NEW PARAHERQUAMIDE ANTIBIOTICS WITH ANTHELMINTIC ACTIVITY†

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Paraherquamide (**1**) an anthelmintic alkaloid, was isolated from a species of *Penicillium* along with three novel analogues **2**, **3** and **4**. Anthelmintic activity at least equal to that of paraherquamide is described for the 14-de-hydroxy compound, VM 54159 (**4**).

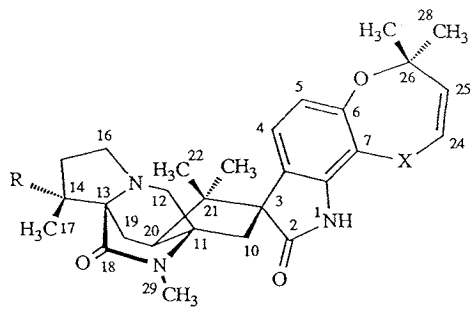
During a screening programme to detect metabolites with anthelmintic activity a strain, identified as a *Penicillium* species, was shown to produce paraherquamide and three, previously unreported, related metabolites.

Paraherquamide (**1**), an oxindole alkaloid originally isolated by YAMAZAKI *et al.*[3] from *Penicillium paraherquei* is structurally related to the marcfortines[4,5] isolated from *Penicillium roquefortii* and the brevianamides[6] initially isolated from *Penicillium brevi-compactum* and subsequently found in *Penicillium viridicatum*[7] and *Penicillium ochraceum*[8].

Paraherquamide shares with marcfortines A and B an unusual dioxygenated 7 membered ring located on the tryptophan unit, whereas marcfortine C has a singly oxygenated, 6 membered pyran ring. In the brevianamides neither ring structure is present.

Brevianamide A has been shown to have insecticidal activity[9] and, since completing the present studies, the anthelmintic activity of paraherquamide has also been reported[10].

Here we report the discovery, isolation, identification and anthelmintic activity of a novel series of paraherquamides including members with the pyran ring found in marcfortine C. The relative stereochemistry of these compounds is shown to be identical to that determined for paraherquamide[11].



Paraherquamide (**1**)	R=OH	X=–O–
VM 55594 (**2**)	R=H	X=Absent
VM 54158 (**3**)	R=OH	X=Absent
VM 54159 (**4**)	R=H	X=–O–

Taxonomic Studies on the Producing Strain

The producing organism, was isolated from a soil sample collected at Kemer, Turkey. For the characterization of the strain the methods and media recommended by PITT[12] were used.

The following morphological observations were made on the cultures grown at 25°C for 7 days

† These metabolites have very recently been described, among others, from a strain of *Penicillium charlesii*[1,2].

on CZAPEK yeast-extract agar[12]; colonies reached up to 30 mm diameter, marginal areas were velutinous and centrally floccose. The mycelium was white in peripheral areas and greyish green at the centre. Some pale yellow patches of mycelium occurred scattered about the colony. A yellowish exudate was present mostly over the central areas. As the culture aged, aerial growth became more grey with a brownish tinge.

Conidiophores with thin cell walls and no foot cells characteristically terminated in well-defined verticils of 3~5 divergent metulae (12~15×2.2~3.0 μm), commonly spathulate. Phialides were borne in compact verticils of 8~12, ampulliform, usually with short collula. Conidia were spheroidal with walls finely roughened, typically borne in long well-defined columns, one per metula.

Based on the morphological characeristics described above, the strain is considered to belong to the genus *Penicillium*.

Fermentation

A conidial suspension was prepared by suspending spores from well-grown colonies on potato-glucose agar in phosphate buffered saline containing glycerol 10% and Tween 80 0.1%.

Two ml of this inoculum was used to spread over the surface of large plates (225×225 mm) containing 300 ml CZAPEK-DOX agar (Oxoid). Plates were incubated in plastic bags at 26°C for a period of 20 days.

Isolation and Purification

The agar from 16 large plate cultures was macerated and extracted with excess acetone, the solids being separated by centrifugation. The solvent extract was concentrated under vacuum and the aqueous residue was extracted with chloroform. Evaporation of the chloroform yielded a yellow oil (4.23 g). This oil was suspended in methanol, the soluble portion was divided in two and each half was chromatographed by preparative reverse phase HPLC. These columns each yielded a paraherquamide rich fraction (identified as the major EHRLICH's positive spot on TLC)–Fraction A, and a later running fraction showing other EHRLICH's positive spots on TLC–Fraction B. Fractions A and B from each column were pooled and weighed (A: 0.93 g, B: 0.18 g).

Fraction A was further purified by sequential normal phase preparative HPLC to yield VM 29919 (117 mg) and VM 54158 (34 mg).

Fraction B was also chromatographed by normal phase HPLC column to produce, in elution sequence, VM 54159 (10.7 mg) and VM 55594 (16.3 mg).

Physico-chemical Properties

VM 29919 was shown to be paraherquamide (**1**), on the basis of the correspondence of the UV, IR, MS as well as the NMR data (Tables 1 and 2) with that reported in the literature[3,11].

VM 55594 was shown to possess structure **2** on the basis of more detailed spectral analysis. The UV spectrum (λ_{max}^{MeOH} nm (ε) 244 (29,900)) was similar to that of marcfortine C[5]. HREI-MS gave a molecular ion (*m/z* 461.2638) corresponding to a molecular formula of $C_{28}H_{35}N_3O_3$. In agreement with this, the ^{13}C NMR spectrum exhibited 28 resonances including, 6 CH_3, 5 CH_2, 2 sp^3CH and 4 sp^2 CH groups as well as 5 sp^3 and 6 sp^2 quaternary carbons. The ^1H NMR spectrum exhibited resonances for 35 protons including 5 singlet and 1 doublet methyl groups and 1 low field NH proton. All these features were similar to those of paraherquamide. The ^1H NMR spectrum was solved with the aid of a 2D ^1H COSY-45 NMR spectrum[13] which revealed a multitude of proton-to-proton connectivities all of which were consistent with structure **2** (Scheme 1). The connectivity between protons and their directly attached carbon atoms

Table 1. ^{1}H and ^{13}C NMR chemical shifts (δ in ppm[a]) for paraherquamide (**1**), VM 55594 (**2**), VM 54158 (**3**) and VM 54159 (**4**).

Atom	δ_H				δ_C			
	Paraherquamide (1)	VM 55594 (2)	VM 54158 (3)	VM 54159 (4)	Paraherquamide (1)	VM 55594 (2)	VM 54158 (3)	VM 54159 (4)
2	—	—	—	—	183.1	185.7	184.3	182.5
C2-NH	7.34	10.09	8.97	7.70	—	—	—	—
3	—	—	—	—	63.2	62.8	62.6	62.9
4	6.81	6.92	6.92	6.82	120.3	125.5	125.6	120.5
5	6.68	6.45	6.46	6.68	117.3	109.5	109.6	117.3
6	—	—	—	—	146.2	153.0	153.1	146.0
7	—	—	—	—	135.4	105.6	105.3	135.2
8	—	—	—	—	132.7	137.5	137.1	132.3
9	—	—	—	—	125.1	121.6	121.3	125.4
10a	2.69	2.66	2.67	2.70	37.1	37.3	37.1	37.5
10b	~1.85	~1.84	1.84	~1.86				
11	—	—	—	—	65.3	65.2	65.3	65.2
12a	3.61	3.60	3.62	3.61	59.2	60.0	59.2	60.1
12b	2.57	2.55	2.58	2.54				
13	—	—	—	—	71.4	67.7	71.4	67.7
14	—	~1.95	—	~1.93	78.1	40.3	78.0	40.3
C14-OH	2.63	—	~2.67	—	—	—	—	—
15a	~1.88	~2.05	~1.9	~2.04	38.2	30.3	38.1	30.2
15b	2.35	~1.84	~2.35	~1.85				
16a	3.21	3.19	3.22	3.19	51.8	53.3	51.9	53.4
16b	2.22	2.26	~2.24	2.26				
17	1.65	~1.43	1.67	1.42	19.2	13.2	19.1	13.1
18	—	—	—	—	171.6	172.4	[c]	172.2
19a	~1.85	~2.06	~1.9-1.8	2.04	22.2	27.7	22.2	27.7
19b	1.79	~1.43		~1.42				
20	3.02	3.06	~3.05	3.05	51.6	52.4	51.7	52.1
21	—	—	—	—	46.4	46.2	46.4	46.3
22	1.10	1.12	1.10	1.10	20.4	20.6	20.4	20.8
23	0.87	0.88	0.86	0.86	23.7	23.9	22.2	24.0
24	6.30	6.48	6.37	6.32	139.1	116.3	116.1	139.0
25	4.89	5.75	5.73	4.89	115.0	131.2	131.3	115.1
26	—	—	—	—	79.8	76.4	76.4	79.8
27	1.45[b2]	1.46[b7]	1.47[b9]	1.45[b8]	29.9[b10]	27.9[b5]	27.9[b4]	30.0[b3]
28	1.44[b2]	1.42[b7]	1.43[b9]	1.43[b8]	29.85[b10]	27.8[b5]	27.8[b4]	29.8[b3]
29	3.05	3.04	3.06	3.03	25.9	25.5	25.9	25.5

[a] Reference: $^{\delta}$TMS = 0 ppm.
[b] Resonance pairs labelled superscript bi may be interchanged.
[c] Resonance not observed — S/N poor.

was established using a 2D $^{1}H,^{13}C$ COSY NMR experiment[13]. The fragments of structure generated by this procedure were then pieced together using a 2D $^{1}H,^{13}C$ COLOC NMR experiment[13], which establishes connectivities between protons and ^{13}C nuclei over 2 to 3 bonds (Table 3). Thus the structure of VM 55594 was established as **2** with close similarities to the structures of known paraherquamides and marcfortines. A similar approach was used to determine the structures of **3** and **4**. The relative stereochemistry of **2** to **4** was deduced to be the same as that of **1** at all centres, on the basis of the close similarity of spectral parameters, especially the ^{13}C NMR chemical shifts (Table 1) with the corresponding values for **1** and the marcfortines[4,5,11]. In addition, the relative stereochemistry at C-14 in **2** was proven

Table 2. A table of proton-to-proton coupling constants, $^nJ_{H,H}$ in Hz for paraherquamide (**1**), VM 55594 (**2**), VM 54158 (**3**), VM 54159 (**4**) in $CDCl_3$-TMS.

$^nJ_{H,H}$	1	2	3[c]	4	$^nJ_{H,H}$	1	2	3[c]	4
$^3J_{4,5}$	8.2	8.2	8.2	8.2	$^3J_{15a,16a}$	9.1	9.1		9.1
$^5J_{4,N2H}$	—	br		0.6	$^3J_{15a,16b}$	4.8	5.4		~5.5
$^5J_{4,24}$	—	0.8		0	$^3J_{15b,16a}$	4.5	4.3		4.1
$^2J_{10,10}$	15.4	15.4	15.4	15.3	$^3J_{15b,16b}$	10.8	10.5		10.2
$^2J_{12,12}$	11.3	11.1		11.0	$^2J_{16,16}$	9.1	9.1		9.1
$^4J_{12b,20}$	1.5	1.6		1.6	$^4J_{N18H,12a}$	—	—	—	—
$^3J_{14,15a}$	—	9.9[b1]	—	[a]	$^2J_{19,19}$	13.0	12.5		12.5
$^3J_{14,15b}$	—	8.8[b1]	—	[a]	$^3J_{19a,20}$	~11[b2]	11.0		11.0
$^3J_{14,17}$	—	6.9	—	6.8	$^3J_{19b,20}$	~10.5[b2]	~9.9		~9.8
$^2J_{15,15}$	13.1	[a]		[a]	$^3J_{24,25}$	7.7	9.9	9.9	7.7

[a] Not clearly observed.
[b] Assignments may be reversed in pairs labelled superscript bi.
[c] Many ^nJ values not observed—spectrum broad.

Table 3. A table of long-range carbon, hydrogen connectivities observed in the 2D $^1H,^{13}C$ COLOC NMR spectrum of VM 55594 (**2**).

Carbon atom	Connectivity	Carbon atom	Connectivity
C-2	10-Ha, 10-Hb	C-14	17-CH_3
C-3	10-Ha, 10-Hb, 22-CH_3, 23-CH_3	C-15	17-CH_3
C-6	4-H	C-18	19-Hb, 29-CH_3
C-7	25-H	C-20	10-Hb, 12-Hb, 22-CH_3, 23-CH_3
C-8	4-H	C-21	10-Ha, 22-CH_3, 23-CH_3
C-9	5-H, 10-Hb	C-22	22-CH_3*, 23-CH_3
C-11	10-Ha, 10-Hb,12-Hb, 29-CH_3/20-H	C-23	22-CH_3, 23-CH_3*
C-12	10-Hb, 12Ha*	C-25	27-CH_3, 28-CH_3
C-13	17-CH_3	C-26	24-H, 25-H, 27-CH_3, 28-CH_3

* Auto-peak due to non-suppression of $^1J_{HC}$ connection.

by ^1H NMR NOE experiments (Table 4). All compounds are shown with the correct relative stereochemistry as determined by our experiments. In addition the compounds are shown with the same absolute stereochemistry as that determined by a recent X-ray study on a heavy-atom derivative of **1**[11]. Some confusion exists in the literature with regards to the stereochemistry of this class of compounds. X-Ray crystallographic analysis of marcfortines A and C has shown that the relative stereochemistry was the same as **1** at all corresponding centres. However, the authors misrepresented the configuration at C-20 in their structural drawings[4,5]. This misrepresentation of marcfortine stereochemistry has been propagated in a very recent publication[14] on the related brevianamide compounds. This latter publication[14] also gives a mirror image representation of **1** even though referring back to the heavy-atom X-ray work[11].

Scheme 1. A map of proton-to-proton connectivities in VM 55594 (**2**) derived from the 2D ^1H COSY-45 NMR spectrum.

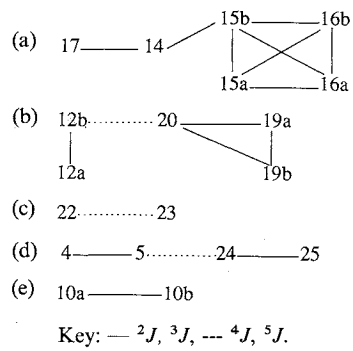


Table 4. A table of proton-to-proton NOE's observed in VM 55594 (**2**).

Proton irradiated	NOE observed
4-H	5-H[a], 10-Hb, 12-Ha[b] (weak), 22-CH_3
10-Ha	10-Hb[a], 29-CH_3
12-Ha	4-H[b], 10-Hb, 12-Hb[a], 22-CH_3
12-Hb	10-Hb, 12-Ha[a], 16-Hb
16-Ha	15-Ha[a], 16-Hb[a]
16-Hb	12-Hb, 15-Hb[a], 16-Ha[a]
17-CH_3	14-H[a], 15-Hb
20-H	19-Ha[a], 19-Hb[a] (weak), 23-CH_3
22-CH_3	4-H, 10-Hb, 12-Ha, 12-Hb[d], 19-Ha[d], 19-Hb, 23-CH_3
23-CH_3	19-Ha (weak), 20-H, 22-CH_3, 24-H (weak)
24-H	25-H[a]
27/28-CH_3	25-H
29-CH_3	10-Ha, 12-Ha[b], 24-H[b,c], 25-H[b,c]

[a] Mixed INDOR/NOE effect.
[b] Corresponding H to H distance in X-ray structure of paraherquamide >3.0Å.
[c] NOE disappeared on addition of CD_3OD.
[d] Negative 3-spin effect.

Table 5. Anthelmintic activity of the paraherquamides.

Compound	*In vitro*[a]	*In vivo*[b]
	MIC_{50} (μg/ml)	% reduction in faecal egg count
Paraherquamide (**1**)	31.2	99.5
VM 55594 (**2**)	>500	62.5
VM 54158 (**3**)	>500	0
VM 54159 (**4**)	15.6	100

[a] *Haemonchus contortus* L_3.
[b] Gerbil-*Trichostrongylus* model, compounds dosed at 4 mg/kg po.

Anthelmintic Activity

The activities of the four metabolites against *Haemonchus contortus* larvae *in vitro* and adult *Trichostrongylus colubriformis* infections in gerbils are shown in Table 5. Structures **1** and **4** (di-oxygenated ring on tryptophan) were clearly more active than **2** and **3** (pyran ring). The corresponding 14-de-hydroxy compounds **2** and **4**, appeared to be marginally more potent than the 14-hydroxy analogues **1** and **3**.

The activity of paraherquamide *in vivo*, is comparable to that recently reported by OSTLIND *et al.*[10].

Experimental

^1H and ^{13}C NMR experiments were conducted on a Bruker AM 400 NMR spectrometer, in a 5-mm $^1H/^{13}C$ dual probe, at 300 K in 0.5 cm^3 $CDCl_3$-TMS solution (0.02 to 0.2 M) using standard software. The 2D and NOE experiments were performed as previously described[15].

Reverse phase preparative chromatography employed a Rainin Dynamax-60A C-18 column, 41.4×250 mm, eluted with methanol-water (75:25) at 20 ml/minute.

Normal phase chromatography was carried out on a Rainin Dynamax-60A silica column, 21,4×250 mm eluted with a dichloromethane-methanol gradient at 8 ml/minute (0 to 10% methanol over 120 minutes for fraction A; fraction B used 0 to 3% over 30 minutes and thereafter isocratic at 3% methanol).

The column eluant was monitored with a UV diode-array detector (Applied Biosystems Ltd., 1000S) with spectral scanning at 0.5 minute intervals which enabled compounds **1** and **4** to be distinguished from **2** and **3** (UV maxima 226 and 244 nm, respectively).

Column fractions were examined by TLC on glass backed silica gel 60 plates (Merck type 5735) developed with $CHCl_3$-MeOH (9:1). Spots were visualised by spraying with EHRLICH's reagent (1% 4-dimethylaminobenzaldehyde in HCl-MeOH (1:3)) and heating. Rf's were as follows: Paraherquamide, 0.49; VM 54158, 0.48; VM 54159 and VM 55594, 0.56.

Anthelmintic *in vivo* tests and *in vitro* MIC_{50}s were obtained by the method of HOOD *et al.*[16].

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