DEMETHYLAVERMECTINS

BIOSYNTHESIS, ISOLATION AND CHARACTERIZATION

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Streptomyces avermitilis normally produces eight avermectins. Avermectin A components contain three methoxyl groups; two on the oleandrose disaccharide and one on the aglycone moiety at C_5. Avermectin B components contain methoxyl groups only on the oleandrose disaccharide. Sinefungin inhibits methylation at all three sites.

Addition of sinefungin to *S. avermitilis* Agly-1, a mutant which produces virtually only avermectin aglycone A components, alters the fermentation and causes an accumulation of avermectin aglycone B components.

Addition of sinefungin to *S. avermitilis* 08, a high producing strain, results in accumulation of 8 new avermectins which lack methoxyl groups on the oleandrose moieties as well as the aglycone. These new avermectins were isolated and shown to possess anthelmintic and insecticidal activity.

The avermectins are oleandrose disaccharide derivatives of 16 membered pentacyclic lactones with potent anthelmintic and insecticidal activity which are produced by *Streptomyces avermitilis*[1,2,3,4]. *S. avermitilis* normally produces 8 major components[3] (Fig. 1). Approximately 50% of the avermectins have a hydroxyl on C5 and are termed B components while the remaining 50% have a methoxyl group on C5 and are termed A components. Conversion of the B components to the A components is catalyzed by the enzyme avermectin B *O*-methyltransferase which transfers the methyl of *S*-adenosylmethionine (SAM) to the C5 hydroxyl group[5]. Sinefungin, an analogue of SAM is a potent inhibitor of this enzyme and other methyltransferases[5,6,7]. We consequently investigated the effect of sinefungin on avermectin production by *S. avermitilis*.

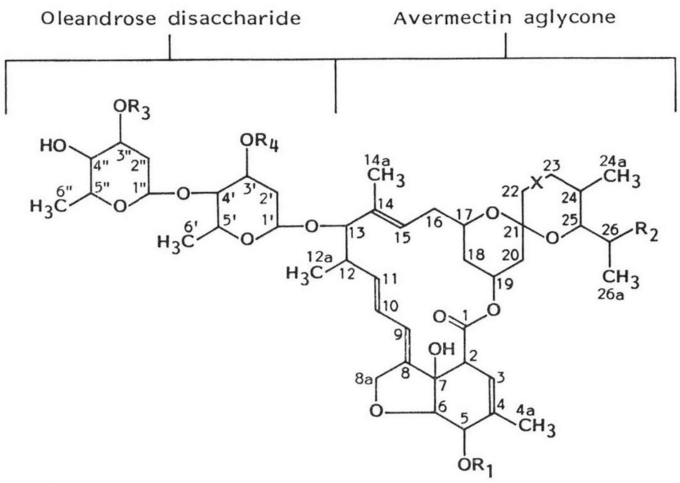
This paper reports the effect of sinefungin on avermectin production by a high producing strain of *S. avermitilis* and by a mutant strain which produces virtually only avermectin aglycones (Fig. 1), *i.e.*, avermectins lacking the oleandrose disaccharide. The isolation, identification and biological activity of new avermectin derivatives (termed demethylavermectins) produced in the presence of sinefungin are described.

Materials and Methods

Microorganisms

Streptomyces avermitilis 08 is a high producing strain derived from *S. avermitilis* MA4848[1]. *S. avermitilis* Agly-1 is a mutant strain which produces virtually only avermectin aglycones A1a and A2a (Fig. 1).

Fig. 1. General structure of the avermectins.



R_1=H, B components; R_1=CH_3, A components
X=CH=CH, 1 components; X=CH_2CHOH, 2 components
R_2=CH_2CH_3, a components; R_2=CH_3, b components
R_3=CH_3, avermectin; R_3=H, 3″-*O*-demethylavermectin
R_4=CH_3, avermectin; R_4=H, 3′-*O*-demethylavermectin.

Fermentation

S. avermitilis was grown in a modified medium B as described by BURG, *et al.*[1] A 5 mM stock solution of sinefungin (Calbiochem-Behring) in distilled water was sterilized by passage through a sterile 0.22 μm filter (Millipore) and different size aliquots were added to the fermentation at 48 hours. Fermentations were continued for an additional 4 days. The quantities of avermectins in the whole broth were monitored with time.

Analysis and Determination of Avermectins

Samples of fermentation broths were brought to 80% saturation with MeOH and shaken vigorously for 15 minutes on an Eberbach reciprocal shaker. The solids were removed by centrifugation and avermectin in the supernatant was determined by HPLC on a Zorbax C-18 column (4.6 mm×25 cm) at 60°C with MeOH - H_2O (85: 15) as the mobile phase[3]. The avermectins were quantitated by measuring absorbance at 247 nM. Avermectin aglycones were determined under identical conditions with MeOH - H_2O (82: 18) as the mobile phase.

Isolation of Demethylavermectins

Cells were harvested by centrifugation at 1,000×*g* for 10 minutes and washed with 50 ml cold deionized water. Avermectins were extracted from the cell paste by vigorous shaking with MeOH for 15 minutes on an Eberbach reciprocal shaker and the solids were removed by centrifugation. The MeOH was concentrated to 1 ml under vacuum and the concentrate was streaked onto Silica Gel-60 F-254 precoated TLC plates (E. M. Laboratories 20×20 cm, 0.25 mm thickness). The TLC plates were developed in CH_2Cl_2 - EtOAc - MeOH (9: 9: 1) for 1 to 1.5 hours. The UV absorbing bands were scraped from the plates, extracted with MeOH, and UV absorption spectra were measured to detect the avermectins. The avermectins were purified by preparative HPLC on a Dupont Zorbax ODS reverse phase column (21.2×25 cm) at room temperature with MeOH - H_2O (85: 15) as the mobile phase at a flow rate of 4.5 ml/minute.

Identification of Demethylavermectins

The structures of the demethylavermectins were determined by NMR and mass spectroscopy. ^1H NMR were recorded in $CDCl_3$ at room temperature (25°C) with a Varian XL-400 instrument (Varian

Table 1. Effect of sinefungin on the formation of avermectin aglycones by *S. avermitilis* Agly-1.

Time	Sinefungin[a] (mM)	Avermectin aglycone (U/ml[b])						
		A1a	A2a	Total A	B1a	B2a	Total B	B/A
96 hours	0	57	43	100.0	2.0	0.5	2.5	0.025
	0.125	10	7.5	17.5	43.5	27.0	70.5	4.0
	0.0625	10	9.5	19.5	42.0	29.5	71.5	3.7
168 hours	0	135	94	229.0	2.5	1.5	4.0	0.017
	0.125	17.5	19	36.5	94.0	52.5	146.5	4.0
	0.0625	21	24	45.0	92.5	49.5	142.0	3.2

[a] Added aseptically to cultures at 48 hours.
[b] Relative units where the total avermectins formed in the absence of sinefungin at 96 hours were set equal to 100. All other avermectins are expressed as a ratio of these values.

Table 2. Demethylavermectin components isolated from *S. avermitilis* 08 treated with sinefungin.

Compound	Emperical formula	MW	Ionic species	MW	
				Calcd	Found
3′-*O*,3″-*O*-bis-Demethylavermectin B1a	$C_{46}H_{68}O_{14}$	844	$(M^a+T_5{}^b)^+$	1,204.6585	1,204.6589
3′-*O*,3″-*O*-bis-Demethylavermectin B2a	$C_{46}H_{70}O_{15}$	862	$(M-H_2O+T_4)^+$	1,132.6190	1,132.6190
3″-*O*-Demethylavermectin B1a	$C_{47}H_{70}O_{14}$	858	$(M-H_2O+T_3)^+$	1,056.5846	1,056.5850
3″-*O*-Demethylavermectin B2	$C_{47}H_{72}O_{15}$	876	$(M-H_2O+T_3)^+$	1,146.6346	1,146.6331
3′-*O*-Demethylavermectin B1a	$C_{47}H_{70}O_{14}$	858	$(M+T_4)^+$	1,146.6346	1,146.6342
3′-*O*-Demethylavermectin B2a	$C_{47}H_{72}O_{15}$	876	$(M-H_2O+T_4)^+$	1,146.6346	1,146.6342
3′-*O*-Demethylavermectin B1a monosaccharide	$C_{40}H_{58}O_{11}$	714	$(M+T_4)^+$	1,002.5560	1,002.5555
3′-*O*-Demethylavermectin A1a monosaccharide	$C_{41}H_{60}O_{11}$	728	$(M+T_3)^+$	944.5321	944.5321

[a] M is the molecular weight of the avermectin component.
[b] T is C_3H_8Si.

Instrument Co., Palo Alto, CA). Low resolution mass spectra were recorded on a Finnigan-Mat212 mass spectrometer in the electron impact mode (EI, 90 eV). Exact mass measurements were made on the same instrument at high resolution by the peak matching method using Ultramark 1600F (U1600F) as internal standard. Avermectin fragmentation patterns were as described by ALBERS-SCHONBERG *et al.*[8]

Assessment of Biological Activity

Anthelmintic activity of the avermectins was determined against *Trichostrongylus colubriformis* in the gerbil (*Meriones uguiculatus*)[9].

Results

Effect of Sinefungin on *S. avermitilis* Agly-1

S. avermitilis Agly-1 produces only avermectin aglycones and of these greater than 97% are A components and only 1~2.5% are B components. Table 1 presents typical results which demonstrate the effect of sinefungin on avermectin production by this culture. Sinefungin inhibited the formation of A components and caused a concomitant accumulation of the B components. The ratio of B components to A components rose from 0.02 to 4.0. Total avermectin production was inhibited 10% to 35% by sinefungin. Sinefungin thus appeared to inhibit the conversion of B components to A components catalyzed by avermectin B *O*-methyltransferase rather specifically.

Table 3. Anthelmintic activity of demethylavermectin components.

Compound	Dose (mg/kg)	Reduction (%)	Worm count[a] activity
3′-*O*,3″-*O*-bis-Demethylavermectin B1a	0.5	96.0	High
	0.125	12.3	None
3′-*O*,3″-*O*-bis-Demethylavermectin B2a	0.5	95.1	High
	0.125	59.2	Slight
3′-*O*-Demethylavermectin B1a	0.5	100.0	High
	0.125	97.3	High
	0.03	0	None
3′-*O*-Demethylavermectin B2a	0.5	92.1	High
	0.125	60.1	Slight
Ivermectin	0.03	100.0	High

[a] Control worm counts averaged 228.2 and ranged from 173 to 280.

Effect of Sinefungin on *S. avermitilis* 08 and Isolation of Demethylavermectins

S. avermitilis 08 normally produces the 8 major avermectin components. Addition of sinefungin (0.125 mM) alters the fermentation and causes the accumulation of new avermectin components. These components were isolated and identified as described above. The high resolution mass spectra values of these components are presented in Table 2. All of these components lack a methoxyl group on either one or both of the oleandrosyl units of the disaccharide (at C′3 and C″3) and all but one are B components. In this case sinefungin inhibits methylation of the oleandrose units as well as conversion of the B components to the A components.

Biological Activity of Demethylavermectins

The anthelmintic and insecticidal activity of the demethylavermectins were determined. The anthelmintic activity is summarized in Table 3. Although the demethylavermectins possessed activity, they were approximately 20-fold less active than ivermectin. Preliminary results indicate all of the demethyl components possess anti-arthropod activity similar to the natural avermectins. The 3′-*O*,3″-*O*-bis-demethylavermectin B2a displayed the least activity.

Discussion

The avermectin A components contain three methoxyl groups, one on the aglycone moiety at C5 and two on the oleandrose disaccharide moiety at C′3 and C″3 (Fig. 1), all of which have been shown to be derived from the *S*-methyl of methionine[10]. The methoxyl at C5 has further been shown to arise *via* an SAM dependent methyltransferase[5]. Although participation of SAM in formation of the methoxyl groups of the oleandrose disaccharide has yet to be shown, this seems probable since methylation of sugars in erythromycin[11] and tylosin[12] has been found to occur *via* SAM dependent methyltransferases. Recently, two classes of methyltransferase mutants of *S. avermitilis* have been identified[13]. One class is unable to methylate the C5 hydroxyl and produces primarily avermectin B components. These mutants lack the enzyme avermectin B *O*-methyltransferase. The other class of mutants does not methylate the C′3 or C″3 of the oleandrose disaccharide and produces demethyl components. These mutants produce demethylavermectins A and B components. These findings indicate that *S. avermitilis* possesses two distinct methyltransferases; one catalyzes methylation of the aglycone moiety while the other catalyzes methylation of the oleandrose disaccharide. The observation that sinefungin, an SAM analogue, inhibits methylation of the avermectins at all three sites indicates that SAM is methyl donor for both enzymes.

The low anthelmintic activity of the demethylavermectin components is surprising. It indicates the stringent structural requirements for biological activity and suggests that the oleandrose disaccharide may be directly involved in binding to the biological receptor.

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