 79.4, respectively. These data suggested that **2** had a 13,14-dihydroxynaphthoquinone, instead of a 13,14-epoxynaphthoquinone moiety.

The relative stereochemistry of **2** is the same as **1**, based upon coupling constants and chemical shift comparisons. As expected, **2** formed an acetonide between the C-3/C-4 *cis* diol.

Although most of the proton and carbon chemical shifts of auxarthrol B were comparable to those of auxarthrol A, there were some significant differences between the NMR spectra of **1** and **2**, notably in the chemical shifts and coupling constants of the alicyclic ring protons of auxarthrol B. These differences reflect the change in magnetic field effects which accompany the formation of the diol at the C-13 and C-14 and the corresponding conformational changes on-going from the half-chair to the chair form. The coupling constant, $J = 2.9$ Hz, between the methine protons at C-3 and C-4 suggested that the C-3 proton is now truly equatorial. The C-4 proton signal is moved slightly upfield and appeared at δ 4.54 and the C-1 equatorial proton is at δ 2.22, upfield ($\Delta\delta$ 0.51) from its position in **1**. The slightly upfield shifts are probably due to the adjacent axial hydroxyl groups which are known to cause shielding of adjacent equatorial protons [5]. The *cis* configuration of the hydroxyl groups at C-13 and C-14 was also deduced from their ^{13}C NMR chemical shifts. The C-13 and C-14 resonance at δ 79.8 and δ 79.9 respectively, suggested that the configuration of both hydroxyl groups are the same. Though this evidence is not unambiguous, it allows us to propose a relative stereochemistry as depicted in **2** of the C-13 and C-14 hydroxyl groups.

In addition, three known pyrrolyloctatetraenyl-α-pyrone pigments, auxarconjugatin A, auxarconjugatin B [1] and rumbrin [2], were also identified from the CPC fractions of the *A. umbrinum* extract. The known pigments were identified by the strategy that we developed for the identification of known compounds present in CPC fractions [13]. The strategy employs a combination of LC-MS photodiode array detection and database search. In this case, identification of auxarconjugatin A, auxarconjugatin B and rumbrin was relatively easy, since these alkaloids yielded very distinct UV spectra. A search of natural products databases (e.g., Dictionary of Natural Products, published by Chapman & Hall) using the UV absorbance maxima and molecular weights for the pigments generated a very narrow list of potential structural matches, leading to the identification of auxarconjugatin A, auxarconjugatin B and rumbrin.

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