FURTHER NOVEL METABOLITES OF THE PARAHERQUAMIDE FAMILY

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Four novel metabolites of a *Penicillium* strain, IMI 332995, which has previously been reported to produce paraherquamide and a number of related metabolites, are herein described[†††]. VM55596 is the first *N*-oxide to be found in this family of compounds. Unusual oxidative substitution is also seen in VM55597. VM55599 appears to be the first documented example of the hexacyclic indole species that have long been postulated as biosynthetic precursors of metabolites of the brevianamide, paraherquamide and marcfortine families.

Since the discovery of the potent anthelmintic activity of paraherquamide much interest has been focused on this class of oxindole alkaloids which includes the marcfortines and brevianamides. All are products of *Penicillium* species and a common metabolic pathway has been proposed[1].

A number of related metabolites have now been isolated by ourselves[2] and others[3,4]. All previously reported paraherquamide metabolites show variation in substitution at C(14) and N(11) in addition to the two variants of the ring system fused to the 6 and 7 positions of the indole. The marcfortine family also show analogous substitutions.

We now describe four further metabolites from our paraherquamide-producing strain including two which, unusually, show additional oxidative substitution at N(12) or C(16) and another which is analogous to the proposed precursor of this class of natural products[1].

Taxonomic Studies

The morphology of the producing strain has been described[2]. This strain has now been deposited in the CAB International Mycological Institute at Kew, UK, under the accession number IMI 332995.

IMI 332995 has been compared (Table 1) with a number of *Penicillium* species obtained from the IMI at Kew. *P. paraherquei* (IMI 68820) is a known producer of paraherquamide, *P. fellutanum* (IMI 40232) was included since ONDEYKA *et al.*[3] typed their paraherquamide producing culture as *P. charlesii* which is included under the species *P. fellutanum*[5]. Early results on IMI 332995 indicated that certain characteristics were most similar to *P. citrinum*. Thus the culture *P. citrinum* IMI 24307 was also examined for comparison.

IMI 332995 differs from *P. paraherquei* in that growth is much slower at 25°C on malt extract and Czapek-yeast extract agars and was also slower on all of the agars tested at 37°C. IMI 332995 differs from *P. fellutanum* in that the penicillus is biverticillate as opposed to monoverticillate and growth was slower on

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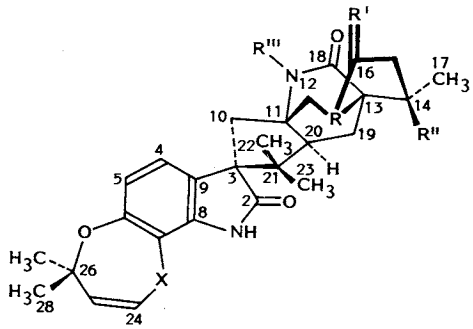
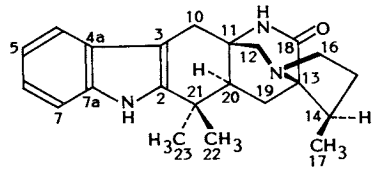
[†††] Metabolites **1**, **2** and **3** are subjects of International Patent Application WO 91/09961.



VM55595 (2)	R=N	R'=H_2	R''=H	R'''=H	X=Absent
VM55596 (3)	R=N^+-O^-	R'=H_2	R''=OH	R'''=Me	X=O
VM55597 (4)	R=N	R'=O	R''=OH	R'''=Me	X=O
Paraherquamide (5)	R=N	R'=H_2	R''=OH	R'''=Me	X=O



VM55599 (1)

Table 1. Comparison of IMI 332995 with known *Penicillium* cultures.

Culture	Type of penicillus	Medium[a]	Diameter of colony at growth temperature (mm)		
			25°C	5°C	37°C
IMI 332995	Biverticillate	MEA	20	No growth	0.6
		CYA	30	No growth	0.7
		G25N	25	No growth	1.2
Penicillium citrinum IMI 24307	Biverticillate	MEA	21	0.3	No growth
		CYA	33	0.4	No growth
		G25N	19	No growth	No growth
P. paraherquei IMI 68820	Mono- and biverticillate	MEA	35	No growth	3.4
		CYA	50	No growth	4.4
		G25N	21	No growth	2.6
P. fellutanum IMI 40232	Monoverticillate	MEA	12	No growth	1.1
		CYA	23	No growth	1.5
		G25N	21	No growth	3.5

[a] MEA; malt extract agar, CYA; Czapek yeast extract agar, G25N; glycerol nitrate agar.

each agar at 37°C.

IMI 332995 is most similar to *P. citrinum* especially in gross morphology but there are differing growth patterns at 25°C and 37°C. It is worth noting that *P. citrinum* strains show an affinity with species in the series *Fellutana*[5].

In view of the morphological differences between IMI 332995 and the other strains tested and the

fact that several new paraherquamides are produced it is believed that IMI 332995 is a novel strain and has simply been designated as a *Penicillium* species at present.

Fermentation

Strain IMI 332995 was inoculated onto Czapek-Dox agar as previously described[2]. Batch sizes and incubation periods were as follows. Batch 1: 5 litres, 20 days; batch 2: 10 litres, 24 days; batch 3: 10 litres, 27 days; batch 4: 20 litres, 26 days.

Isolation and Purification

Each fermentation batch was separately extracted in excess acetone, filtered, concentrated under vacuum and partitioned into chloroform. The chloroform extract of batches 1 and 2 were separately purified by reverse phase HPLC (41.4 mm diameter column) into two bulk fractions: A (containing paraherquamide and VM54158) and B (containing VM54159 and VM55594) which were, in turn, chromatographed by normal phase HPLC (21.4 mm diameter column) as before[2].

VM55595 (9.6 mg) was isolated from the normal phase chromatography of fraction B of fermentation batch 1. It eluted from the column after VM55594.

VM55596 was found as a late eluting peak from the chromatography of fraction A of batch 2. This material was further purified using a normal phase column (10 mm diameter) eluted with a linear gradient of CH_2Cl_2 with increasing MeOH. Pure VM55596 (10 mg) eluted at 93 : 7 (CH_2Cl_2 - MeOH).

Chloroform extracts of fermentation batches 3 and 4 were pooled and concentrated to dryness. The MeOH soluble portion of this extract (9.4 g) was applied to a column (25 cm × 10 cm diameter) packed with HP20SS (Mitsubishi Chemicals, Japan) previously equilibrated with 70% MeOH in water. This column was eluted with increasing concentrations of MeOH as follows: 6.5 litres at 70%, 3.5 litres at 75%, 3.5 litres at 80%, 6.5 litres at 85% and 8.0 litres at 90%. Half litre fractions were collected sequentially from the start. Individual fractions were examined by TLC and combined appropriately into bulks A ~ J. These bulks included D (fractions 12 ~ 19), E (20 ~ 32) and G (40 ~ 46). After concentration each bulk was chromatographed by normal phase HPLC (41.4 mm diameter column eluted with a linear gradient of CH_2Cl_2 - MeOH from 100 : 0 to 94 : 6 over 60 minutes). In bulks D and E the component eluting just after the main paraherquamide peak was collected. This material was pooled and VM55597 (10.2 mg) was isolated after further normal phase HPLC (10 mm diameter column; hexane - 2-propanol linear gradient 98 : 2 to 78 : 22 over 120 minutes).

Chromatography of bulk G yielded a number of components, the first to elute was rechromatographed by normal phase HPLC (10 mm diameter column using a hexane - 2-propanol gradient from 98 : 2 to 96 : 4 over 12 minutes then held at 96 : 4) to yield 5.0 mg of VM55599.

Physico-chemical Properties

VM55599 (**1**)

Elucidation of Structure: VM55599 exhibited a positive EHRLICH's reaction typical of the paraherquamides[2] but displayed a somewhat different UV spectrum λ_{max}^{MeOH} (ε) 226 (29,700) and 280 (5,600). Mass spectrometry (EI and FAB) indicated a molecular weight of 349 and high-resolution EI-MS gave a molecular ion at *m/z* 349.2123 corresponding to a molecular formula of $C_{22}H_{27}N_3O$. In agreement with this the ^{13}C NMR spectrum exhibited 22 resonances including 3 CH_3, 5 CH_2, 2 sp^3CH and 4 sp^2CH groups as well as 3 sp^3 and 5 sp^2 quaternary carbons, including only one C=O group, and thus indicating the

Table 2. ^{1}H and ^{13}C NMR chemical shifts (δ in ppm[a]) for VM55599 (**1**), VM55595 (**2**), VM55596 (**3**), VM55597 (**4**) and paraherquamide (**5**).

Atom	δ_H					δ_C			
	VM55599 (**1**)	VM55595 (**2**)	VM55596 (**3**)	VM55597 (**4**)[d]	Paraherquamide (**5**)	VM55599 (**1**)	VM55595 (**2**)	VM55596 (**3**)	Paraherquamide (**5**)
N1-H	8.77	9.42	~8.5	7.39	7.34	—	—	—	—
2	—	—	—	—	—	141.2	184.9	182.2	183.1
3	—	—	—	—	—	104.0	62.5	62.9[b6]	63.2
4	7.43	6.88	6.86	6.82	6.81	117.7	125.6	120.5	120.3
4a	—	—	—	—	—	126.8	—	—	—
5	7.07	6.43	6.70	6.72	6.68	119.0	109.4	117.7	117.3
6	7.15	—	—	—	—	121.3	153.0	146.4	146.2
7	7.33	—	—	—	—	110.6	105.4	135.4	135.4
7a	—	—	—	—	—	136.5	—	—	—
8	—	—	—	—	—	—	137.5	132.4	132.7
9	—	—	—	—	—	—	121.8	124.1	125.1
10	2.82 (a)	2.25 (a)	2.69 (a)	2.79 (a)	2.69 (a)	30.0	40.0	36.0	37.1
	2.94 (b)	1.91 (b)	1.88 (b)	2.03 (b)	~1.85 (b)				
11	—	—	—	—	—	55.6	61.8	62.8[b6]	65.3
11-NH	6.47	6.85	—	—	—	—	—	—	—
12	3.49 (a)	3.64 (a)	4.09 (a)	3.67 (a)	3.61 (a)	58.8	60.2	76.2	59.2
	2.29 (b)	2.59 (b)	3.87 (b)	3.54 (b)	2.57 (b)				
13	—	—	—	—	—	66.3	68.2	83.8	71.4
14	~3.00	~1.95	—	—	—	30.2	39.9	77.9	78.1
14-OH	—	—	[c]	[c]	2.63	—	—	—	—
15	~1.42 (a)	~2.05 (a)	~2.5 (a)	2.77 (a)	~1.88 (a)	33.0	30.3	39.1	38.2
	~2.15 (b)	~1.82 (b)	~2.5 (b)	2.52 (b)	2.35 (b)				
16	~3.00 (a)	3.19 (a)	4.05 (a)	—	3.21 (a)	53.5	52.8	69.4	51.8
	~2.15 (b)	2.37 (b)	3.33 (b)		2.22 (b)				
17	1.05	~1.40	1.74	1.84	1.65	17.4	13.1	22.2	19.2
18	—	—	—	—	—	174.8	174.3	167.7	171.6
19	1.78 (a)	~2.05 (a)	1.85 (a)	~2.0 (a)	~1.85 (a)	26.8	27.6	14.5	22.2
	2.00 (b)	~1.40 (b)	3.11 (b)	~2.0 (b)	1.79 (b)				
20	~2.15	3.07	3.29	3.23	3.02	46.7	53.4	51.0	51.6
21	—	—	—	—	—	34.2	46.2	46.8	46.4
22	1.44	1.07	1.20	1.10	1.10	23.9	20.6	21.1	20.4
23	1.35	0.85	0.91	0.89	0.87	30.5	24.1	23.3	23.7
24	—	6.44	6.32	6.31	6.30	—	116.3	138.9	139.1
25	—	5.74	4.90	4.90	4.89	—	131.2	115.2	115.0
26	—	—	—	—	—	—	76.3	79.9	79.8
27	—	1.46[b1]	1.46[b2]	1.47[b3]	1.45[b4]	—	28.0[b5]	29.9[b7]	29.9[b8]
28	—	1.41[b1]	1.44[b2]	1.43[b3]	1.44[b4]	—	27.8[b5]	29.7[b7]	29.85[b8]
29	—	—	3.07	3.03	3.05	—	—	27.1	25.9

[a] Solvent: $CDCl_3$ except for (**1**) which was $CDCl_3$ plus 1 drop DMSO-d_6 to aid solubility and (**3**) which was $CDCl_3$ plus 1 drop of CD_3OD to aid solubility. Reference: $\delta_{TMS}=0$ ppm. See Fig. 3 for the definitions of Ha and Hb. [b] Signal pairs may be interchanged. [c] Not clearly observed. [d] No ^{13}C data available due to low sample mass.

Table 3. Proton-proton coupling constants, $^{n}J_{H,H}$ in Hz, for VM55599 (**1**), VM55595 (**2**), VM55596 (**3**), VM55597 (**4**) and paraherquamide (**5**) in $CDCl_3$-TMS.

$^{n}J_{H,H}$	VM55599 (**1**)[a]	VM55595 (**2**)	VM55596 (**3**)	VM55597 (**4**)	Paraherquamide (**5**)
$^{3}J_{4,5}$	7.7	8.2	8.2	8.2	8.2
$^{5}J_{4,N1H}$	0.8	br	—	[c]	—
$^{5}J_{5,24}$[d]	—	0.7	—	—	—
$^{2}J_{10,10}$	15.1	15.1	15.8	15.6	15.4
$^{2}J_{12,12}$	10.2	10.9	13.0	12.0	11.3
$^{4}J_{12b,20}$	1.9	1.4	1.8	[c]	1.5
$^{3}J_{14,15a}$	[c]	[c]	—	—	—
$^{3}J_{14,15b}$	[c]	[c]	—	—	—
$^{3}J_{14,17}$	7.2	6.9	—	—	—
$^{2}J_{15,15}$	[c]	[c]	[c]	17.0	13.1
$^{3}J_{15a,16a}$	[c]	9.1	[c]	—	9.1
$^{3}J_{15a,16b}$	[c]	5.4	[c]	—	4.8
$^{3}J_{15b,16a}$	[c]	4.4	[c]	—	4.5
$^{3}J_{15b,16b}$	[c]	10.5	[c]	—	10.8
$^{2}J_{16,16}$	[c]	9.2	[c]	—	9.1
$^{2}J_{19,19}$	13.2	12.4	14.2	[c]	13.0
$^{3}J_{19a,20}$	11.4	10.8	11.0	11.7[b]	~11.0
$^{3}J_{19b,20}$	4.3	10.1	10.2	9.4[b]	~10.5
$^{3}J_{24,25}$	—	9.9	7.7	7.7	7.7

[a] In $CDCl_3$-TMS plus 1 drop DMSO-d_6. In addition to the above, the following coupling constants were also observed: $^{4}J_{4,6}\sim{}^{5}J_{4,7}\sim{}^{4}J_{5,7}\sim1.1$, $^{3}J_{5,6}\sim7.0$ and $^{3}J_{6,7}\sim8.1$ Hz.
[b] Assignments may be reversed in pairs labelled superscript b.
[c] Not clearly observed.
[d] Note that the corresponding coupling constant in Table 2 of ref 2 was erroneously labelled $^{5}J_{4,24}$.

Table 4. A table of connectivities observed in the 2D ^{1}H, ^{13}C COLOC NMR spectrum of VM55599 (**1**).

Carbon	Connectivity to proton
C2	10a, 10b, 22, 23
C3	10a, 10b
C11	10a, 10b
C12	10a, 12b
C13	17, 19b
C15	17
C18	19b
C20	10b, 22, 23
C21	19b, 22, 23
C22	22, 23
C23	22, 23

presence of 25 non-exchangeable protons. The 1D ^{1}H NMR spectrum indicated the presence of 27 protons, including 2 NH groups (Tables 2 and 3). A 2D ^{1}H, ^{13}C COSY NMR spectrum was used to obtain an unambiguous assignment of the ^{13}C resonances of all the protonated carbons in VM55599. Nine structural fragments were constructed on the basis of connectivities observed in

Fig. 1. Proton-proton connectivities observed in the 2D ^{1}H COSY-45 NMR spectrum of VM55599 (**1**).

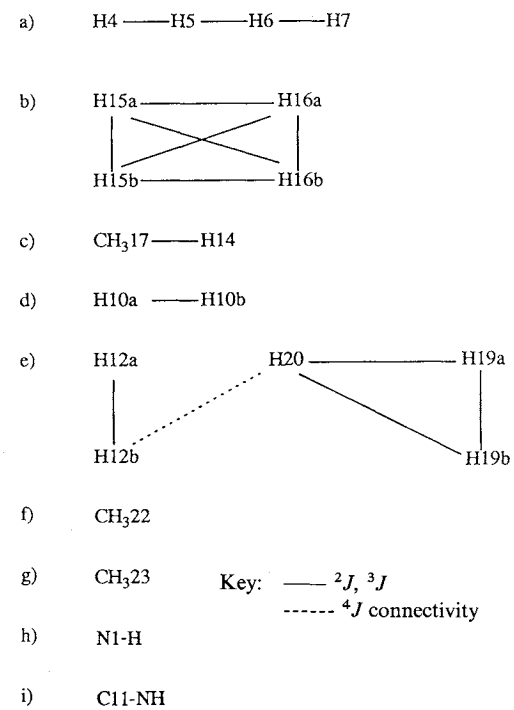
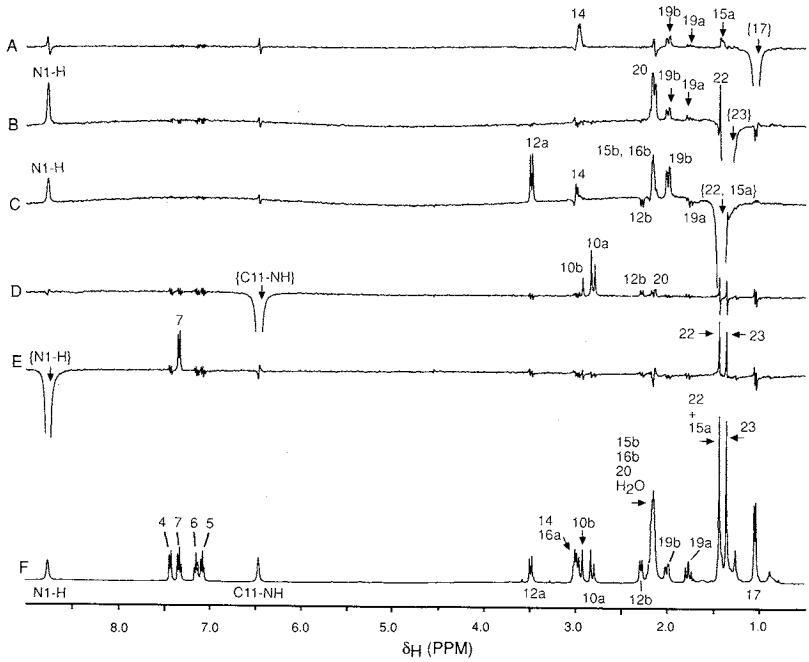


Fig. 2. A series of ^1H NOE difference NMR spectra of VM55599 (**1**) produced by irradiation of A) $CH_3$17, B) $CH_3$23, C) $CH_3$22 and 15Ha, D) C11-NH and E) N1-H. The difference spectra are expanded vertically ×32 relative to the normal spectrum (F).



the 2D ^1H COSY-45 NMR spectrum (Fig. 1) and these were then pieced together into the final structure on the basis of 2- and 3-bond proton-to-carbon connectivities observed in the 2D ^1H, ^{13}C COLOC NMR spectrum (Table 4), as well as through-space proton-to-proton connectivities observed in NOE difference spectra (Table 5, Fig. 2). The following connectivities were particularly important. The observation of COLOC connectivities from H22 and H23 to C2, C20, C21, C22 and C23 allowed the construction of the C2~C21 (C22, C23)–C20 fragment as sp^2C–sp^3C(CH_3)$_2$–sp^3C. N1-H was connected to both C2 in this fragment and to C7a on the aromatic ring on the basis of the observation of NOE's from N-1H to H22 and H23 and to H7, respectively (Fig. 2E). The observation of COLOC connectivities from H10a and H10b to C2, C3, C11, C12 and C20 allowed the construction of the indole nucleus and the fused 6-membered ring containing C10. The rest of the structure was pieced together in a similar fashion. Thus VM55599 has structure **1**.

Table 5. ^1H NOE's observed in VM55599 (**1**).

Proton irradiated	NOE observed at
N1-H	7, 22, 23
4	5, 10b[a]
10a	11-NH
C11-NH	10a, 10b, 12b, 20
12a	12b, 22
12b	10b, 12a
14	15b, 17, 19a[a]
16a	12a[a], 15a, 16b
17	14, 15a, 19a[a], 19b, 22[a]
19a	14[a], 17[a], 19b, 20, 22[b]
19b	17, 19a, 22, 23[a]
22	N1-H, 12a, 12b[b], 19b, 19a[b]
23	N1-H, 19b, 19a[a], 20, 22

[a] Weak.
[b] Weak negative NOE due to a 3-spin effect.

The "left-hand-side" of **1** is analogous to the "left-hand-side" of the aristotelines[6], whilst the "right-hand-side" is analogous to a des-hydroxylated paraherquamide[2] and in particular VM55595 (**2**).

Fig. 3. A molecular model of VM55599 produced using CHEM-X[10]. The oxygen atom is shaded, the nitrogen atoms are stippled and the dot on a hydrogen atom indicates that it is Ha. Note that our definitions of 12Ha, 15Ha and 16Ha are *opposite* to those of LIESCH and WICHMANN[4]. Our definitions have been used consistently for all the molecules reported both here and previously[2].

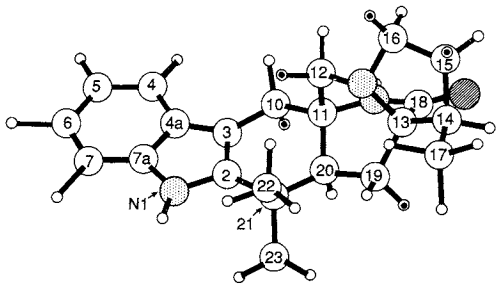


Table 6. ^{1}H NOE's observed in VM55596 (**3**).

Proton irradiated	NOE observed at
4	10b, 22
10a	10b[a], 29 (weak)
10b	4, 10a[a], 12a, 12b
12a	10b, 22
12b	10b, 16b
15a, b	16a[a], 16b[a], 17
16a	15[a], 16b[a]
16b	12b, 15[a], 16a[a]
17	15a, b
19a	19b[a], 20[a]
19b	19a[a], 22
20	19a[a], 23 (weak)
22	4, 12a, 12b[b], 19b, 23
23	20, 22
27, 28	5, 25
29	10a

[a] Mixed INDOR/NOE effect.
[b] Negative, 3-spin effect.

Indeed, the ^{13}C NMR chemical shifts of C2 to C7a are within 1.4 ppm of the values quoted for aristolasicol[6] ($\Delta\delta = 0.0$ to 1.4, average 0.5 ppm), if the quoted values for C2 and C7a are reversed. VM55599 also appears to be the first documented example of the hexacyclic indole species that have long been postulated as biosynthetic precursors of the brevianamides, marcfortines and paraherquamides[1].

Elucidation of Stereochemistry: It is assumed that the absolute stereochemistry at C20 is the same as that in paraherquamide[7] on biogenic grounds and all structure are drawn on this basis. The subsequent discussion is concerned with relative stereochemistry only. H20 and C12 must be *trans-diaxial* in view of $^4J_{20,12b}$ and the strong NOE from H22 to H12a *i.e.* NOE[22]12a (Fig. 2C). Thus *assuming C20 is S*, as drawn, then C11 is *S* and C13 is forced to be *R* by the constraints of ring closure. However, there must be some change in stereochemistry in the 5-membered C12-N to C13 ring in view of the differing ^{13}C and ^{1}H NMR chemical shifts in the region C13 to C19 between **1** and both VM55594 and VM54159[2]. It was concluded from the pattern of NOE's that the stereochemistry at C14 was *R*: inverted from the C14 *R* configuration in paraherquamide itself[7]. This *epi* C14 configuration was confirmed by the low- and high-frequency shifts of the ^{1}H NMR signals of $CH_3$17 and H14, respectively: an effect which has been previously observed in some synthetic *epi* C14 paraherquamide analogues[8]. The observation of only a weak NOE[16a]12a and NOE[17]19b > NOE[17]19a was consistent with the stereochemistry at C12-N being *S*, as observed in the X-ray crystal structure of paraherquamide itself.

An unusual feature of the ^{1}H NMR spectrum of **1** was revealed during the course of running some ^{1}H inversion-recovery experiments. The relaxation rate of $CH_3$22 ($R_{1null} \sim 1.2\,s^{-1}$) was only half that of $CH_3$23 ($R_{1null} \sim 2.4\,s^{-1}$). Inspection of a model of VM55599 (Fig. 3) revealed that $CH_3$22 is in close spatial contact with C12-N, H12a, H19b and $CH_3$23 and that there is little opportunity to relieve this strain. Thus it is possible that the slow relaxation rate of $CH_3$22 is due to fast rotation (relative to $CH_3$23) caused by raising of the ground state energy level for rotation of $CH_3$22.

VM55595 (**2**), VM55596 (**3**) and VM55597 (**4**)

Similar approaches were used to determine the structures of **2** to **4**. The relative stereochemistries

are the same as paraherquamide at all centres as judged by ^{1}H and ^{13}C NMR chemical shifts and by ^{1}H NOE data (Tables 2, 3 and 6). Note that the assignment for H15a, H15b given here and previously[2] for paraherquamide is opposite to that given by the Merck group[4,8]. Our assignments are unambiguous and based on a careful analysis of δ, J and NOE values in the compounds reported here and previously[2]. Note also that the assignment of H12a and H12b for paraherquamide by the Merck group changes between references 4 and 8. The values given here agree with reference 8 and are definitive. Finally, our definitions of Ha and Hb for the geminal protons of these molecules differ in part from those of the Merck group[4] (see Fig. 3).

In the case of VM55597 (**4**), the structure was confirmed when synthetic material produced subsequently by the Merck group[7] was shown to be identical to our natural product by ^{1}H NMR (chemical shifts in agreement to within 0.03 ppm except for H20 [0.09] and N1-H [0.21 ppm], see Table 2). In the case of VM55596 (**3**) the structure was confirmed by chemical means. Reduction of **3** with Ph_3P produced a single product which was identical to paraherquamide by TLC and HPLC. Compound **3** is the first *N*-oxide to be isolated in this series of compounds.

Anthelmintic Activity

The activities of the four metabolites, and of paraherquamide itself against adult *Trichostrongylus colubriformis* in gerbils was determined by the method of HOOD *et al.*[11].

Paraherquamide (**5**) and its *N*-oxide (**3**) gave 100 and 94% reductions in faecal egg counts respectively when dosed at 2.0 mg/kg whereas **1**, **2** and **4** each lacked significant activity in this model when closed at 4.0 mg/kg.

Experimental

All NMR experiments were performed in the 5 mm ^{1}H, ^{13}C dual probe (normal geometry) of a Bruker AM400 spectrometer at 300 K in 0.5 cm^3 $CDCl_3$-TMS solution (0.003 to 0.04 M). For VM55599, the experiments were conducted in $CDCl_3$-TMS solution to which 1 drop of $(CD_3)_2SO$ had been added to aid solubility. In addition, for VM55599, the ^{1}H NOE experiments were conducted twice; before and after addition of C_6D_6 to improve spectral dispersion. For VM55596, the solvent was $CDCl_3$-TMS plus 1 drop of CD_3OD. The 2D, T_1 and NOE experiments were performed as previously described[9]. MS data was obtained on a VG ZAB 1F in EI and FAB modes (using thioglycerol and a saturated solution of sodium acetate in 3-nitro-benzyl alcohol as the FAB matrices).

All HPLC employed 250 mm Rainin Dynamax-60A columns of either silica (normal phase) or bonded C-18 (reverse phase). Flow rates were as follows, 41.2 mm diameter: 20 ml/minute, 21.4 mm: 8 ml/minute, 10 mm: 4 ml/minute.

The TLC system was as described previously[2].

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representation of paraherquamide and represents marcfortine with the wrong C20 stereochemistry.

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