Direct Production of 5-Oxo Derivatives of Avermectins by a Recombinant Strain of *Streptomyces avermitilis*

Sir:

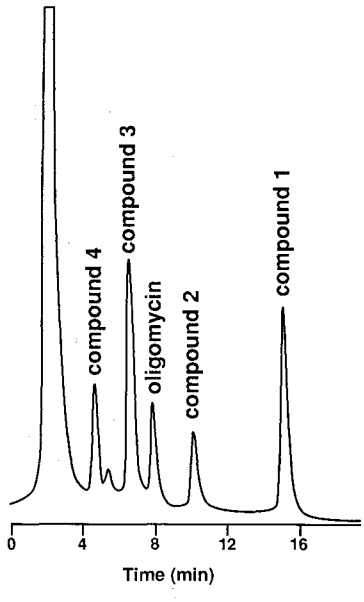
Avermectins are a family of oleandrose disaccharide derivatives of sixteen-membered macrolides with potent anthelmintic and insecticidal activities produced by *Streptomyces avermitilis*[1]. A large number of avermectin derivatives have been prepared by a variety of techniques, including mutagenesis[2], biotransformation[3], mutational biosynthesis[4] and chemical modification[5]. The main purposes for preparation of such derivatives are enhanced potency, broader spectrum and lower toxicity. Ivermectin, 22,23-dihydroavermectin B1, has been prepared chemically from avermectin B1 components to attain enhanced potency. Milbemycins[6], which are structurally related to avermectins, have been also modified for similar purposes. 5-Oxime derivatives of milbemycins have enhanced potency[7,8]. 5-Oxime derivatives of avermectins have also more potent activities. The synthesis of 5-oxime derivatives of the above both compounds needs at least two steps, in which the hydroxyl group at the C-5 position of the aglycone moiety is selectively oxidized by active manganese dioxide to form 5-oxo derivatives and then the keto residue is reacted with hydroxylamine to convert to 5-oxime derivatives[7]. In these steps, oximation of 5-oxo derivatives is a quantitative reaction but selective oxidation of the hydroxyl group at C-5 of avermectins and milbemycins gives a lower yield[7,9]. The yield of the selective oxidation of the hydroxyl residue at C-5 of avermectin B components was 40 to 60%. If we could get a strain that produces 5-oxoavermectins directly, 5-oxime derivatives of avermectins would be efficiently and easily constructed.

The biosynthetic pathway to avermectins after the formation of the aglycones has been proposed by us[10] and the Merck group[5,11]. After condensation of fatty acids by polyketide synthase, 6,8a-seco-6,8a-deoxy-5-oxoavermectin aglycone is formed by the gene products of *aveAI*, *AII* and *aveC*. The cyclization takes place between C-6 and C-8a positions catalyzed by the gene product of *aveE* to form 5-oxoavermectin aglycone. The keto group at C-5 position is reduced to form avermectin aglycone. Glycosylation at the C-13 position by the gene product of *aveB* and *O*-methylation at C-5 by the gene product of *aveD* then take place to form avermectins. Almost all mutants have been obtained, however, the mutant which lacks the activity of reduction of the keto residue at C-5 position of the aglycone has never been isolated. Such mutant would be expected to produce 5-oxoavermectins because *aveE* mutant produces 6,8a-seco-6,8a-deoxy-5-oxoavermectin B glycosylated at the C-13 position[12].

During analysis of the gene cluster for avermectin biosynthesis, we have found the region responsible for the reduction at the C-5 keto residue[13]. We have attempted a frame shift mutation in this region on the chromosome of the wild type strain *Streptomyces avermitilis* K139. The resulting recombinant strain did not produce any natural avermectins, but did produce new components different from natural avermectins (Fig. 1). To elucidate their structures, the recombinant strain (K2082) was cultivated in a liquid production medium as described previously[14~16] and the new components were isolated from the cultured broth. The mycelia from 1 liter of the cultured broth were extracted with 200 ml of acetone and the extract, in which mycelia were removed by filtration, was evaporated under reduced pressure. About 100 ml of the concentrate was suspended in 100 ml of deionized water and then was extracted twice with 100 ml of methylene chloride. About 0.6 g of oily material was obtained by passage of the extract through 4 g of silica gel to remove impurities. Four components, compound **1** (24 mg), compound **2** (5 mg), compound **3** (28 mg) and compound **4** (6 mg), were obtained by purification by preparative thin layer chromatography and reversed phase high performance liquid chromatography in a manner similar to that described previously[17].

Each compound was subjected to spectral analyses. From the results of IR analysis (Table 1), a new absorption at 1685 cm^{-1} which was not detected in natural avermectins was observed in spectra of all four compounds. This indicated the presence of an α,β-unsaturated ketone in addition to the lactone carbonyl (1735 cm^{-1}). Their ultraviolet absorptions were not similar to those of natural avermectins (λ_{max}: 243 nm) but were similar to 4'-deoleandrosyl-6,8a-seco-6,8a-deoxy-5-oxoavermectin B1a and B2a[12]. These structures were

Fig. 1. Chromatogram of analytical HPLC of the mycelial extracts from the recombinant strain K2082 derived from K139.



The separation conditions were described in ref 17.

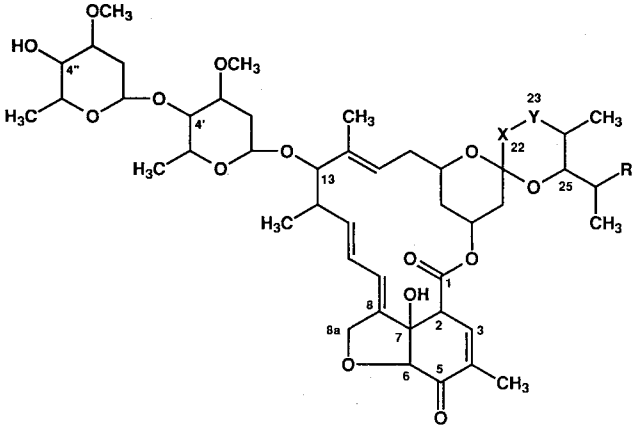
Table 1. Physico-chemical properties of 5-oxoderivatives of avermectins.

	Compound **1**	Compound **2**	Compound **3**	Compound **4**
IR (cm^{-1})	3440, 1730, 1685	3440, 1730, 1685	3440, 1735, 1685	3440, 1735, 1685
UV μ_{max}^{MeOH} nm (ε)	240 (29,000), 251 sh (20,600)	240 (29,200), 251 sh (20,600)	240 (26,500), 251 sh (20,800)	240 (26,400), 251 sh (20,700)
FAB-MS (m/z)				
$(M+Na)^+$	893	879	911	897
$(M+DEA)^+$*	976	962	994	980
Elementary analysis				
Found:	H 8.20	H 7.80	H 8.21	H 7.96
	C 65.77	C 66.71	C 64.63	C 64.95
Calcd:	H 8.10	H 8.00	H 8.16	H 8.06
	C 66.19	C 65.87	C 64.84	C 64.51
Formula	$C_{48}H_{70}O_{14}$	$C_{47}H_{68}O_{14}$	$C_{48}H_{72}O_{15}$	$C_{47}H_{70}O_{15}$
MW	871.1	857.0	889.1	875.1

* DEA; diethylamine.

Fig. 2. Structural formulae for 5-oxoavermectins.

Both sugars are α-L-oleandrose.



5-Oxoavermectins	X–Y	R
B1a	CH=CH	C_2H_5
B1b	CH=CH	CH_3
B2a	CH_2–CH(OH)	C_2H_5
B2b	CH_2–CH(OH)	CH_3

determined by comparing their NMR spectra with those of natural avermectins. From the analysis of 400-MHz ^1H NMR, all compounds isolated from the recombinant strain K2082 were shown to lack a proton at the C-5 position, and identical downfield shifts in the chemical shifts of H-2, H-3, and H-6 indicated the conversion of an allylic alcohol to an α,β-unsaturated ketone. The mass spectra of these compounds also agree with loss of two hydrogen atoms from C-5 and its substituent OH. Other NMR data were similar to those of natural avermectins. These results show that the above four compounds **1**, **2**, **3** and **4** obtained from the recombinant strain K2082 are identical with 5-oxoavermectins B1a, B1b, B2a and B2b, respectively (Fig. 2), which have been chemically synthesized by CHABALA *et al.*[9].

Thus, a producer (K2082) of 5-oxoavermectins was constructed successfully by the specific disruption of the gene for the C-5 keto reduction step without affecting the productivity in the avermectin producer *S. avermitilis*. 5-Oxoavermectins are directly produced by cultivation of the recombinant strain without any chemical process. It should be easy to convert 5-oxoavermectins to 5-oxime derivatives by chemical oximation.

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References

1) BURG, R. W.; B. M. MILLER, E. E. BAKER, J. BIRNBAUM, S. A. CURRIE, R. HARTMAN, Y. KONG, R. L. MONAGHAN, G. OLSON, I. PUTTER, J. B. TUNAC, H. WALLICK, E. O. WALLICK, R. ŌIWA & S. ŌMURA: Avermectins, new family of potent anthelmintic agents: producing organism and fermentation. Antimicrob. Agents Chemother. 15: 351～367, 1979
2) GOEGELMAN, R. T.; V. P. GULLO & L. KAPLAN: Novel C-076 compounds. US Patent 4,378,353, 1983
3) GOEGELMAN, R. T.; E. S. INAMINE & R. F. WHITE: Avermectin bioconversion products. US Patent 4,666,937, 1987
4) DUTTON, C. J.; S. P. GIBSON, A. C. GOUDIE, K. S. HOLDOM, M. S. PACEY, J. C. RUDDOCK, J. D. BU'LOCK & M. K. RICHARDS: Novel avermectins produced by mutational biosynthesis. J. Antibiotics 44: 357～365, 1991
5) CAMPBELL, W. C. (*Ed.*). "Ivermectin and Abamectin" Springer-Verlag, New York, NY, 1989
6) TAKIGUCHI, Y.; H. MISHIMA, M. OKUDA, M. TERAO, A. AOKI & R. FUKUDA: Milbemycins, a new family of macrolide antibiotics: Fermentation, isolation and physicochemical properties. J. Antibiotics 33: 1120～1127, 1980

7) TSUKAMOTO, Y.; K. SATO, S. MIO, S. SUGAI, T. YANAI, N. KITANO, S. MURAMATSU, Y. NAKADA & J. IDE: Synthesis of 5-keto-5-oxime derivatives of milbemycins and their activities against microfilariae. Agric. Biol. Chem. 55: 2615~2621, 1991

8) WADE, C. G.; S. H. MERCER, D. I. HELPER & T. M. CRAIG: Effect of milbemycin oxime against *Ancylostoma caninum* in dogs with naturally acquired infection. Am. J. Vet. Res. 52: 951~953, 1991

9) CHABALA, J. C.; A. ROSEGAY & M. A. R. WALSH: Tritium labeling of avermectins B1a and B2a via stereoselective reduction of 5-ketoavermectins. J. Agric. Food Chem. 29: 881~884, 1991

10) IKEDA, H. & S. ŌMURA: Genetic aspects of the selective production of useful components in the avermectin producer *Streptomyces avermitilis*. Actinomycetol. 7: 133~144, 1993

11) FISHER, M. H. & H. MROZIK: Chapter 14. The avermectin family of macrolide-like antibiotics. In "Macrolide Antibiotics. Chemistry, Biology and Practice" (*Ed.* S. ŌMURA), pp. 553~606, Academic Press, 1984

12) PANG, C.-H.; K. MATSUZAKI, H. IKEDA, H. TANAKA & S. ŌMURA: Production of new 6,8a-seco-6,8a-deoxy derivatives of avermectins by a mutant strain of *Streptomyces avermitilis*. J. Antibiotics 48: 59~66, 1995

13) IKEDA, H.; Y. TAKADA, C.-H. PANG, H. TANAKA & S. ŌMURA: *manuscript in preparation*

14) IKEDA, H.; H. KOTAKI & S. ŌMURA: Genetic studies of avermectin biosynthesis in *Streptomyces avermitilis*. J. Bacteriol. 169: 5615~5621, 1987

15) IKEDA, H.; H. KOTAKI, H. TANAKA & S. ŌMURA: Involvement of glucose catabolism in avermectin production in *Streptomyces avermitilis*. Antimicrob. Agents Chemother. 32: 282~284, 1988

16) IKEDA, H.; Y. TAKADA, C.-H. PANG, H. TANAKA & S. ŌMURA: Transposon mutagenesis by Tn*4560* and applications with avermectin-producing *Streptomyces avermitilis*. J. Bacteriol. 175: 2077~2082, 1993

17) ŌMURA, S.; H. IKEDA & H. TANAKA: Selective production of specific components of avermectins in *Streptomyces avermitilis*. J. Antibiotics 44: 560~563, 1991