Production of 6,8a-Seco-6,8a-deoxy Derivatives of Avermectins by a Mutant Strain of *Streptomyces avermitilis*

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(Received for publication June, 9, 1994)

Streptomyces avermitilis produces the anthelmintic and insecticidal secondary metabolite avermectins. Following mutagenesis of a recombinant strain, *S. avermitilis* K2038 (*aveD X*) with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a derivative strain K2057 (*aveD aveE X*), was isolated, which produced seven avermectin-related compounds different from the eight components of avermectins. Four among these seven compounds from mutant K2057 were found to be new metabolites. Their structures were 4'-deoleandrosyl-6,8a-seco-6,8a-deoxyavermectin B1a, 4'-deoleandrosyl-6,8a-seco-6,8a-deoxy-5-oxoavermectin B1a, 4'-deoleandrosyl-6,8a-seco-6,8a-deoxy-5-oxoavermectin B2a and 6,8a-seco-6,8a-deoxy-2,5-didehydroavermectin B2a, all of which lack the furan ring at C-6, C-8a. The mutation affecting the formation of the furan ring (*aveE*) is located in the center of the gene cluster for avermectin biosynthesis.

The avermectins are oleandrose disaccharide derivatives of 16-membered pentacyclic lactones produced by *Streptomyces avermitilis*[1] with potent anthelmintic and insecticidal activities. During studies of the biosynthesis of avermectin, some biosynthetically blocked mutants of *S. avermitilis* have been isolated and characterized. CHEN and INAMINE[2] isolated a mutant which produced 6,8a-deoxy-avermectin derivatives and found that the mutant was deficient in the formation of the furan ring at C-6 to C-8a on the avermectin aglycone; the mutant accumulates seven avermectin-related compounds in mycelia. Recently, we isolated a recombinant strain of *S. avermitilis* (K2038) which produces only two components, avermectins B1a and B2a[3], which have most potent anthelmintic activity. The recombinant strain possesses two phenotypes; loss of avermectin B2 5-*O*-methyltransferase activity (*aveD*, accumulating "B" components) and efficient incorporation of L-isoleucine or its keto acid into the avermectin aglycone (*X*, accumulating "a" components). We have isolated mutants which are unable to form the furan ring from the recombinant strain.

This paper deals with mutant isolation, structure elucidation of new compounds from the mutant K2057, and the location of mutation in gene cluster for avermectin biosynthesis.

Materials and Methods

Microorganisms

Streptomyces avermitilis K2038 derived by protoplast fusion between K2021 and K2034[3]. Mutant K2057 from strain K2038 was used for the isolation of new compounds.

Mutagenesis

S. avermitilis K2038 was mutagenised with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described previously[4]. Mutants were screened by examining the mycelial extract of each colony by silica gel TLC or reversed phase HPLC as described previously[5,6].

Media and Fermentation

YMS agar[4] was used for sporulation. Liquid media[5] were used for the seed and fermentation culture. Spores of mutant K2057 were inoculated into a 500-ml flask containing 100 ml of seed medium and cultured on the rotary shaker (200 rpm) for 2 days at 28°C. Two milliliters of the seed culture was inoculated into a 500-ml flask containing 100 ml of production medium and incubated on the rotary shaker (180 rpm) for 7 days at 28°C.

Extraction of Avermectin-related Compounds

Fermentation cultures of sixty-five flasks (6 liters) was filtered to remove the culture filtrate. The residual mycelia were washed with 1 liter of deionized water and harvested. One liter of acetone was added to the washed mycelia and mixed vigorously shaking for 30 minutes at room temperature and filtered to remove mycelia. The mycelia were reextracted with 500 ml of acetone and the combined acetone extracts were evaporated under the reduced

pressure. Five hundred milliliters of deionized water was added to the concentrate and the mixture was extracted twice with 150 ml of methylene chloride and methylene chloride extracts were evaporated to dryness. The oily extract (3.02 g) was dissolved in 5.0 ml of ethyl acetate, the solution passed through 25 g of dry silica gel to remove oily material and the eluate was evaporated to dryness. The crude material (2.43 g) was further purified by silica gel TLC and reversed phase HPLC to separate individual components.

Complementation Studies

A cosmid library of *S. avermitilis* K139 prepared with the *E. coli-Streptomyces* bifunctional cosmid vector pKU206[7] was introduced into *S. avermitilis* K2057. DNA fragments were subcloned with plasmid vector pKU109[6,8]. Transformants were patched (1 cm^2) onto YMS agar containing 15 μg of thiostrepton per ml. After incubating for 8 days at 30°C, the patches were cut out and extracted with 0.5 ml of methanol. The extract was analyzed by silica gel TLC or reversed phase HPLC[3~6].

Results

Isolation of Mutant K2057 Producing Avermectin-related Compounds

Among over 2,000 colonies obtained after the treatment of *S. avermitilis* K2038 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, only one colony was found to produce avermectin-related compounds different from avermectins B1a and B2a, the products of the parent strain K2038. The mutant strain was named K2057. As shown in Fig. 1, mutant K2057 produced seven avermectin-related compounds. The retention times of the compounds in reversed phase HPLC were different from those avermectins[1]. Production levels were similar to that of the parent K2038.

Isolation of Avermectin-related Compounds from the Mutant Strain K2057

Seven avermectin-related compounds were isolated from a 6 liter culture of K2057 as follows. The crude extract from the mycelia was applied to a column chromatography of Sephadex LH-20 (40 i.d. × 500 mm) which was developed with *n*-hexane - methylene chloride - methanol (10 : 10 : 1). Fractions 28~35 contained compounds **V** and **VII** (260 mg), fractions 36~40 contained compounds **IV** and **VI** (50 mg), fractions 41~54 contained compounds **I**, **II**, **III** and **IV** (1.15 g), and fraction 55~62 contained 70 mg of oligomycin.

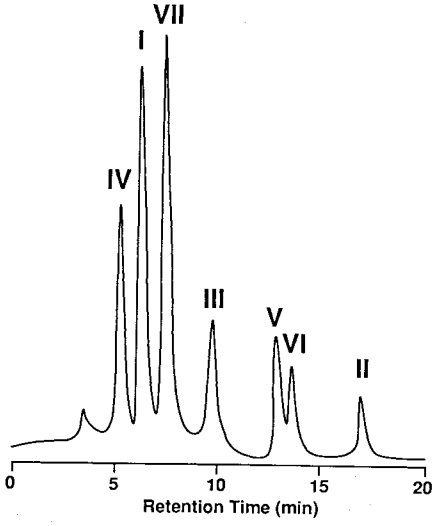
The mixture of compounds **I**, **II**, **III** and **IV** (1.15 g) obtained by Sephadex LH-20 column chromatography was further applied to flash silica gel column chromatography (35 i.d. × 300 mm) which was developed with *n*-hexane - 2-propanol (85 : 15). Fractions 43~70 contained compounds **II** and **IV** (46 mg), fractions 74~85 contained compound **II** (10 mg), fraction 86~124 contained compounds **I** and **II** (41 mg), and fractions 125~145 contained compound **I** (31 mg).

Finally, each compound was isolated from the corresponding partially purified fraction by preparative silica gel TLC using *n*-hexane - 2-propanol (85 : 15) as developing solvent except compounds **V** and **VII**. The mixture of compounds **V** and **VII** (260 mg) obtained by Sephadex LH-20 column chromatography was applied to preparative silica gel TLC to give 27 mg of compound **V**-rich and 23 mg of compound **VII**-rich fractions. Furthermore, these fractions were applied to preparative HPLC to give purified compounds **V** and **VII**. Purified compounds obtained were as follows: 31 mg of compound **I**, 16 mg of **II**, 13 mg of **III**, 34 mg of **IV**, 4.6 mg of **V**, 13 mg of **VI**, and 4.2 mg of **VII**.

Physico-chemical Properties of Compounds **I** to **VII**

Physico-chemical properties of the seven compounds are shown in Table 1. The ultraviolet absorption of compounds **I**, **II** and **III** were similar to those of natural avermectins[9]. However, UV absorption spectra of compounds **IV**, **VI** and **VII** were different from those of natural avermectins; compound **V** was distinctly different. The presence of an α,β-unsaturated ketone in compounds **IV**, **VI** and **VII** was assumed by the presence

Fig. 1. Analytical HPLC of a mycelial extract of mutant K2057.



Culture conditions were described previously[4]. The mycelium from a 10-ml culture was extracted with 5 ml of methanol, after removal methanol, products were extracted with 1 ml of chloroform. The organic layer was harvested and evaporated to dryness. The crude mycelial extract was dissolved in methanol. A portion of the extract was analyzed by reversed-phase HPLC as described previously[5].

Table 1. Physico-chemical properties of avermectin-related compounds **I** to **VII** produced by strain K2057.

	I	II	III	IV	V	VI	VII
IR ν_{max}^{MeOH} (cm^{-1})	3440, 1705	3440, 1705	3440, 1705	3440, 1705, 1675	3440, 1705	3440, 1705, 1675	3440, 1705, 1675
UV λ_{max}^{MeOH} (nm)	241.5	240	242	236	249	235	238
(ε)	28,800	28,400	28,800	33,800	—	34,100	—
$[\alpha]_D^{25}$ MeOH	+24.1	+31.4	+52.8	+0.1	—	+7.0	—
(c)	0.26	1.0	1.0	1.0	—	1.0	—
FAB-MS (m/z)							
$(M+Na)^+$	899	881	737	753	879	735	591
$(M+DEA)^+$*	982	964	820	836	962	818	674
Elementary analysis							
Found:	H 8.69	H 8.54	H 8.69	H 8.49	H 8.36	H 8.40	H 8.47
	C 65.92	C 67.33	C 69.00	C 67.40	C 67.38	C 69.16	C 71.90
Calcd:	H 8.73	H 8.68	H 8.74	H 8.55	H 8.47	H 8.48	H 8.51
	C 65.73	C 67.11	C 68.88	C 67.37	C 67.27	C 69.08	C 71.80
Formula	$C_{48}H_{76}O_{14}$	$C_{48}H_{74}O_{13}$	$C_{41}H_{62}O_{10}$	$C_{41}H_{62}O_{11}$	$C_{48}H_{72}O_{13}$	$C_{41}H_{60}O_{10}$	$C_{34}H_{48}O_7$
MW	877.1	859.1	714.9	730.9	857.1	712.9	568.8

* DEA; diethylamine.

of IR absorption at 1675 cm^{-1} in addition to the lactone carbonyl absorption at 1705 cm^{-1}. Mass spectra and elementary analysis support the molecular mass and formula of each compound (Table 1).

Structures of Compounds I to VII

The structures of these compounds were determined by comparison of their spectral properties with those of natural avermectins and their derivatives[9]. The ^1H and ^{13}C NMR spectra (Tables 2 and 3) were particularly informative. The structures of compounds **I**, **II**, **III**, **IV**, **VI** and **VII** were considered to be similar to those of avermectins B2a, B1a, 4′-deoleandrosylavermectin B1a, B2a, B1a and avermectin B1a aglycone, respectively, based on spectral data. ^{13}C NMR showed that loss of the oxymethine carbon at C-6 (79.3~79.4 ppm) and oxymethylene carbon at C-8a (68.2~68.3 ppm) had occurred in these compounds with addition of the methyl carbon (13.3~14.0 ppm) and the methylene carbon (48.0~48.7 ppm). Moreover, new proton signals, 3.44~3.80 ppm (2H, m) of H-6 and 1.55~1.77 ppm (3H, s) of H-8a, were detected in their ^1H NMR spectra. These results indicate the absence of a furan ring formed from C-6, C-7, C-8 to C-8a, which is present in the avermectins. The presence of an α,β-unsaturated ketone in these compounds was confirmed by gain of a carbonyl carbon (196.3~196.5 ppm) and loss of a proton in the C-5 position. Consequently, compounds **I**, **II** and **VII** were identified with 6,8a-seco-6,8a-deoxyavermectins B2a, B1a and 6,8a-seco-6,8a-deoxy-5-oxo avermectin B1a aglycone, respectively, which had been previously reported[2,10]. On the other hand, compounds **III**, **IV**, **V** and **VI** were novel derivatives of avermectins. Compounds **III**, **IV** and **VI** lack one oleandrose moiety and compounds **IV** and **VI** contain an α,β-unsaturated ketone, suggesting that compounds **III**, **IV** and **VI** are new derivatives of avermectins and are 4′-deoleandrosyl-6,8a-seco-6,8a-deoxyavermectin B1a, 4′-deoleandrosyl-6,8a-seco-6,8a-deoxy-5-oxoavermectin B2a and 4′-deoleandrosyl-6,8a-seco-6,8a-deoxy-5-oxoavermectin B2a, respectively (Fig. 2). The structure of compound **V** was determined by comparison with that of compound **I**, 6,8a-seco-6,8a-deoxyavermectin B2a. Their ^1H and ^{13}C NMR spectra (Fig. 3) were particularly informative. Two proton signals, C-2-H and C-5-H, and one of two protons at C-6 observed with compound **I** disappeared, and the other proton appeared in a downfield with compound **V**. Furthermore, the signals of six carbons at C-2 to C-7 were shifted downfield region (Table 3). The above results indicate that a 6-membered ring at C-2 to C-7 is aromatized. Ultimately, the structure of compound **V** was determined as 6,8a-seco-6,8a-deoxy-2,5-didehydroavermectin B2a.

Determination of the Region for the Furan Ring Formation Step on the Cloned DNA Fragment

Since all products of mutant K2057 were 6,8a-seco-6,8a-deoxy derivatives of avermectins, the mutant contained mutation(s) affecting furan ring formation at C-6 to C-8a and we defined the genotype as *aveE*. We have cloned entire gene cluster for avermectin biosynthesis[6], and cosmid clones were used to determine the location of *aveE* by complementation of the mutant with wild type DNA fragments to restore avermectin production. The transformants produced avermectins B1a and B2a which were products of the parent strain K2038. As shown in

Table 2. ^{1}H NMR (400 MHz) data for compounds **III**, **IV**, **V** and **VI**.

No.	III	IV	V	VI
2	3.45 (s)	3.78 (d, 2.0)	—	3.80 (dd, 2.0)
3	5.29 (d, 1.5)	6.43 (t, 1.5)	7.40 (d, 1.0)	6.43 (t, 1.5)
4-CH_3	1.85 (s)	1.87 (s)	2.07 (d, 1.0)	1.87 (dd, 2.5, 1.5)
5	4.47 (t, 7.0)	—	—	—
6-Ha	2.13 (dd, 13.0, 7.0)	2.78 (d, 16.5)	—	2.78 (d, 16.0)
6-He	1.86 (t, 13.0)	2.49 (d, 16.5)	—	2.49 (d, 16.0)
6	—	—	6.62 (s)	—
8-CH_3	1.71 (s)	1.77 (s)	2.24 (s)	1.77 (s)
9	6.26 (d, 11.0)	6.28 (d, 11.0)	5.72 (d, 11.0)	6.29 (d, 11.0)
10	5.99 (dd, 15.0, 11.0)	5.98 (dd, 15.0, 11.0)	6.09 (dd, 15.0, 11.0)	5.98 (dd, 15.0, 11.0)
11	5.57 (dd, 14.0, 10.0)	5.61 (dd, 15.0, 10.0)	4.49 (dd, 15.0, 10.0)	5.60 (dd, 15.0, 10.0)
12	2.44 (m)	2.45 (m)	2.54 (m)	2.45 (qn, 7.0)
12-CH_3	1.17 (d, 7.0)	1.18 (d, 7.0)	1.18 (d, 7.0)	1.18 (d, 7.0)
13	3.95 (br, s)	3.97 (br, s)	3.99 (br, s)	3.95 (br, s)
14-CH_3	1.53 (s)	1.55 (s)	1.58 (s)	1.54 (s)
15	4.83 (br, dd, 10.0, 3.0)	4.80 (t, 7.0)	4.90 (br, d, 10.0)	4.83 (br, dd, 10.0, 3.0)
16-Ha	*	2.32 (m)	2.33 (m)	*
16-He	2.27 (m)	*	*	2.27 (dd, 24.0, 12.5)
17	3.89 (m)	3.74 (m)	3.77 (m)	3.89 (m)
18-Ha	0.77 (q, 12.0)	0.79 (q, 12.0)	0.77 (q, 12.0)	0.81 (q, 12.0)
18-He	1.77 (br, t)	1.79 (m)	1.95 (m)	1.80 (m)
19	5.37 (tt, 12.0, 4.5)	5.34 (tt)	5.44 (m)	5.42 (tt, 11.5, 5.0)
20-Ha	1.48 (m)	1.50 (t, 12.0)	1.44 (t, 12.0)	1.56 (t, 12.0)
20-He	1.91 (ddd, 12.0, 4.5, 1.5)	1.93 (ddd, 12.0, 4.5, 1.5)	1.91 (m)	1.96 (ddd, 12.0, 4.5, 1.5)
22-Ha	—	1.67 (dd, 14.0, 3.0)	1.67 (d, 3.0)	—
22-He	—	1.98 (dd, 14.0, 3.0)	1.98 (d, 3.0)	—
22	5.75 (dd, 9.8, 1.5)	—	—	5.77 (dd, 10.0, 1.5)
23	5.55 (dd, 10.0, 2.5)	3.76 (m)	3.77 (m)	5.56 (dd, 10.0, 2.5)
24	2.27 (m)	1.60 (m)	1.60 (m)	2.27 (m)
24-CH_3	0.90 (d, 6.5)	0.93 (d, 7.0)	0.93 (dd, 7.2)	0.91 (d,7.5)
25	3.47 (m)	3.54 (m)	3.56 (dd, 11.0, 1.0)	3.47 (dd, 11.0, 1.0)
26	1.54 (m)	1.51 (m)	1.51 (m)	1.49 (m)
26-CH_3	0.87 (d, 6.5)	0.85 (d, 7.0)	0.87 (d, 6.5)	0.88 (d,7.0)
27	1.44 (m, 7.0)	1.45 (m, 7.0)	1.49 (m)	1.44 (m, 7.0)
28	0.89 (t, 7.0)	0.90 (t, 7.0)	0.90 (t, 7.0)	0.90 (t, 7.0)
1′	4.78 (d, 3.0)	4.77 (d, 3.5)	4.77 (d, 3.0)	4.79 (d, 3.5)
2′	*	*	*	*
3′	3.59 (ddd, 11.0, 9.0, 5.0)	3.58 (ddd, 11.0, 9.0, 5.0)	3.65 (m)	3.59 (m)
3′-OCH_3	3.52 (s)	3.51 (s)	3.41 (s)	3.48 (s)
4′	3.16 (t, 9.0)	3.16 (t, 9.0)	3.24 (t, 9.0)	3.16 (t, 9.0)
5′	3.88 (m)	3.89 (m)	3.85 (m)	3.89 (m)
5′-CH_3	1.27 (d, 6.0)	1.27 (d, 6.0)	1.28 (d, 6.0)	1.27 (d, 6.0)
1″	—	—	5.40 (d, 3.5)	—
2″	—	—	*	—
3″	—	—	3.49 (m)	—
3″-OCH_3	—	—	3.48 (s)	—
4″	—	—	3.17 (dd, 9.0)	—
5″	—	—	3.79 (m)	—
5″-CH_3	—	—	1.26 (d, 6.5)	—

Spectra were recorded in $CDCl_3$ solution; chemical shifts are given in ppm; coupling constants are given in hertz. Abbreviations: a = axial, e = equatorial.

* Unresolved in spectra.

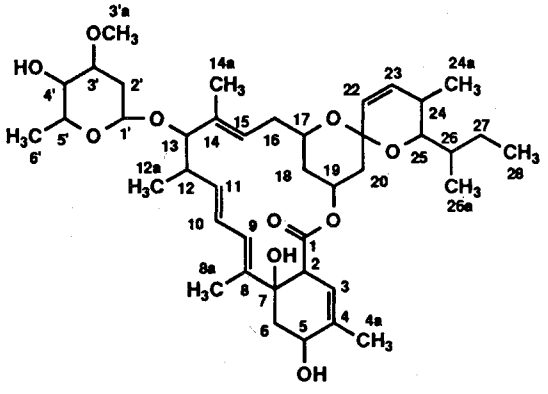
Fig. 4(A), seven cosmid clones were introduced into mutant K2057; transformants derived from four different cosmids produced avermectins B1a and B2a. Since the 4.9 kbp *Bam*HI fragment in the middle of the gene cluster for avermectin biosynthesis overlapped in these cosmid clones, five subclones carrying, respectively the 4.90 kbp *Bam*HI(BB), 2.62 kbp *Bam*HI/*Xho*I(BX), 2.95 kbp *Bam*HI/*Sac*I(BS), 3.15 kbp *Pst*I(PP) and 2.25 kbp *Nsi*I/*Bam*HI(BN) fragments, were constructed (Fig. 4(B)). Two of the transformants carrying the 4.90 kbp *Bam*HI or 3.15 kbp *Pst*I fragments produced abundant avermectins B1a and B2a, however, transformants carrying

Table 3. ^{13}C NMR chemical shifts of compounds III, IV, V and VI.

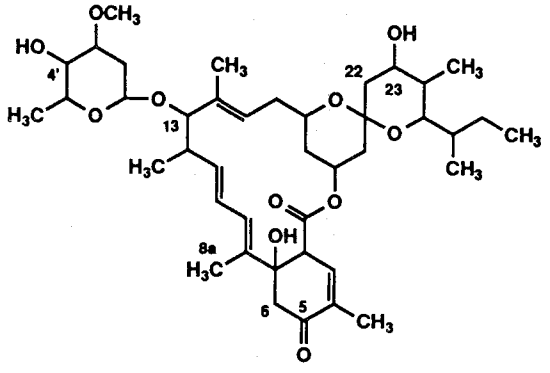
No.	III	IV	V	VI	No.	III	IV	V	VI
1	174.1	172.5	169.5	172.7	21	95.7	99.6	99.9	95.7
2	41.2	47.4	122.4	47.4	22	136.2	41.3	41.3	136.2
3	118.4	136.0	132.1	134.5	23	127.9	69.9	70.0	127.7
4	136.2	135.1	134.7	136.4	24	30.6	35.8	35.8	30.6
4a	19.1	15.7	18.4	15.7	24a	16.4	13.7	13.8	16.4
5	68.0	196.3	155.6	196.5	25	74.9	70.9	71.1	75.0
6	48.0	48.7	144.2	48.7	26	35.2	35.2	35.1	35.1
7	76.8	79.2	144.3	79.2	26a	12.9	12.4	12.5	12.9
8	139.2	137.3	123.8	137.3	27	27.5	27.3	27.3	27.5
8a	13.3	14.0	15.3	14.0	28	12.0	11.7	11.6	12.0
9	124.7	125.9	128.5	125.9	1′	95.1	95.0	94.9	95.0
10	126.2	126.0	126.9	126.0	2′	33.9	34.1	34.2	33.8
11	136.7	137.2	135.9	137.1	3′	78.3	78.3	79.3	78.3
12	40.4	40.8	40.9	40.4	3′a	56.8	56.7	56.4	56.8
12a	20.1	19.9	19.7	19.9	4′	76.2	76.2	80.6	76.2
13	83.3	83.0	83.0	83.2	5′	68.5	68.0	67.0	68.0
14	134.5	134.9	134.8	134.9	5′a	17.7	17.7	17.8	17.7
14a	15.6	15.6	15.6	15.7	1″	—	—	98.6	—
15	118.1	117.6	118.0	118.3	2″	—	—	34.5	—
16	34.2	33.9	33.3	34.2	3″	—	—	78.2	—
17	68.6	68.5	68.6	68.8	3″a	—	—	56.8	—
18	36.4	36.2	36.4	36.3	4″	—	—	76.1	—
19	68.5	68.1	68.1	68.5	5″	—	—	67.3	—
20	40.7	40.7	41.0	40.7	5″a	—	—	18.2	—

Spectra were obtained in $CDCl_3$ solutions; chemical shifts are given in ppm.

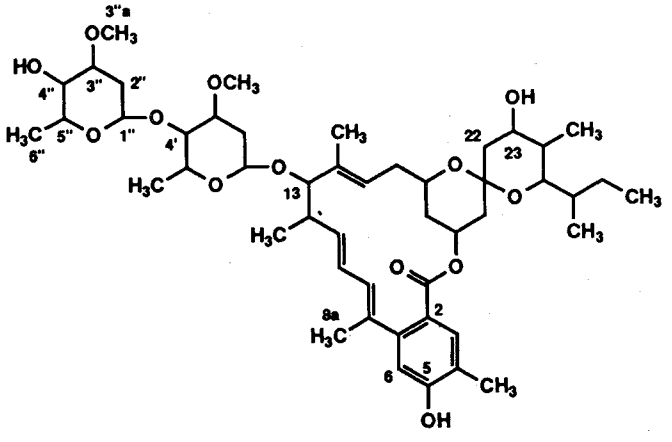
Fig. 2. Structures of new derivatives of avermectins which lack a furan ring at C-6 to C-8a.



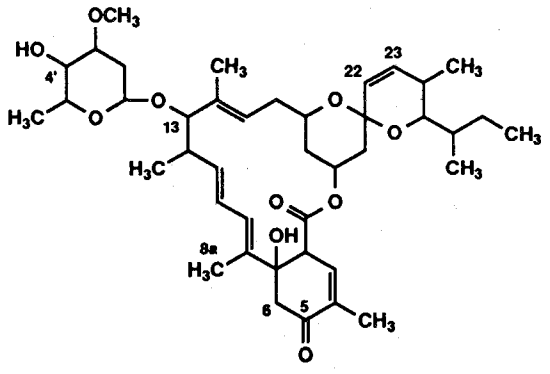
Compound III
(4′-Deoleandrosyl-6,8a-seco-6,8a-deoxyavermectin B1a)



Compound IV
(4′-Deoleandrosyl-6,8a-seco-6,8a-deoxy-5-oxoavermectin B2a)



Compound V
(6,8a-Seco-6,8a-deoxy-2,5-didehydroavermectin B2a)



Compound VI
(4′-Deoleandrosyl-6,8a-seco-6,8a-deoxy-5-oxoavermectin B1a)

Fig. 3. 400-MHz ^{1}H NMR spectral assignments for compounds I[2,10] and V.

Abbreviations are used as follows; s=singlet, d=doublet, t=triplet.

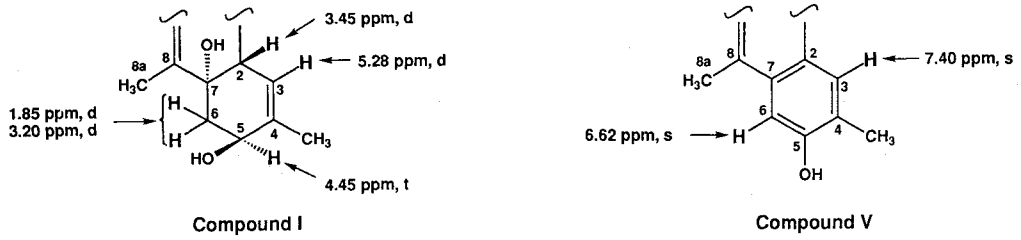
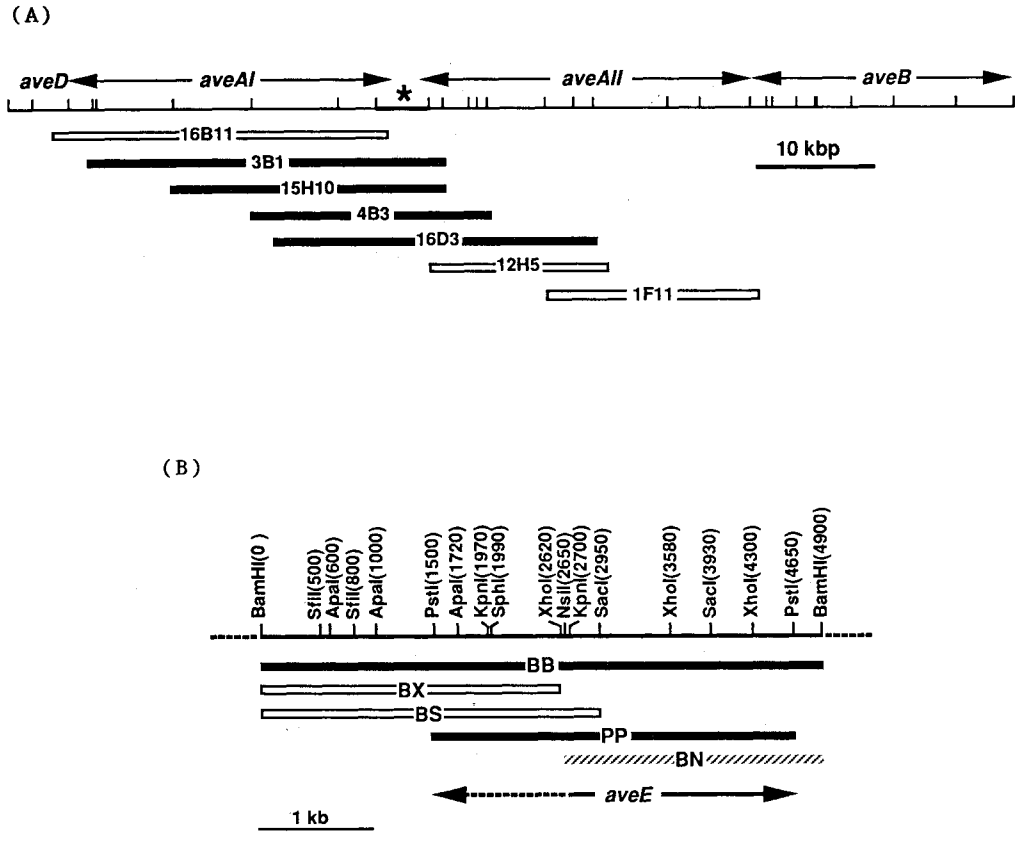


Fig. 4. Analysis of the genetic complementation of mutant K2057.

(A) *Bam*HI restriction map of gene cluster for avermectin biosynthesis in *S. avermitilis* K139. Asterisk means 4.9 kbp *Bam*HI fragment. Each genetic marker has been described previously[6,11]. (B) The restriction map of the 4.9 kbp *Bam*HI fragment in the center of the gene cluster for avermectin biosynthesis.

Open column; complementation was not observed, closed column; complementation was strongly observed and hatched column; complementation was observed but the restoration of avermectin production was not enough.

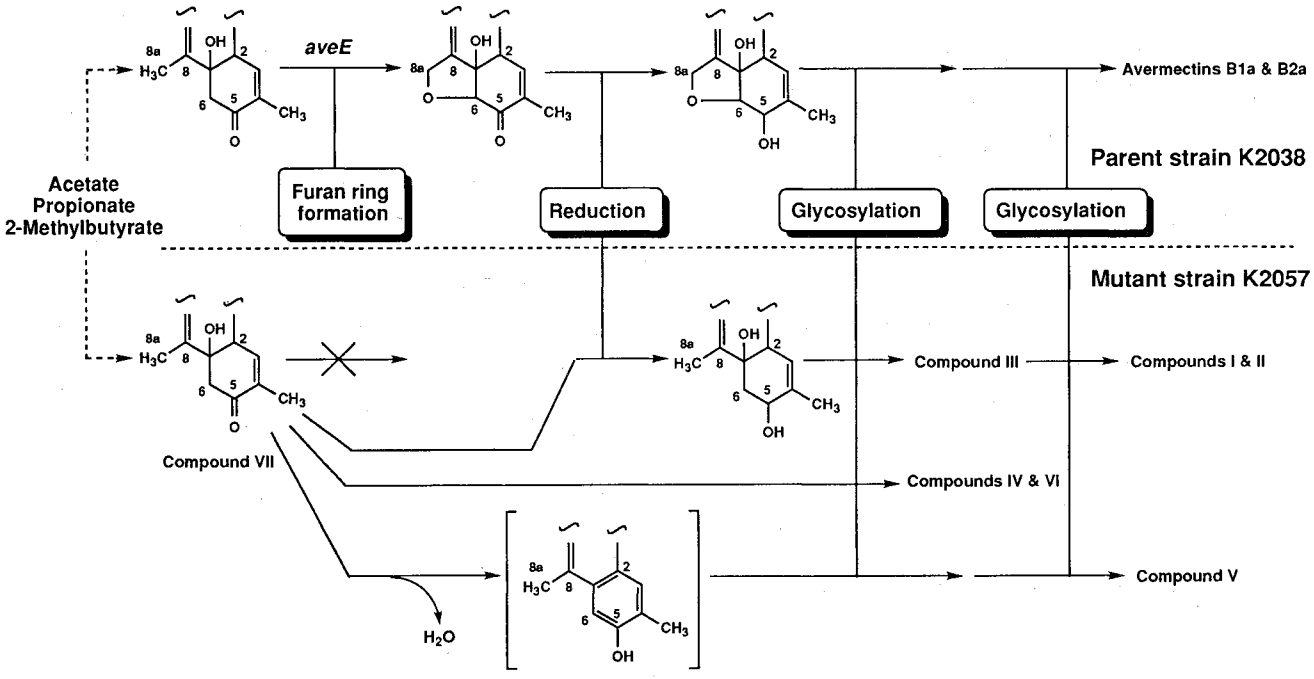


2.25 kbp *Nsi*I/*Bam*HI fragment produced 6,8a-seco-6,8a-deoxy derivatives of avermectins and small amount of avermectins B1a and B2a (Fig. 4(B)).

Discussion

Mutants affecting furan ring formation at C-6 to C-8a in the avermectins have been reported by CHEN and INAMINE[2] and GOEGELMAN *et al.*[10]. MA-5218, which was derived from the wild type strain *S. avermitilis* ATCC31271, produced at least seven 6,8a-seco-6,8a-deoxyavermectin derivatives[10]. On the other hand, we obtained mutant K2057 affecting furan ring formation derived from a selective producer K2038, which produces the most effective components B1a and B2a[3]. Mutant K2057 produced seven different 6,8a-seco-6,8a-deoxy derivatives of avermectins which were structurally different from those of the mutant MA-5218. Four out of seven compounds were new compounds. The genotype of mutant K2057 was *aveE* (furan ring formation) *aveD* (C-5 *O*-methylation) *X* (selective incorporation of branched-chain fatty acid) and that of mutant MA-5218 would be *aveE* alone. Thus, mutant K2057 was unable

Fig. 5. Proposed pathway for biosynthesis of avermectins and their derivatives in the parent and its mutant strains, K2038 and K2057, respectively.



to produce C-5 *O*-methylated and "b" components of avermectins, but did produce other avermectin-related antibiotics. Determinants other than *aveE* could give rise to accumulation of above-mentioned components of 6,8a-seco-6,8a-deoxy derivatives of avermectins.

Since all avermectin derivatives from mutant K2057 lacked the furan ring at C-6 to C-8a, the mutation would be located in the step for furan ring formation in avermectin biosynthesis. Restoration of the avermectin production by introducing DNA fragments involved in avermectin biosynthesis from the wild type strain confirmed that the region involved in furan ring formation occurs in a 3.15 kbp *Pst*I fragment. Interestingly, transformants carrying 2.25 kbp *Nsi*I/ *Bam*HI fragment produced small amount of avermectins. Although the sequence of this region has not determined, the *Nsi*I/*Bam*HI fragment would not contain complete region for furan ring formation.

Since mutant K2057 accumulates the above-mentioned seven 6,8a-seco-6,8a-deoxy derivatives of avermectins, we can propose the following biosynthetic pathway following formation of 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycones including compound **VII** (6,8a-seco-6,8a-deoxy-5-oxoavermectin B1a aglycone; Fig. 5). The first step is the formation of a furan ring at C-6 to C-8a of the aglycone to generate 5-oxoavermectin aglycones. The second step is the reduction of C-5 keto residue to form avermectin aglycones. The final steps are two rounds of glycosylation at C-13 and C-4′ OH. In mutant K2057 lacking the furan ring forming activity, an aglycone in 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycones is reduced to form 6,8a-seco-6,8a-deoxyavermectin aglycones. The aglycones are then glycosylated at C-13 OH to form compound **III** (4′-deoleandrosyl-6,8a-seco-6,8a-deoxyavermectin B1a) and the second glycosylation takes place at C-4′ OH to form compounds **II** (6,8a-seco-6,8a-deoxyavermectin B1a) and **I** (6,8a-seco-6,8a-deoxyavermectin B2a). On the other hand, 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycones are glycosylated at C-13 OH without reduction of the keto residue to form compound **IV** (4′-deoleandrosyl-6,8a-seco-6,8a-deoxy-5-oxoavermectin B2a) and **VI** (4′-deoleandrosyl-6,8a-seco-6,8a-deoxy-5-oxoavermectin B1a). Probably, since both compounds **IV** and **VI** would not be suitable substrates for the second glycosylation at C-4′ OH, disaccharide derivatives of 6,8a-seco-6,8a-deoxy-5-oxo derivatives would not be accumulated. We assumed the biosynthetic route for the formation of compound **V** to be as follows; (1) 6,8a-seco-6,8a-deoxy-5-oxoavermectin B2a aglycones would be dehydrated between C-7 OH and C-2 or C-6 protons, enolization would occur at C-5 keto residue and the 6-membered ring (C-2 to C-7) would be aromatized to form 6,8a-seco-6,8a-deoxy-2,5-didehydroavermectin B2a aglycones. (2) Two rounds of glycosylation at C-13 and C-4′ OH would take place to form compound **V**. The question of whether the dehydration reaction is enzymatic or spontaneous remains to be clarified.

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