ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Tetrahedron 60 (2004) 3999–4005

Tetrahedron

Genome-inspired search for new antibiotics. Isolation and structure determination of new 28-membered polyketide macrolactones, halstoctacosanolides A and B, from *Streptomyces halstedii* HC34

Shigehiro Tohyama,[a] Tadashi Eguchi,[a,*] Rabindra P. Dhakal,[b] Tomoyoshi Akashi,[b] Miyuki Otsuka[b] and Katsumi Kakinuma[b,*]

[a]*Department of Chemistry and Materials Science, Tokyo Institute of Technology, O-okayama, Meguro-ku, Tokyo 152-8551, Japan*
[b]*Department of Chemistry, Tokyo Institute of Technology, O-okayama, Meguro-ku, Tokyo 152-8551, Japan*

Received 8 January 2004; revised 20 February 2004; accepted 8 March 2004

Abstract—During the search for polyketide synthase (PKS) in the genome of *Streptomyces halstedii* HC34, we found clustered new genes which appeared to encode typical Type 1 PKSs beyond the cluster harboring the genes for the biosynthesis of antitumor antibiotic vicenistatin. The deduced domain configuration of these putative PKS genes allowed to predict a corresponding partial structure of polyketide, which was in turn materialized by isolation of new polyketide macrolactone halstoctacosanolides A and B from the fermentation broth of *S. halstedii* HC34. The structures of these metabolites were determined by spectroscopic means to have a novel 28-membered macrolactone structure. The partial structure deduced from the genetic data was completely compatible to the structures of halstoctacosanolides A and B. This success clearly demonstrates the present new approach of genome-inspired search for new antibiotics promising. Halstoctacosanolides A and B showed moderate antimicrobial activity against several microorganisms.
© 2004 Elsevier Ltd. All rights reserved.

1. Introduction

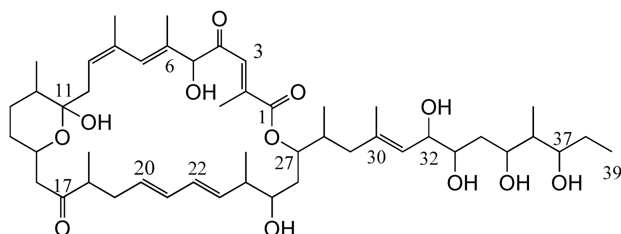
Over the last several decades, a lot of antibiotics were isolated from various *Streptomecetes* and other microorganisms, and importance of antibiotics is well-recognized throughout the medicinal and agricultural fields.[1] Particularly, serious issue of resistance of pathogenic bacteria against commonly used antibiotics urged to develop more effective drugs of natural and synthetic origin. In addition to the conventional bioassay-guided approach including high-throughput screening, various methodologies particularly employing microbial genetic technology have emerged. Among those genome-driven approaches, combinatorial biosynthesis has recently been gaining relevance for generation of new structures. This technology utilizes combination of pertinent genes involved in the already known but different biosynthetic pathways to produce novel or modified metabolites.[2] Another approach to access to interesting natural products concerns to uncultured microbes, which involves initial isolation of DNA directly from soil (environmental DNA, eDNA) using PCR and its subsequent expression in the established expression systems. Although a successful achievement has recently been reported by this approach,[3] there are still some difficulties particularly in dealing with functionally unknown DNA of unknown origin. The third approach relies on the genetic information compiled by extensive genome projects.

Streptomyces bacteria and closely related genera are still extraordinary rich sources to be searched for a wide variety of secondary metabolites as lead to new drug candidates. Completion of the genome projects of *Streptomyces coelicolor*[4] and *Streptomyces avermitilis*[5] revealed the presence of a large number of gene clusters harboring the biosynthetic enzymes of secondary metabolites in the genome of these strains. Among these clusters are the biosynthetic genes for the previously identified secondary metabolites as well as those for the unidentified products, which have yet to be discovered from nature. It should be pointed out that, according to a mathematical modeling, only 3% of all biologically active metabolites produced by *Streptomycetes* have been identified so far.[6] A large number of antibiotics from this genus still await to be discovered.

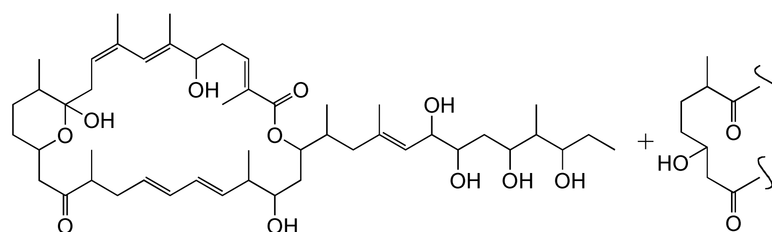
In the last decade, general methods and appropriate probes for cloning of antibiotic biosynthetic gene clusters in

Keywords: 28-Membered macrolactone; Isolation; Structure determination; Polyketide synthase.
* Corresponding authors. Tel./fax: +81-3-5734-2631;
e-mail address: eguchi@cms.titech.ac.jp

0040–4020/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2004.03.027



Halstoctacosanolide A (**1**)



Halstoctacosanolide B (**2**)

Figure 1. Structures of halstoctacosanolides A (**1**) and B (**2**).

Streptomycetes and related microorganisms have been well developed.[7] Recent advances in these area are extremely helpful us to understand whole biosynthetic scenarios of various biologically important natural products, especially of polyketides, and large knowledge bases successfully compiled to date are apparently useful for correlating the DNA base sequences to the polyketide products.[8] It is thus possible nowadays to estimate chemical natures of a starter- and extender units of a polyketide directly from the DNA sequence data of the corresponding biosynthetic gene cluster without knowing the actual product.[8,9] Therefore, an emerging rational approach to search for new antibiotics, particularly in *Streptomycetes*, may rely apparently on the accumulated DNA sequence information.

Streptomyces halstedii HC34 produces an antitumor antibiotic vicenistatin, the structure of which is comprised of a macrocyclic 20-membered lactam aglycon and an amino-sugar vicenisamine.[10,11] We reported recently that the extender units of the aglycon are derived from acetate and propionate in a standard polyketide biosynthetic pathway, whereas the aglycon precursor is primed by an unusual

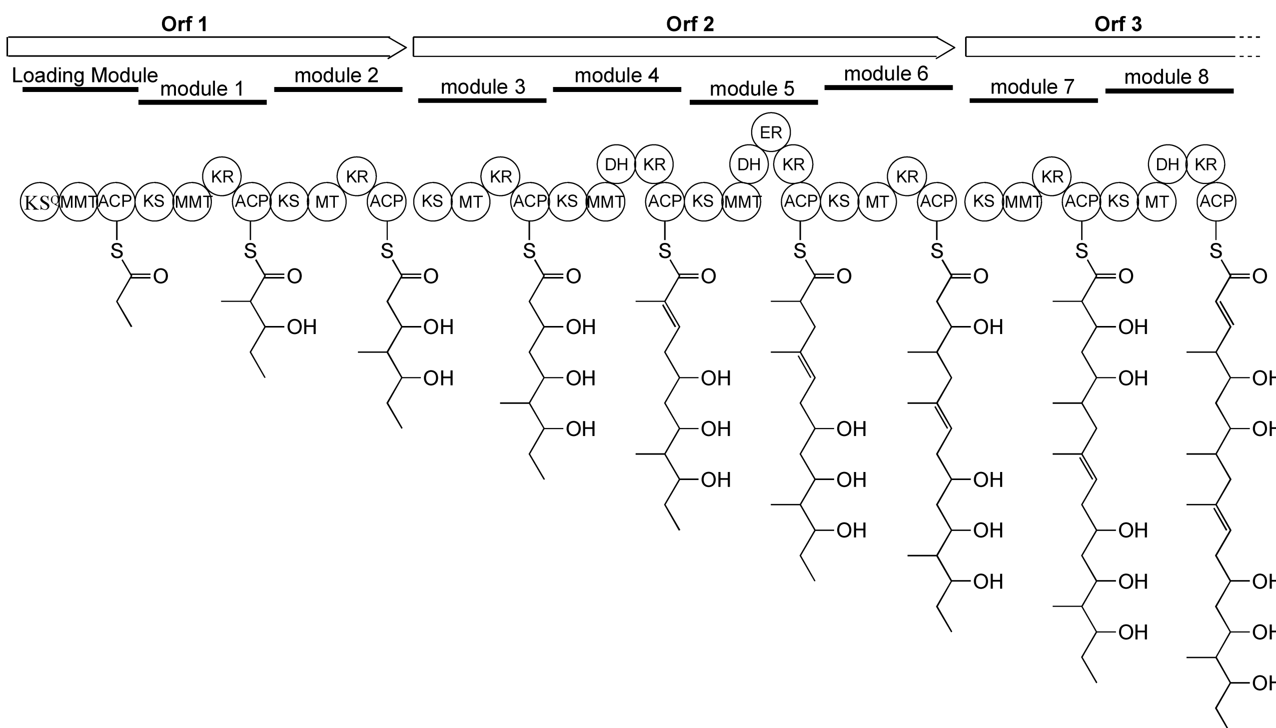


Figure 2. The PKS genes from *S. halstedii* HC34. Each circle represents an enzymatic domain in the PKS multifunctional proteins. MM, malonyl transferase; MMT, methylmalonyl transferase; ACP, acyl carrier protein; KS, ketoacyl-ACP synthase; KS^Q, inactive KS; KR, ketoacyl ACP reductase; DH, hydroxylthioester dehydratase; ER, enoyl reductase.

starter unit derived from 2-methylaspartate.[12–14] More recently, we have described cloning, sequencing and functional analysis of the biosynthetic gene cluster for vicenistatin.[15]

During the search for the polyketide synthase (PKS) genes in *S. halstedii* HC34, we found that this microorganism possesses a series of typical Type 1 PKS genes which are different from those responsible for the vicenistatin biosynthesis. These findings and the deduced sequence information strongly indicated possible production of so-far unidentified polyketide metabolite(s), which prompted us to start isolation and chemical studies. In this paper, we describe the isolation and structure determination of two new 28-membered macrolactones, which we named halstoctacosanolides A and B (Fig. 1).

2. Results and discussion

Based on the extensive analysis of the cosmid library of *S. halstedii* HC34,[15] we were able to identify a portion of a biosynthesis gene cluster of new polyketide (total ca. 45 kbp so far). These genes are composed of three open reading frames which appear to encode typical Type 1 PKS containing nine modules. From the homology search for the deduced amino acid sequences of these genes (DDBJ; accession No. AB158460), the starter unit and the extender units as well as the oxidation states of each unit could be clearly predicted. It was thus anticipated that these PKSs catalyze the biosynthesis of a polyketide chain containing a partial structure as shown in Figures 2 and 3. The most important point at this stage was that an anticipated molecule having this partial structure was shown to be unprecedented. Thus, we started its isolation and chemical studies.

To obtain new polyketide(s) from the fermentation broth of *S. halstedii* HC34, the same culture conditions as those for vicenistatin were appropriate.[10] After culture for 3 days, both supernatant and mycelium cake were separately extracted with ethyl acetate. After combining and concentrating the extracts, two new compounds were isolated and purified to homogeneity through repeated chromatography. We named these compounds as halstoctacosanolides A (**1**) and B (**2**), the structures of which were determined as follows.

The physico-chemical properties of halstoctacosanolide A (**1**) are summarized in Table 1. The molecular formula of **1** was established as $C_{48}H_{76}O_{12}$ on the basis of HRFAB-MS data. In the IR spectrum, **1** showed strong bands at 3410 and 1710 cm^{-1}, which revealed the presence of hydroxyl and carbonyl groups. The ^{1}H NMR spectrum in $CDCl_3$ (Fig. 4) indicated the presence of 10 methyl and 8 olefinic protons. Further, the ^{13}C NMR spectrum showed 48 carbon signals including two ketonic (δ 198.9, 215.4), an ester (δ 165.8), an acetal (δ 98.9), 12 olefinic, and 8 hydroxylated carbons. All carbon signals were divided into 10 methyl, 9 methylene, 21 methine, and 8 quaternary carbons by DEPT experiments as indicated in Table 2.

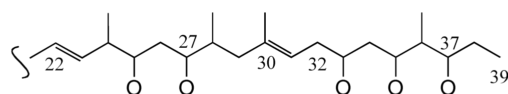


Figure 3. Partial structure deduced from genetic analysis.

Table 1. Physico-chemical properties of halstoctacosanolides A (**1**) and B (**2**)

	1	**2**
Appearance	Colorless powder	Colorless powder
Mp	79–80 °C	79–80 °C
$[\alpha]_D^{22}$	+43.2 (*c* 1.0, $CHCl_3$)	−22.3 (*c* 1.0, $CHCl_3$)
Molecular formula	$C_{48}H_{76}O_{12}$	$C_{48}H_{78}O_{11}$
HRFAB-MS		
Calcd: (*m/z*)	867.5234 $(M+Na)^+$	853.5442 $(M+Na)^+$
Found: (*m/z*)	867.5247	853.5393
UV λ_{max} (ε) (in MeOH)	233 nm (64,000)	226 nm (48,000)
IR ν (KBr); cm^{-1}	3410 (br), 2960, 2930, 1710	3420 (br), 2960, 2930, 1700
Elemental Anal.		
Calcd	C: 68.22; H: 9.06	C: 69.37; H: 9.46
Found	C: 68.17; H: 8.99	C: 69.12; H: 9.76

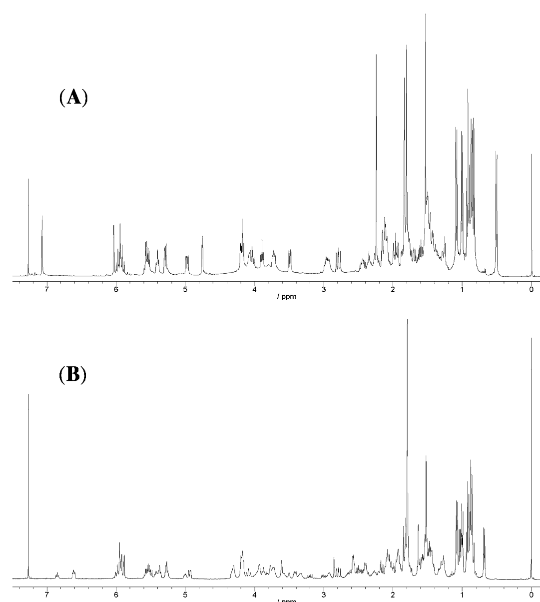


Figure 4. ^{1}H NMR spectra (400 MHz, $CDCl_3$) of (A); halstoctacosanolide A (**1**) and (B) halstoctacosanolide B (**2**).

By ^{1}H–^{1}H COSY and HMBC experiments, one polyketide chain composed of nonadeca-ketides was established as shown in Figure 5. Since the long range coupling between 27-H and C-1 ester carbon was clearly detected in a HMBC experiment, **1** was found to have a 28-membered macrolactone structure. The geometries of the double-bonds at C-20 and C-22 were determined to be *E* by their spin–spin coupling constants ($J_{20,21}$=14.6 Hz and $J_{22,23}$=14.9 Hz). The other double-bond geometries were confirmed by NOE experiments. While essentially no NOE was observed between 3-H and 2-CH_3, the NOE between 2-CH_3 and 5-H was clearly detected, therefore the double bond at C-2 was determined to be *E*. Furthermore, the NOEs between 6-CH_3 and 8-CH_3, and 8-CH_3 and 9-H were observed, thus the double-bond geometries at C-6 is to be *E* and at C-8 to be *Z*. The double bond at C-30 was proved to be *E* by observation of the NOE between 32-H and 30-CH_3.

Table 2. NMR data of **1**, **3** and **4** in $CDCl_3$

No.	Halstoctaisaconolide A (**1**)		Compound **3**		Compound **4**	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	165.79 (s)		166.51 (s)		167.74 (s)	
2	145.50 (s)		144.94 (s)		129.38 (s)	
3	126.70 (d)	7.07 (d, 1.6)	126.68 (d)	7.14 (d, 1.4)	136.93 (d)	6.69 (t, 6.5)
4	198.87 (s)		199.82 (s)		34.80 (t)	2.62/2.54 (m)
5	82.38 (d)	4.75 (d, 4.0)	83.16 (d)	4.67 (s)	74.81 (d)	4.30 (t, 5.9)
6	133.71 (s)		133.27 (s)		138.49 (s)	
7	129.40 (d)	6.04 (brs)	130.13 (d)	6.12 (s)	125.23 (d)	5.94 (s)
8	134.92 (s)		132.42 (s)		133.25 (s)	
9	122.56 (d)	5.41 (dd, 6.5, 7.4)	124.50 (d)	5.37 (m)	122.66 (d)	5.29 (t, 6.6)
10	39.91 (t)	1.89/1.97 (m)	34.05 (t)	1.98/2.23 (m)	33.58 (t)	2.03/2.28 (m)
11	98.92 (s)		101.01 (s)		101.21 (s)	
12	35.71 (d)	1.35 (m)	36.34 (d)	1.51 (m)	35.93 (d)	1.64 (m)
13	27.42 (t)	1.38/1.51 (m)	27.16 (t)	1.38/1.60 (m)	27.16 (t)	1.41/1.68 (m)
14	38.78 (t)	1.27/1.52 (m)	31.77 (t)	1.39/1.57 (m)	31.74 (t)	1.29/1.58 (m)
15	67.51 (d)	4.04 (brt, 10.7)	67.63 (d)	4.05 (brt, 11.0)	67.69 (d)	4.03 (t, 11.1)
16	48.15 (t)	2.13 (m)	47.82 (t)	2.23 (m)	47.80 (t)	2.25 (m)
	2.89 (dd, 10.5, 13.5)		2.78 (dd, 10.8, 14.4)		2.76 (dd, 10.4, 14.3)	
17	215.38 (s)		212.98 (s)		213.19 (s)	
18	48.05 (d)	2.97 (ddq, 10.0, 13.8, 7.2)	47.77 (d)	2.72 (dq, 13.4, 6.8)	47.80 (d)	2.65 (m)
19	34.75 (t)	2.15 (m)	34.92 (t)	2.18 (m)	34.98 (t)	1.97/2.57 (m)
	2.44 (ddd, 7.8, 10.0, 13.8)		2.53 (quintet, 7.1)			
20	130.68 (d)	5.57 (m)	129.79 (d)	5.56 (quintet, 7.1)	129.68 (d)	5.54 (dt, 14.2, 7.3)
21	131.47 (d)	5.97 (dd, 10.2, 14.6)	132.12 (d)	5.98 (m)	132.55 (d)	6.02 (m)
22	129.36 (d)	5.92 (ddd, 0.7, 10.2, 14.9)	130.39 (d)	5.95 (m)	130.10 (d)	5.98 (m)
23	136.22 (d)	5.55 (m)	135.20 (d)	5.34 (m)	135.63 (d)	5.37 (dd, 8.2, 14.7)
24	41.14 (d)	2.13 (m)	43.41 (d)	2.03 (m)	43.60 (d)	2.08 (m)
25	68.72 (d)	3.50 (d, 11.1)	71.88 (d)	3.19 (dd, 2.2, 9.8)	72.31 (d)	3.14 (t, 8.4)
26	31.98 (t)	1.46 (m)	33.62 (t)	1.49/1.63 (m)	33.74 (t)	1.43/1.64 (m)
	1.71 (dd, 11.2, 13.7)					
27	75.73 (d)	4.99 (dd, 2.5, 10.6)	75.72 (d)	5.12 (dd, 2.2, 9.8)	73.94 (d)	5.01 (dd, 2.4, 10.5)
28	31.68 (d)	2.35 (m)	32.92 (d)	2.16 (m)	33.00 (d)	2.16 (m)
29	44.93 (t)	1.95/2.11 (m)	44.69 (t)	1.98/2.07 (m)	44.66 (t)	2.00/2.06 (m)
30	136.15 (s)		137.30 (s)		137.69 (s)	
31	128.13 (d)	5.29 (d, 8.8)	127.47 (d)	5.27 (d, 9.0)	126.99 (d)	5.24 (d, 8.5)
32	72.25 (d)	4.18 (dd, 6.0, 8.8)	72.20 (d)	4.18 (m)	72.31 (d)	4.17 (m)
33	71.44 (d)	3.73 (ddd, 2.2, 6.0, 9.6)	71.50 (d)	3.72 (dt, 1.8, 8.1)	71.61 (d)	3.70 (t, 8.2)
34	32.34 (t)	1.48/1.79 (m)	38.19 (t)	1.41/1.86 (m)	38.13 (t)	1.43/1.83 (m)
35	73.77 (d)	4.19 (m)	73.52 (d)	4.17 (m)	73.29 (d)	4.13 (m)
36	40.63 (d)	1.51 (m)	41.10 (d)	1.49 (m)	41.19 (d)	1.50 (m)
37	79.08 (d)	3.90 (dt, 1.3, 6.9)	78.41 (d)	3.81 (dt 1.5, 6.1)	77.83 (d)	3.78 (t, 6.6)
38	28.08 (t)	1.47/1.59 (m)	28.13 (t)	1.43/1.53 (m)	27.88 (t)	1.40/1.55 (m)
39	10.44 (q)	0.92 (t, 7.4)	10.40 (q)	0.91 (t, 7.4)	10.48 (q)	0.90 (t, 7.4)
2-CH_3	14.95 (q)	2.25 (d, 1.4)	15.03 (q)	2.26 (d, 1.4)	12.75 (q)	1.82 (s)
6-CH_3	14.74 (q)	1.54 (d, 1.1)	14.98 (q)	1.55 (s)	14.60 (q)	1.54 (s)
8-CH_3	24.31 (q)	1.84 (s)	23.86 (q)	1.79 (s)	24.22 (q)	1.73 (s)
12-CH_3	16.13 (q)	0.52 (d, 6.7)	16.02 (q)	0.67 (d, 6.3)	16.26 (q)	0.80 (d, 6.1)
18-CH_3	16.46 (q)	1.09 (d, 7.2)	15.01 (q)	1.06 (d, 6.8)	14.62 (q)	1.04 (d, 4.9)
24-CH_3	11.20 (q)	1.01 (d, 7.0)	15.24 (q)	1.01 (d, 6.9)	16.15 (q)	1.02 (d, 5.1)
28-CH_3	14.23 (q)	0.87 (d, 6.9)	14.62 (q)	0.89 (d, 6.9)	14.62 (q)	0.85 (d, 6.8)
30-CH_3	16.28 (q)	1.81 (s)	16.41 (q)	1.79 (s)	16.57 (q)	1.77 (s)
36-CH_3	4.23 (q)	0.84 (d, 7.1)	4.63 (q)	0.82 (d, 7.1)	5.00 (q)	0.82 (d, 7.3)
11-OCH_3			47.63(q)	3.02 (s)	47.42 (q)	3.06 (s)

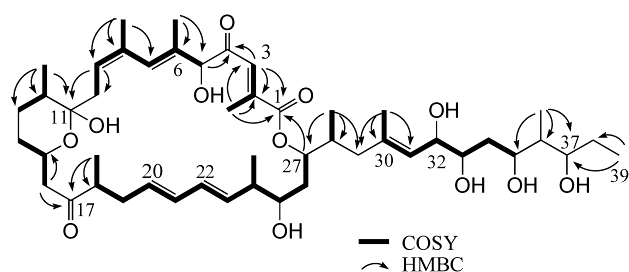


Figure 5. $^1H-^1H$ COSY and HMBC correlations of halstoctacosanolide A (**1**).

The remaining structure to be determined was the position of the oxygen functionalities forming a hemi-acetal ring. However, no direct information was available from the NMR spectra of **1** so far analyzed. To circumvent this difficulty, **1** was derivatized into its methyl acetal **3**. Thus, treatment of **1** with pyridinium *p*-toluenesulfonate in methanol gave a methyl acetal derivative **3** as a single product (Fig. 6). In **3**, an NOE between the introduced methoxy protons (δ 3.02) and 15-H was clearly observed, and the presence of a 6-membered cyclic acetal moiety in **3** was thus established as shown in Figure 6. All the data discussed above allowed us to deduce the planar structure of **1** as shown in Figure 1. The resulting structure of **1** strongly suggested that **1** is biosynthesized through rather regular

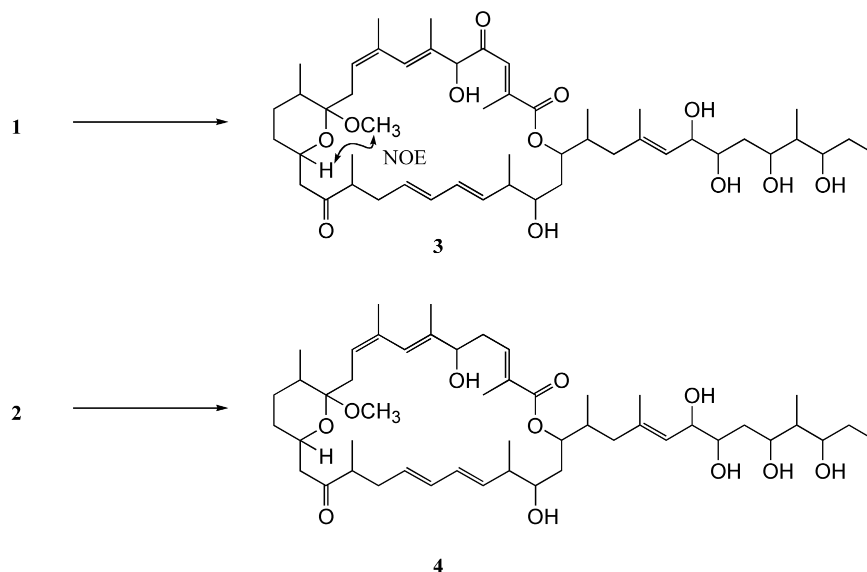


Figure 6. Derivatization of **1** and **2** into methyl acetal derivatives **3** and **4**, respectively.

polyketide pathway catalyzed by Type 1 PKSs, except for two features. The oxygen functionalities at the C-4 and C-32 positions of **1** appear to be irrelevant to the simple PKS pathway and may probably be introduced by post-PKS modifications. The partial structure (Figs. 2 and 3) deduced from the genetic data of new Type 1 PKSs is apparently incorporated into the structure of **1** (C-22 to C-38).

The physico-chemical data of **2** are also shown in Table 1. The molecular formula of **2** was established as $C_{48}H_{78}O_{11}$ on the basis of HRFAB-MS. The molecular formula suggested that the structure of **2** was a deoxygenated derivative of **1**. However, the structure determination of **2** was not straightforward because **2** was obtained as an inseparable mixture of two components. Although these two compounds could be separated by HPLC, each fraction spontaneously turned out to become similar mixture again. Thus, **2** was suggested to exist as an equilibrium mixture of two isomers. As was anticipated, the ^{1}H and ^{13}C NMR spectra were extremely complex. In the ^{1}H NMR spectrum (Fig. 4), the paired signals were observed and the ratio of each signal changed depending on the solvent (1:2 in $CDCl_3$ and 1:5 in CD_3OD), which well supported the state of this compound as an equilibrium mixture of two isomers in solution. In the ^{13}C NMR spectrum, more than 90 signals were observed. However, a key clue to solve this problem came from the observation that three ketonic carbon signals (δ 213.4, 214.7, 215.2) and one acetal signal (δ 99.1) were observed in the ^{13}C NMR spectrum. Thus, **2** appeared to exist in equilibrium of keto-hemiacetal isomerization. Since further structural analysis of natural **2** seemed to be difficult, methyl acetalization was performed again as in the case of **1**. The resulting methyl acetal derivative **4** (Fig. 6) showed rather simple spectra in various NMR experiments, and the spectra turned out to be quite similar to those of **3** as summarized in Table 2. Straightforward analysis of the NMR spectra of **4** including HMBC and NOE experiments (data not shown) indicated that **4** was a deoxo-derivative of **3** at C-4 as shown in Figure 6. Therefore, the natural **2** is a mixture of the ketonic and hemi-acetal forms as shown in Figure 1.

The planar structures of **1** and **2** were determined to be nonadeca-ketides of same biosynthetic origin having a 28-membered macrocyclic lactone ring as shown in Figure 1. A few examples containing a 28-membered lactone ring structure are known in nature as classical polyene antibiotics.[16–27] Halstoctacosanolides are the first examples containing such a 28-membered lactone of non-polyene antibiotics. Stereochemical analyses of **1** and **2** are now in progress.

Antibacterial activities of **1** and **2** were preliminarily tested, and the MIC (μg/ml) values are shown in Table 3. These compounds were moderately active against *Moraxella catarrhalis*.

As described above, halstoctacosanolides A and B appear to belong to the standard polyketides biosynthesized mainly by Type 1 PKSs with some post-PKS modifications. Particularly interesting is that the relevant partial structure of halstoctacosanolides A and B was first predicted from the

Table 3. MIC value (μg/mL) of **1** and **2**

Test organism	1	2	Midecamycin	Azithromycin
Staphylococcus aureus 209P JC-1	>64	64	0.5	0.25
Micrococcus luteus ATCC9341	32	32	0.06	0.03
Haemophilus influenzae Rd/*acrB*::*Km*	64	32	0.5	0.5
Streptococcus pneumoniae 1913	64	32	0.25	0.06
Streptococcus pyogenes Cook	32	32	0.13	0.06
Moraxella catarrhalis W-0506	0.5	<0.25	2	0.03

genomic data and it was in fact found in the natural products. Certain proof by means, for example, of gene disruption and phenotype analysis should be necessary to conclude the relationship between the genetic information and the resulting metabolites, and the present case is by no means an exception. Genetic studies will be described elsewhere. However, the present observation of complete agreement between the predicted partial constitution and the actual structure may convince of direct relationship between the deduced DNA sequence and halstoctacosanolides A and B. Most significant in the present study are to show that genomic analysis of microorganisms, particularly of *Streptomycetes* and related species, is extremely beneficial to directed search for new microbial metabolites and to chemical studies thereof, and to stimulate an emerging field of natural product chemistry.

More than 20,000 secondary metabolites have been described so far from *Streptomycetes* and other *Actinomycetes*. Particularly, the genus *Streptomyces* and closely related genera are still an extraordinary rich source of a wide variety of secondary metabolites as lead compounds for the development of new successful drugs. For example in *S. avermitilis*, 30 different secondary metabolite gene clusters were assigned[28] and several unidentified secondary metabolites were predicted from the genetic data,[5] although isolation of the predicted products has not necessarily been described fully. The findings provided by us and others apparently demonstrate strongly the potential of genetic information in *Streptomycetes* to unveil yet unknown substances.

In conclusion, we successfully demonstrated the isolation of new bioactive compounds, halstoctacosanolides A and B, by genome-inspired search. These compounds in fact escaped from attention with conventional activity-based screening. This approach appears to be promising to search for new antibiotics and useful in natural product chemistry.

3. Experimental

3.1. General procedures

Optical rotations were measured with a JASCO DIP-360 spectrometer. Mass spectra were obtained on a JEOL JMS-700 in FAB mode using 3-nitrobenzyl alcohol as matrix. UV and IR spectra were recorded on a Shimadzu UV-160A spectrophotometer and Horiba FT-710 spectrophotometer, respectively. NMR spectra were measured by using JEOL LA-400 and Bruker DRX-500 spectrometers. Chemical shifts are reported in δ values relative to internal tetramethylsilane (δ 0.00). Column chromatography was carried out with a Silica gel 60 (70–230 or 230–400 mesh, Merck) and preparative TLC was performed on PLC plate (Silica gel 60 F_{254}, Merck, 0.5 mm thickness).

3.2. Fermentation

The vicenistatin production medium was used as described,[10] containing potato starch 3%, soya flake 1.5%, yeast extract 0.2%, corn steep liquor 0.5%, NaCl 0.3%, $MgSO_4\cdot 7H_2O$ 0.05%, $CoCl_2\cdot 6H_2O$ 0.0005% and $CaCO_3$ 0.3%, the pH being adjusted to 7.1 before sterilization with 2 M NaOH. A 10 μL of spore suspension of *S. halstedii* HC 34 was added to an autoclaved 100 mL of this medium in a 500 mL baffled flask equipped with cotton plug. The culture was grown at 27 °C for 2 days with shaking at 200 rpm on a rotary shaker. Vegetative cultures (125 mL×12) of the production medium having the same composition were inoculated with 1 mL of the pre-culture, and were grown under the same conditions for 3 days.

3.3. Isolation and purification

The fermentation broth (1.5 L) was centrifuged to obtain a mycelium cake and supernatant. The mycelium cake was extracted with acetone (500 mL). The extract was filtered through a pad of Celite and the filtrate was evaporated to an aqueous suspension. The suspension, after being adjusted to pH10, was extracted three times with ethyl acetate. The combined organic layer was washed with brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The supernatant was also extracted three times with ethyl acetate after being adjusted to pH10. The combined organic layer was washed with brine, dried over $MgSO_4$, and concentrated in vacuo. Then, the crude extracts thus obtained were combined and subjected to flash chromatography with silica gel, which was eluted with 10% methanol in ethyl acetate to afford a mixture of halstoctacosanolides. The mixture was further purified by preparative TLC (90% ethyl acetate in hexane) to give halstoctacosanolide A (**1**) (33 mg) and halstoctacosanolide B (**2**) (110 mg).

3.3.1. Preparation of halstoctacosanolide A methyl acetal (3). A solution of **1** (19.1 mg, 0.022 mmol) in methanol (3.0 mL) was treated with pyridinium *p*-toluenesulfonate (3 mg) for 3 h at room temperature. The reaction mixture was diluted with Tris–HCl buffer (1 mol/L, pH 8), and the resulting mixture was evaporated to remove methanol, whereupon the rest was extracted three times with ethyl acetate. The combined organic extracts was washed with brine, dried over $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by preparative TLC (10% methanol in $CHCl_3$) to afford **3** (13.6 mg, 70%) as a pale yellow powder. Mp 84.8–85.5 °C; $[\alpha]_D^{29}=+34.9$ (c 1.23, $CHCl_3$); IR (KBr): 3400 (br), 2960, 2930, 1710 cm^{-1}; NMR data of **3** are shown in Table 3; HRFAB-MS calcd for $C_{49}H_{78}O_{12}Na$: *m/z*; 881.5391 ($M+Na^+$). Found: *m/z*; 881.5434.

3.3.2. Preparation of halstoctacosanolide B methyl acetal (4). The compound **2** was treated as in the same manner as described in the preparation of **3**, and a methyl acetal derivative **4** was obtained as colorless powder (27.7 mg, 78%). Mp 80.9–82.5 °C; $[\alpha]_D^{27}=-36.8$ (c 1.0, $CHCl_3$); IR (KBr): 3420(br), 2960, 2930, 1700 cm^{-1}; NMR data of **4** are shown in Table 3; HRFAB-MS calcd for $C_{49}H_{80}O_{11}Na$: *m/z*; 867.5598 ($M+Na^+$). Found: *m/z*; 867.5566.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research and the COE21 program from the Ministry of Education, Culture, Sports, Science and

Technology, Japan. The authors are grateful to Meiji Seika Kaisha Co Ltd. for the assay of antimicrobial activity.

References and notes

1. Demain, A. L. *Nat. Biotechnol.* **2002**, *20*, 331.
2. Cropp, T. A.; Kim, B. S.; Beck, B. J.; Yoon, Y. J.; Sherman, D. H.; Reynolds, K. A. *Biotechnol. Genet. Engng Rev.* **2002**, *19*, 159–172.
3. (a) Knight, V.; Sanglier, J. J.; DiTullio, D.; Braccili, S.; Bonner, P.; Waters, J.; Hughes, D.; Zhang, L. *Appl. Microbiol. Biotechnol.* **2003**, *62*, 446–458. (b) Brady, S. F.; Chao, C. J.; Clardy, J. *J. Am. Chem. Soc.* **2002**, *124*, 9968–9969.
4. Bentley, S. D.; Chater, K. F.; Cerdeno-Tarraga, A. M.; Challis, G. L.; Thomson, N. R.; James, K. D.; Harris, D. E.; Quail, M. A.; Kieser, H.; Harper, D.; Bateman, A.; Brown, S.; Chandra, G.; Chen, C. W.; Collins, M.; Cronin, A.; Fraser, A.; Goble, A.; Hidalgo, J.; Hornsby, T.; Howarth, S.; Huang, C. H.; Kieser, T.; Larke, L.; Murphy, L.; Oliver, K.; O'Neil, S.; Rabbinowitsch, E.; Rajandream, M. A.; Rutherford, K.; Rutter, S.; Seeger, K.; Saunders, D.; Sharp, S.; Squares, R.; Squares, S.; Taylor, K.; Warren, T.; Wietzorrek, A.; Woodward, J.; Barrell, B. G.; Parkhill, J.; Hopwood, D. A. *Nature* **2002**, *417*, 141–147.
5. Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Shinose, M.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; Hattori, M.; Omura, S. *Nat. Biotechnol.* **2003**, *21*, 526–531.
6. Watve, M. G.; Tickoo, R.; Jog, M. M.; Bhole, B. D. *Arch. Microbiol.* **2001**, *176*, 386–390.
7. Kieser, T.; Bibb, M. J.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. *Practical Streptomyces genetics*; John Innes Foundation: Norwich, England, 2000.
8. Staunton, J.; Weissman, K. J. *Nat. Prod. Rep.* **2001**, *18*, 380–416.
9. Yadav, G.; Gokhale, R. S.; Mohanty, D. *J. Mol. Biol.* **2003**, *328*, 335–363.
10. Shindo, K.; Kamishohara, M.; Odagawa, A.; Matsuoka, M.; Kawai, H. *J. Antibiot.* **1993**, *46*, 1076–1081.
11. Arai, H.; Matsushima, Y.; Eguchi, T.; Shindo, K.; Kakinuma, K. *Tetrahedron Lett.* **1998**, *39*, 3181–3184.
12. Otsuka, M.; Eguchi, T.; Shindo, K.; Kakinuma, K. *Tetrahedron Lett.* **1998**, *39*, 3185–3188.
13. Otsuka, M.; Fujita, M.; Matsushima, Y.; Eguchi, T.; Shindo, K.; Kakinuma, K. *Tetrahedron* **2000**, *56*, 8281–8286.
14. Nishida, H.; Eguchi, T.; Kakinuma, K. *Tetrahedron* **2001**, *57*, 8237–8242.
15. Ogasawara, Y.; Minami, A.; Katayama, K.; Otsuka, M.; Eguchi, T.; Kakinuma, K. *Chem. Biol.* **2004**, *11*, 79–86.
16. Cope, A. C.; Bly, R. K.; Burrows, E. P.; Ceder, O. J.; Ciganek, E.; Gillis, B. T.; Porter, R. F.; Johnson, H. E. *J. Am. Chem. Soc.* **1962**, *84*, 2170–2178.
17. Dhar, M. L.; Thaller, V.; Whiting, M. C. *J. Chem. Soc.* **1964**, 842–861.
18. Cope, A. C.; Burrows, E. P.; Derieg, M. E.; Moon, S.; Wirth, W. D. *J. Am. Chem. Soc.* **1965**, *87*, 5452–5460.
19. Pandey, R. C.; Narasimhachari, N.; Rinehart, K. L., Jr.; Millington, D. S. *J. Am. Chem. Soc.* **1972**, *94*, 4306–4310.
20. Falkowski, L.; Golik, J.; Zielinski, J.; Borowski, E. *J. Antibiot.* **1976**, *29*, 197–198.
21. Pozsgay, V.; Tamas, J.; Czira, G.; Wirthlin, T.; Levai, A. *J. Antibiot.* **1976**, *29*, 472–476.
22. Pandey, R. C.; Rinehart, K. L., Jr. *J. Antibiot.* **1977**, *30*, 146–157.
23. Hirota, H.; Itoh, A.; Ido, J.; Iwamoto, Y.; Goshima, E.; Miki, T.; Hasuda, K.; Ohashi, Y. *J. Antibiot.* **1991**, *44*, 181–186.
24. Bortolo, R.; Spera, S.; Guglielmetti, G.; Cassani, G. *J. Antibiot.* **1993**, *46*, 255–264.
25. Schlingmann, G.; Milne, L.; Borders, D. B.; Carter, G. T. *Tetrahedron* **1999**, *55*, 5977–5990.
26. Gupte, T. E.; Chatterjee, N. R.; Nanda, R. K.; Naik, S. R. *Indian J. Chem., Sec. B* **2000**, *39B*, 936–940.
27. Fukuda, T.; Kim, Y. P.; Iizima, K.; Tomoda, H.; Omura, S. *J. Antibiot.* **2003**, *56*, 454–458.
28. Omura, S.; Ikeda, H.; Ishikawa, J.; Hanamoto, A.; Takahashi, C.; Shinose, M.; Takahashi, Y.; Horikawa, H.; Nakazawa, H.; Osonoe, T.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; Hattori, M. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 12215–12220.