Novel Anthelmintic Metabolites from an *Aspergillus* Species; the Aspergillimides

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Two members of a novel class of anthelmintics, the aspergillimides, have been isolated from the *Aspergillus* strain IMI 337664. This novel fungus also produced two known and one structurally novel paraherquamide. This paper describes the fermentation, isolation, structure elucidation and anthelmintic activity of aspergillimide (VM55598, **1**), 16-keto aspergillimide (SB202327, **2**), and the paraherquamides VM54159 (**3**), SB203105 (**4**) and SB200437 (**5**). The aspergillimides are equivalent to paraherquamides which have lost both the dioxygenated 7-membered ring and the phenyl ring to which this is fused; gaining in their place a C8-keto group. SB203105 is the first example of a 4-substituted paraherquamide.

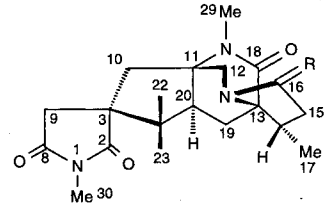
The paraherquamides are oxindole alkaloids originally isolated by YAMAZAKI *et al.*[1], which have aroused much interest because of their anthelmintic properties[2]. Novel paraherquamides have been recently isolated by ourselves[3,4] and other groups[5,6]. The paraherquamides are related to the marcfortines and brevianamides; all have been isolated from *Penicillium* species and all are proposed to share a common metabolic pathway[4,7].

In our quest for novel, natural product anthelmintics with improved properties, we investigated the metabolites produced by the *Aspergillus* species IMI 337664. We now describe two members of a new class of anthelmintics, the aspergillimides, which we isolated from this organism along with three paraherquamides; the first time paraherquamides have been isolated from an organism outside the *Penicillia*.

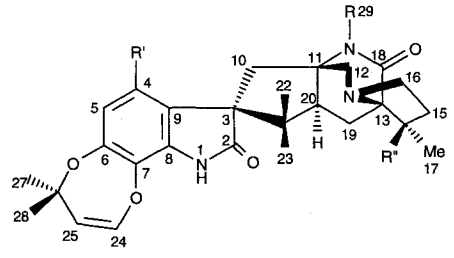
Taxonomy

Aspergillus sp. IMI 337664 is believed to comprise a previously unreported strain in the genus *Aspergillus*. It has been deposited in the C.A.B. International Mycological Institute, Ferry Lane, Kew, Surrey, TW9 3AF, England. The characteristics of *Aspergillus* sp. IMI 337664 were as follows:

Colonies on CZAPEK's solution agar reached a diameter of 3~3.5 cm in 10 days at 28°C. The basal mycelium was fairly loose, white and largely submerged bearing abundant conidial structures which were deep brownish-black and covered the entire colony except for a narrow growing margin. The conidial heads were large and brown-black in colour. At first they were globose, but later split into two or more reasonably well-defined columns. Conidiophores had comparatively thick walls (2.0~2.5 μm) and were brownish in shade particularly in the upper half. The vesicles were globose or nearly so, and the metulae were quite long, with short phialides. Conidia were globose and brownish which became echinulate as they matured. Colonies on potato dextrose agar grew more rapidly, reaching 4~5 cm, in 10 days at 28°C. Basal mycelia were flat and cream yellow, heavily



VM55598 (**1**)	aspergillimide	R = H, H
SB202327 (**2**)	16-keto aspergillimide	R = O



VM54159 (**3**)		R = Me	R' = H	R" = H
SB203105 (**4**)		R = Me	R' = OH	R" = H
SB200437 (**5**)		R = H	R' = H	R" = H
VM29919 (**6**)	paraherquamide	R = Me	R' = H	R" = OH

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sporulating in black shades. Pink sclerotia were produced in moderation (0.8~1.2 mm diameter). IMI 337664 is believed to belong to the *Aspergillus niger* group.

Fermentation

For the production of aspergillimide, VM55598 (**1**) and VM54159 (**3**), fermentation of strain IMI 337664 was carried out as previously described[3] except that the incubation period was 39 days. SB200437 (**5**) and SB202327 (**2**) were produced by fermentation of strain IMI 337664 in the same fashion but over approximately 30 days in Potato Dextrose agar (Oxoid Ltd., Basingstoke, UK). SB203105 (**4**) was produced by fermentation of strain IMI 337664 in the same fashion but over 26 days in PMG (peptonised milk glycerol) agar.

Isolation and Purification

Aspergillimide, VM55598 (1) and VM54159 (3)

A batch of large plate Czapek-Dox agar cultures of IMI 337664 (total 5 litres) was homogenised in acetone (6 litres) centrifuged and the solids further acetone extracted with 2×6 litre washes. The acetone was concentrated by vacuum rotary evaporation to yield an aqueous residue. This was extracted three times with 2 volumes of $CHCl_3$. The organic phase was evaporated under vacuum to yield 0.96 g of solid.

The solid was dissolved in methanol (10 ml) and chromatographed in three runs on a 21.4 mm diameter preparative HPLC silica column. The column was eluted (10 ml/minute) with dichloromethane (DCM) and a linear gradient of methanol from 0~20% over 90 minutes. The column eluent was monitored at 240 and 288 nm. The majority of UV absorbing peaks eluted in 97-3 (DCM-MeOH) and therefore the gradient was held at these proportions and then recommenced. Fractions were collected and assayed for anthelmintic activity.

Anthelmintic activity in each of the 3 column runs eluted in two bands corresponding to peaks of UV absorbance (240 nm); peak A (28 to 34 minutes) and peak B (major component, 42 to 52 minutes). Both eluted after a strong band of yellow-orange pigment. Fractions containing A and B from all three runs were combined separately to yield material for further purification. The B fractions were combined in two separate samples, B1 and B2, representing the leading and tailing sides of the B peak respectively.

Combined A fractions were concentrated to dryness (17 mg), redissolved in methanol, and rechromatographed on a 21.4 mm diameter silica HPLC column, eluted at 10 ml/minute using DCM and a linear methanol gradient from 0~10% over 90 minutes. Anthelmintic activity was detected in fractions 48~52 (10 ml each) which were combined and evaporated to dryness under vacuum.

This material was again dissolved in methanol and chromatographed on a 10 mm C-18 HPLC column, eluted at 4 ml/minute with methanol-water (70:30). Anthelmintic activity was detected in fractions 18~27 (4 ml each). Fractions 23~25 were combined and dried under vacuum to yield 0.8 mg of VM54159 (**3**).

The combined B1 fractions from 3 silica column runs were evaporated to dryness and rechromatographed on the 21.4 mm diameter silica HPLC column eluting (10 ml/minute) with DCM and a linear methanol gradient up to 10% over 90 minutes. Fractions 43~56 (10 ml each) showed anthelmintic activity and these were combined and evaporated to dryness. This material was redissolved in methanol and injected onto a 21.4 mm HPLC column eluted at 10 ml/minute with methanol-water (60:40). Anthelmintic activity was detected in fractions 12~23 (10 ml each); the peak fractions 14 and 15 were combined and evaporated to dryness under vacuum to yield 11.0 mg of VM55598 (aspergillimide, **1**).

SB200437 (5) and SB202327 (2)

Ten litres of agar was homogenised in methanol and concentrated by vacuum rotary evaporation to yield an aqueous residue, which was extracted with chloroform to yield 4 g of solid. The solid was chromatographed on a silica HPLC column (41.4 mm dia.), eluting (15 ml/minute) with DCM and a linear gradient of methanol from 0~10% over 90 minutes. Fractions were collected by hand corresponding to peaks in UV absorbance (240 and 280 nm). Anthelmintic activity was detected in fractions 13 and 14, which eluted at 5% methanol, and in two later peaks. Fraction 13 was concentrated to dryness, redissolved in methanol, and rechromatographed on the 21.4 mm silica HPLC column, eluted at 10 ml/minute using hexane:ethyl acetate, 1:1 and a linear propan-2-ol gradient from 0~10% over 90 minutes. Fractions (10 ml) were examined by TLC and for anthelmintic activity. Fractions 52 to 66 were combined, evaporated to dryness and rechromatographed on the 21.4 mm silica HPLC column eluting (10 ml/minute) with DCM and a linear methanol gradient up to 10% over 90 minutes. The main anthelmintic activity resided in fractions 53 to 56 (10 ml each) which were combined and evaporated to dryness to yield 3.0 mg of SB200437 (**5**).

Fraction 14 (~200 mg) from the 41.4 mm silica HPLC

column was run in 2 batches on a 21.4 mm phenyl HPLC column (column 1; 10 ml/minute, eluting with a linear gradient from 65% to 95% methanol over 180 minutes; column 2; 10 ml/minute eluting at 68% methanol for 60 minutes and then a linear gradient from 68% to 95% methanol over 120 minutes). A new active component was found in fractions 10 and 11 from columns 1 and 2 respectively. These fractions (8 ml) were combined and evaporated to dryness to yield 2.0 mg of SB202327 (**2**).

SB203105 (**4**)

10 litres of agar was homogenised with acetone and concentrated by vacuum rotary evaporation to an aqueous residue, which was extracted with chloroform to yield 2.2 g of solid. The solid was chromatographed in two halves on a 41.4 mm silica HPLC column, eluting (20 ml/minute) with DCM and a linear gradient of methanol from 0~10% over 90 minutes, collecting 8 ml fractions. Fractions 128 to 147 from column 1 were combined with fractions 125 to 133 of column 2 to yield sample A. Fractions 148 to 175 of column 1 were combined with fractions 134 to 162 of column 2 to yield sample B. Samples A and B were separately chromatographed on a 21.4 mm phenyl HPLC column with methanol/5 mM pH 7.0 sodium phosphate (sample A; 10 ml/minute, eluting with linear gradients from 60% to 80% methanol over 120 minutes and from 80% to 100% methanol over 30 minutes; sample B; 10 ml/minute eluting with linear gradients from 50% to 80% methanol over 90 minutes and from 80% to 100% methanol over 30 minutes). A new active component was detected in both fractions 89 to 91 from sample A and fraction 86 from sample B. These fractions (8 ml) were combined and evaporated to dryness to yield 6.8 mg of SB203105 (**4**).

Physico-chemical Properties

Aspergillimide (VM55598, **1**) and 16-keto aspergillimide (SB202327, **2**)

Elucidation of structure: aspergillimide (**1**) had a UV_{max} at 206 nm and produced a molecular ion at *m/z* 359 by EI-MS, the molecular weight being confirmed by

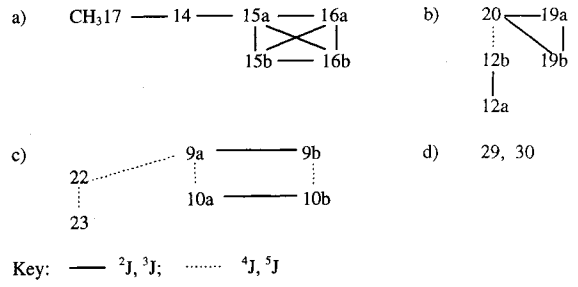
Table 1. ^{1}H and ^{13}C NMR chemical shifts (δ in ppm[a]) and ^{1}H, ^{1}H coupling constants ($^{n}J_{H,H}$ in Hz) for aspergillimide (VM55598, **1**) and 16-keto aspergillimide, SB202327 (**2**), in $CDCl_3$-TMS.

Atom	δ_H		δ_C	$^{n}J_{HH}$	
	VM55598 (**1**)	SB202327 (**2**)	VM55598 (**1**)	VM55598 (**1**)	SB202327 (**2**)
2	—	—	181.7	—	—
3	—	—	57.9	—	—
8	—	—	175.4	—	—
9	a) 2.96	a) ~2.93	38.4	a)	a) c
	b) 2.51	b) 2.55		b) 18.4	b) 18.4
10	a) 2.77	a) 2.84	39.2	a) 14.9	a) 15.4
	b) 1.61	b) 1.77		b) 15.0	b) 15.5
11	—	—	64.6	—	—
12	a) 3.33	a) 3.36	59.3	a) 11.0	a) 12.0
	b) 2.43	b) 3.43		b) 11.0, 1.8	b) 12.0, 1.6
13	—	—	67.2	—	
14	1.90	~2.30	40.3	6.9, c	~11.5[b], ~7.5[b], c
15	a) 1.82	a) ~2.51[d2]	30.1	a) 10.3, 4.2, c	a) 16.1[b], 7.5[b]
	b) 2.01	b) ~2.39[d2]		b) 5.7, c	b) 15.9[b], 11.5[b]
16	a) 3.15	a) —	53.3	a) 9.0, 9.0, 4.2	a) —
	b) 2.21	b) —		b) 10.5, 9.1, 5.5	b) —
17	1.40	1.53	13.0	6.6	6.9
18	—	—	172.1	—	—
19	a) 2.03	a) 2.18	27.9	a) 12.6, 11.0	a) 12.9, 10.6
	b) 1.37	b) 1.52		b) 12.5, 9.5	b) 12.9, 10.4
20	2.83	3.06	53.4	11.1, 9.5, 1.8	10.5, 10.5, 1.7
21	—	—	44.6	—	
22	1.08	1.04	20.1	—	
23	0.87	0.89	23.9	—	
29	2.98	2.99[d1]	24.8[d3]	—	
30	2.98	2.96[d1]	25.4[d3]	—	

[a] Reference; $\delta_{TMS} = 0$ ppm.
[b] Approximate values from first order analysis of second order spin system.
[c] Signal obscured preventing full analysis.
[d] Assignments may be interchanged between pairs labelled di.

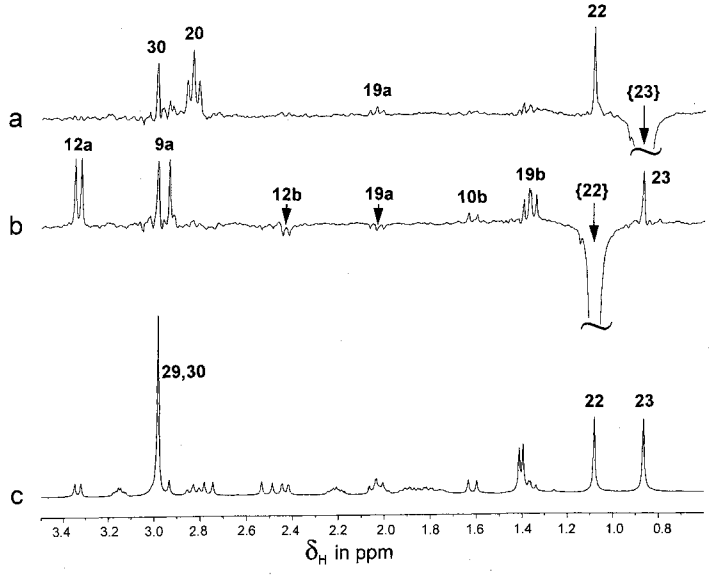
FAB-MS. The ^{13}C NMR spectrum (Table 1) displayed 20 resonances including 5 CH_3, 6 sp^2 CH_2 and 2 sp^3 CH groups as well as 4 sp^3 quaternary carbons and 3 carbonyl groups, thus indicating 29 non-exchangeable hydrogen atoms. No exchangeable protons were observed in the ^1H NMR spectrum and it was therefore deduced that the molecular formula was $C_{20}H_{29}N_3O_3$. Inspection of the ^1H NMR spectrum revealed a strange similarity to those of VM55594 and VM54159[3] but no olefinic or aromatic resonances were observed. Analysis of the 2D ^1H COSY-45 NMR spectrum (Fig. 1) confirmed that the proton-to-proton connectivities in aspergillimide were exactly the same as in VM55594[3], except that: i) no aromatic or unsaturated protons were present, and ii) new, weak, long-range connectivities were observed from CH_3-22 and H10a to a new methylene proton, the partner of which was weakly connected to H10b. The resonances of the protonated carbons of aspergillimide were unambiguously assigned using a 2D ^1H, ^{13}C HETCOR NMR experiment, and the quaternary carbon resonances C11, C13 and C18 were assigned by reference to VM54159 and VM55594[3]. The chemical shifts of the ^{13}C resonances of C11 to C19 were within 0.8 ppm of the corresponding values for VM54159 and VM55594[3]. Furthermore, all observed ^1H coupling constants were within 0.5 Hz of the corresponding values in VM55594[3]. These two observations confirmed that the structure and relative stereochemistry of aspergillimide were the same as those of VM55594 on the "right-hand-side" of the molecule. The IR spectrum shows carbonyl absorptions at 1770, 1695 and 1646 cm^{-1}, as expected for imide and amide groups[8].

Fig. 1. The proton-to-proton connectivities in aspergillimide (VM55598, **1**) observed in the 2D ^1H COSY-45 NMR spectrum.



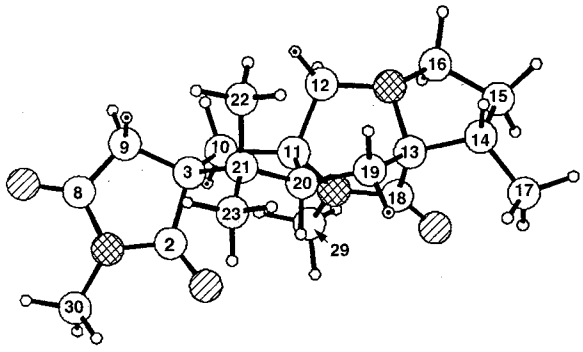
^1H NOE difference spectroscopy (Fig. 2) further confirmed the structure, and the relative stereochemistry in region of the C3 C10 C11 C20 C21 ring (see Fig. 3). The NOEs observed upon irradiation of CH_3-22 and CH_3-23 were exactly the same as for VM55594[3] except that the NOEs to H4 and H24 were missing and the novel NOEs-NOE [22]9a and NOE [23]30 were observed. No NOE [22]9b was observed although $r_{22,9b}$ ~2.3 Å minimum (Dreiding models). This is probably due to the cancellation of the direct positive NOE by the weak, negative NOE relayed from H9a ($r_{22,9a}$ ~1.5 Å minimum!). Although $r_{23,30}$ ~3.6 Å is relatively long, the NOE [23]30 observed is a reflection of the relative isolation of the CH_3-30 protons. Thus the relative stereochemistry of aspergillimide is the same as paraherquamide (**6**) at all centres. It was thus concluded that the structure of aspergillimide was **1**, VM55598.

Fig. 2. A series of NOE difference spectra of aspergillimide (VM55598, **1**) produced by irradiation of a) CH3-23 and b) CH3-22. The NOE difference spectra are plotted over the normal spectrum (c).



For SB 202327 (**2**) EI MS gave no useful data, but FAB MS indicated a molecular weight of 373 Daltons, 14 Daltons higher than aspergillimide (**1**). The 400 MHz ^{1}H NMR spectrum exhibited resonances for 5 CH_3, 5 CH_2, and 2 CH groups; 1 CH_2 group less than for **1**. Inspection of the spectrum and comparison with that of **1** showed that **2** exhibited all the spectral features of **1** with the exception of the C16 methylene resonances. The spectrum was assigned (Table 1) by comparison with that of **1**, and exhibited changes in chemical shift consistent with the structural change from 16-CH_2 to 16-CO. Homodecoupling and 2D ^{1}H COSY-45 NMR experiments were used to confirm the proton-to-proton connectivities deduced from the 1D NMR spectrum and confirm the structure of SB 202327 as 16-keto-aspergillimide, (**2**). The COSY spectrum exhibited the same, unexpected, stereospecific $^5J_{9a,22}$ observed in VM55598 (**1**) and in addition, the conversion of 16-CH_2 to 16-CO resulted in the appearance of connectivities due to $^5J_{12a,15a}$ and $^5J_{12a,15b}$; all three couplings being unresolved even in the resolution enhanced 1D NMR spectrum.

Fig. 3. A molecular model of aspergillimide (VM55598, **1**) produced using CHEM-X[11].



The oxygen atoms are shaded, the nitrogen atoms are stippled, and the dot on a hydrogen atom indicates that it is Ha. Note that our definition of Ha and Hb are different from those of LIESCH and WICHMANN[6]. Our definitions have been used consistently for all the molecules reported here and previously[3,4].

Paraherquamides VM54159 (3), SB 203105 (4) and SB 200437 (5)

Elucidation of structure. The structure of VM54159

Table 2. ^{1}H NMR chemical shifts (δ in ppm[a]) for VM54159 (**3**), SB203105 (**4**), SB200437 (**5**) and paraherquamide (VM29919, **6**) in $CDCl_3$-TMS.

Atom	VM54159 (3)	SB203105 (4)	SB200437 (5)[d]	Paraherquamide VM29919 (6)[c]
C2-NH	7.76	7.35	7.59	7.34
4	6.81	—	6.80	6.81
5	6.68	6.11	6.67	6.68
C5-OH	—	~8.9 (br)	—	—
10a	2.70	2.55	2.26	2.69
10b	1.86	2.42	1.96	~1.85
C11-NH	—	—	6.46	—
12a	3.62	4.02	3.66	3.61
12b	2.55	2.49	2.60	2.57
14	~1.92	~1.7	~1.95	—
C14-OH	—	—	—	2.63
15a	~2.04	~2	~2.07	~1.88
15b	~1.85	~2	~1.84	2.35
16a	3.20	~3.22 (br)	3.19	3.21
16b	2.26	~2	2.36	2.22
17	1.42	1.48	1.40	1.65
19a	~2.04	~2.10	~2.07	~1.85
19b	~1.41	~1.5?	~1.43	1.79
20	3.05	2.79	3.06	3.02
22	1.10	1.16	1.08	1.10
23	0.86	0.93	0.87	0.87
24	6.31	6.27	6.31	6.30
25	4.89	4.78	4.88	4.89
27	1.45[b1]	1.46[b2]	1.45[b3]	1.45[b4]
28	1.43[b1]	1.41[b2]	1.43[b3]	1.44[b4]
29	3.03	3.04	—	3.05

[a] Reference; $\delta_{TMS} = 0$ ppm.
[b] Assignment uncertain between pairs labelled bi.
[c] Data from reference 3.
[d] $^2J_{10,10} \sim 14.7$, $^2J_{12,12} \sim 11.6$, $^3J_{14,17} \sim 6.6$, $^3J_{24,25} \sim 7.7$ Hz.

(**3**) was simply solved on the basis of identical MS and NMR data (Table 2) to that reported previously[3]. The structures of SB203105 (**4**) and SB200437 (**5**) were solved by comparison of their spectral data (Table 2) with that already reported for related paraherquamides[3,4]. The NMR spectrum of SB200437 in $CDCl_3$/TMS showed unusual and interesting behaviour. The linewidth of the CH_3-22 signal was significantly greater than that of CH_3-23 (peak height ratio *ca.* 0.5 : 1), and in a CPMG experiment, the signal for CH_3-22 relaxed rapidly to zero intensity, whereas the signal for CH_3-23 was only moderately attenuated. In addition, the resonances of the H-10, H-12, H-14, H-15, H-16, H-19 and H-20 protons were relatively broad Addition of d_6-acetone to the solution made these resonances even broader. However, the ^1H NMR spectrum appeared normal in CD_3OD/TMS solution. This behaviour indicated that SB200437 was undergoing some dynamic process in $CDCl_3$/TMS solution, possibly due to aggregation. By contrast, the spectra of VM54159, were normal in $CDCl_3$/TMS solution. For SB203105 (**4**), the addition of one drop of d_6-acetone to the $CDCl_3$ solution caused a significant broadening of many parts of the spectrum. Upon the further addition of one drop of CD_3OD, the H10a, H10b AB quartet changed into a broad singlet a δ 2.45 ppm. It is possible that these effects were also due to the formation of intermolecular complexes in solution. Unusual long-range NOEs that disappeared upon addition of CD_3OD have already been observed for VM55594 in $CDCl_3$/TMS solution[3], and paraherquamide itself shows NOEs between CH_3-29 and H24 which are unexpected on the basis of the molecular structure (J. R. EVERETT, unpublished observations). It should be noted that SB203105 (**4**) is the first paraherquamide to be discovered with C-4 substitution. Merck have reported the isolation of C-5 hydroxylated compounds after biotransformation of both paraherquamide and dihydroparaherquamide.[12]

Anthelmintic Activity

The oral activities of the four new metabolites, paraherquamide and VM54159 against adult *Trichostrongylus colubriformis* infections in gerbils were determined by the method of HOOD *et al.*[10] (Table 3). During bioassay-guided extraction (*Haemonchus contortus* L_3 larvae), the 16-keto analogue of aspergillimide (**2**) showed evidence of activity *in vitro* but not *in vivo*.

Experimental

All NMR experiments were performed in the 5 mm ^1H, ^{13}C dual probe (normal geometry) of a Bruker AM400 spectrometer at 300 K in 0.5 ml $CDCl_3$-TMS solution. The approximate solution concentrations were 0.056, 0.011, 0.037, 0.028 and 0.013 M for compounds **1** to **5** respectively. 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).

The preparative HPLC system comprised a Waters 660 solvent delivery system and ABI 1000S photodiode-array detector; columns were Dynamax-60A, 250 mm in length, (Rainin Instrument Co., USA). Fractions were selected on the basis of *in vitro* anthelmintic activity and positive EHRLICH's reaction on TLC. Anthelmintic activity was detected by the inhibition of the motility of *H. contortus* L_3 larvae as described by HOOD *et al.*[10]. Column fractions or dilutions thereof were dried into wells in microtitre plates, 0.1 ml of larvae was added and the motility assessed visually after standing at room temperature for approximately 1 hour.

The HPLC retention times of all metabolites are shown in Table 4.

Table 3. Oral *in vivo* anthelmintic activities.

Compound	% Reduction in faecal egg count at stated dose				
	0.5 mg/kg	1 mg/kg	4 mg/kg	10 mg/kg	20 mg/kg
VM55598, aspergillimide (**1**)	—	—	—	44	98
SB202327 (**2**)	—	0	—	—	—
VM54159 (**3**)	88	96	99	—	—
SB203105 (**4**)	—	—	73	67	—
SB200437 (**5**)	—	—	—	86[a]	—
VM29919, paraherquamide (**6**)	19	70	99	—	—

[a] Dosed orally at 7.7 mg/kg.

Table 4. HPLC retention times.

Compound	Rt in minutes			
	1[a)]	2[a)]	3[a)]	4[a)]
Aspergillimide, VM55598 (**1**)		5.4		4.0
SB202327 (**2**)			5.2	
VM54159 (**3**)	9.3	11.6		9.6
SB203105 (**4**)				5.8
SB200437 (**5**)	9.4			
Paraherquamide (**6**)	12.8	9.4		

[a)]: 1, Microsorb C18 column, 4.6×250 mm, 90:10 methanol: 0.1 M acetic acid pH 3.5, 1.0 ml/minute. 2, Microsorb Phenyl column, 4.6×250 mm, 75:25 methanol: water, 1.0 ml/minute. 3, Microsorb Phenyl column, 4.6×250 mm, 70:30 methanol: water, 1.0 ml/minute. 4, Microsorb Phenyl column, 4.6×250 mm, 50:50 acetonitrile: 5 mM pH 7.0 phosphate buffer, 1.0 ml/minute.

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