SYNTHESIS AND ANTIMICROBIAL ACTIVITY OF SPERABILLIN DERIVATIVES

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Modification of sperabillins was carried out. The 2-amidinoethylamino moiety was removed by brief acidic hydrolysis. The 2,4-hexadienoyl moiety was hydrogenated to the hexanoyl moiety and this was cleaved by an enzymatic reaction using the cells of *Pseudomonas acidovorans* IFO 13582. The 2-amidinoethylamino and the 2,4-hexadienoyl moieties were replaced with other groups. The derivative which was prepared by condensation of two molar amounts of dehexadienoylsperabillin A with (*E,E*)-muconic acid showed better protective effects than sperabillin A against Gram-negative bacteria.

Sperabillin A (**1**), B (**2**), C (**3**) and D (**4**) are novel antibiotics isolated from the culture broth of *Pseudomonas fluorescens* YK-437[1]. They were active against Gram-positive and Gram-negative bacteria including the methicillin-, aminoglycoside- or macrolide-resistant strains. Moreover, they showed better protective effects against bacteria in experimentally infected mice than were predicted from *in vitro* potencies[1].

As reported previously, their unique structures include 2,4-hexadienoic acid, 3,6-diamino-5-hydroxyhexanoic (or -heptanoic) acid and 2-aminoethanamidine as shown in Fig. 1[2].

We have been interested in the novel structures and biological activities of these antibiotics, and have studied their modification and structure-activity relationship with the hope of obtaining more potent derivatives. In this paper, we describe the preparation of sperabillin derivatives by a semi-synthetic approach and their biological activities.

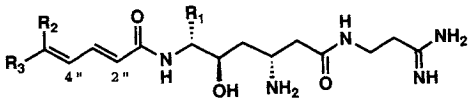
Results and Discussion

Chemistry

Modification of the 2-Amidinoethylamino Moiety

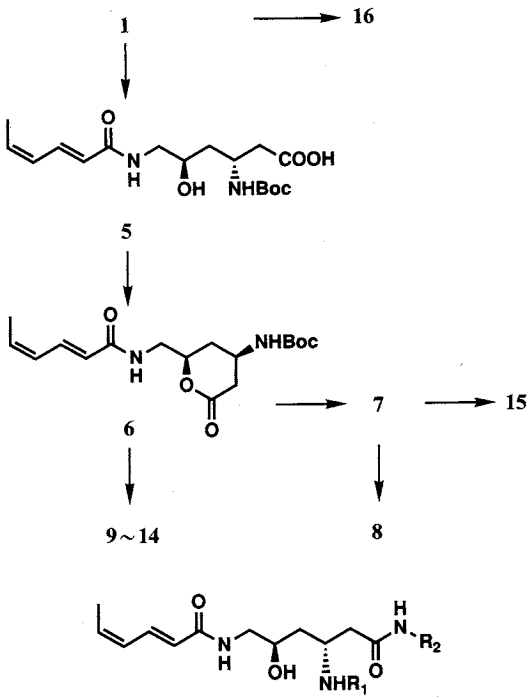
We first investigated the modification of the 2-amidinoethylamino moiety of **1** as shown in Scheme 1. Selective acidic hydrolysis followed by protection of the amino group to afford **5** was done in a previous study[2]. Compound **5** was treated with acetic anhydride and trifluoroacetic acid (TFA) to give a lactone (**6**), and then the lactone ring was opened by reaction with excess 1,2-diaminoethane leading to **7**. Subsequent deprotection with TFA gave **8**. In a similar manner, a series of derivatives (**9**～**14**) was prepared. Among them, compound **10**, which was designated as sperabillin P, has been isolated from

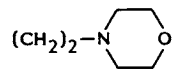
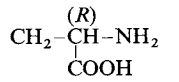
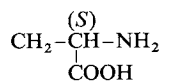
Fig. 1. Structures of sperabillins (**1**～**4**).



Sperabillin	R_1	R_2	R_3
A (**1**)	H	CH_3	H
B (**2**)	CH_3	CH_3	H
C (**3**)	H	H	CH_3
D (**4**)	CH_3	H	CH_3

Scheme 1.



	R_1	R_2
7	Boc	$(CH_2)_2NH_2$
8	H	$(CH_2)_2NH_2$
9	H	$(CH_2)_3NH_2$
10	H	$(CH_2)_4NH_2$ (Sperabillin P)
11	H	
12	H	
13	H	
14	H	CH_2CH_3
15	H	$(CH_2)_2NH(C=NH)NH_2$
16	H	$(CH_2)_2CONH_2$

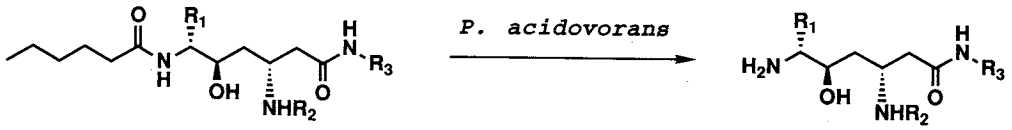
the fermentation broth of the organism producing sperabillins (data not shown). Before the deprotection, the primary amino group in **7** was treated with *S*-methylisothiourea to yield the guanidino derivative **15**. The amidine function in **1** was labile in alkaline solution, and easily converted into the carbamoyl derivative (**16**), with liberation of ammonia, by passage through a column of Dowex 1 (OH^-).

Modification of the 2,4-Hexadienoyl Moiety

Enzymatic reaction was tried for selective hydrolysis of this moiety of sperabillins (Scheme 2). For this purpose, 47 strains of *Pseudomonas* species were tested for their acylase activity. Among them, *Pseudomonas acidovorans* IFO 13582 (TAMAOKA *et al.* proposed to reclassify this as *Comamonas acidovorans*[3]) and *Pseudomonas pertucinogena* IFO 14163 hydrolyzed the tetrahydro derivative (**17**), which was obtained by catalytic hydrogenation of **1** and **3**, to afford the desired compound (**18**), while none of the strains examined directly hydrolyzed the 2,4-hexadienoylamino group of **1**. The enzymatic reaction of **17** was carried out with the cells of *P. acidovorans* IFO 13582 in phosphate buffer (pH 7.0) at 37°C and completed overnight, giving **18** in 65% yield. This reaction was very convenient for preparation of the deacyl derivatives, for example, the reaction proceeded when compounds **19**~**21**, which have the hexanoyl moiety, were used as substrates to afford **22**~**24**, respectively. It has been reported that *Pseudomonas* sp. M-6-3 produces polymyxin acylase and that this strain is related to *P. acidovorans* but is different in some characteristics[4,5]. In addition, the production of the acylase from *P. acidovorans* which we used here was inducible (data not shown), but that of the polymyxin acylase was not[4]. At present, we are studying the isolation, characterization and substrate specificity of the enzyme.

Condensation of the primary amino group of **22** and **23** with (2*E*,4*E*)-2,4-hexadienoic acid in the presence of dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) followed by deprotection with TFA gave the minor components, sperabillin C (**3**) and D (**4**), respectively. In a similar manner, sperabillin derivatives having the cinnamoyl (**25**), crotonyl (**26**), (*E*,*E*)-muconyl (**27**) or palmitoyl (**28**) moiety instead of the 2,4-hexadienoyl moiety were prepared (Scheme 2).

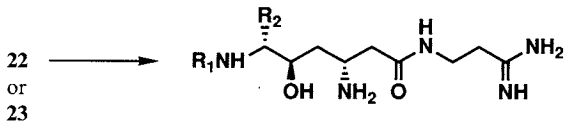
Scheme 2.



17, 19~21

18, 22~24

	R_1	R_2	R_3
17, 18	H	H	$(CH_2)_2C(=NH)NH_2$
19, 22	H	Boc	$(CH_2)_2C(=NH)NH_2$
20, 23	CH_3	Boc	$(CH_2)_2C(=NH)NH_2$
21, 24	H	Boc	$(CH_2)_2NHBoc$



3, 4, 25~28

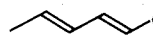
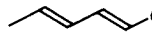
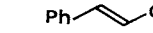
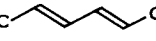
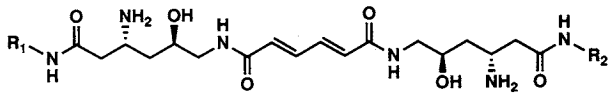
	R_1	R_2
3		H
4		CH_3
25		H
26		H
27		H
28	$C_{15}H_{31}CO$	H

Fig. 2.

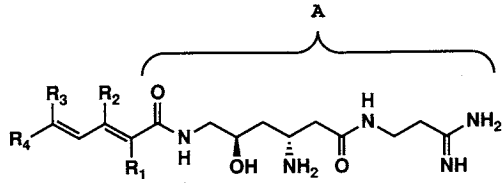


	R_1	R_2
29	$CH_2CH_2C(=NH)NH_2$	$CH_2CH_2C(=NH)NH_2$
30	$CH_2CH_2NH_2$	$CH_2CH_2NH_2$
31	$CH_2CH_2C(=NH)NH_2$	$CH_2CH_2NH_2$

Condensation of Muconic Acid with Two Molar Amounts of 22

When both carboxyl groups of (*E*,*E*)-muconic acid were condensed using two molar amounts of **22**, compound **29** was obtained after deprotection. This compound had improved antibacterial activity and thus, we tried to modify its amidino group. Compound **30** was prepared from muconic acid and **24** by the same method. Stepwise condensation of muconic acid with **24** and **22** gave **31** (Fig. 2). Instead of muconic acid, several muconic acid analogues

Fig. 3.



	R_1	R_2	R_3	R_4
32	CH_3	H	H	A
33	H	CH_3	H	A
34	CH_3	H	CH_3	A
35	CH_3	H	A	CH_3

Table 1. Antibacterial activities of sperabillin derivatives.

Organism[b]	MIC[a] (μg/ml)							
	1	**8**	**28**	**29**	**30**	**31**	**32**	**34**
Staphylococcus aureus FDA 209P	50	>100	6.25	6.25	>100	25	3.13	6.25
S. epidermidis IFO 3762	>100	>100	12.5	12.5	>100	25	6.25	12.5
Bacillus subtilis NIHJ PCI 219	>100	>100	3.13	>100	>100	>100	>100	>100
Escherichia coli NIHJ JC-2	>100	>100	25	50	100	50	100	50
Salmonella typhimurium IFO 12529	>100	>100	100	25	50	50	50	50
Citrobacter freundii IFO 12681	>100	>100	100	12.5	25	12.5	25	25
Klebsiella pneumoniae IFO 3317	>100	>100	50	12.5	12.5	12.5	12.5	12.5
Pseudomonas aeruginosa IFO 3080	25	50	100	25	50	50	>100	>100
Alcaligenes faecalis IFO 13111	3.13	50	>100	50	100	100	>100	>100
Acinetobacter calcoaceticus IFO 13006	25	>100	6.25	25	>100	50	50	25

[a] MIC values were determined by an agar dilution method using DYAB medium[9].
[b] Inoculum size was 10^6 cfu/ml.

were coupled with **22** to afford **32**~**35** (Fig. 3). In the case of (*Z*,*Z*)-2,5-dimethylmuconic acid, one of the double bonds isomerized to the *E*-form during the reaction yielding **35**.

Biological Activity

Antibacterial activities of the sperabillin derivatives were examined by an agar dilution method (Table 1). When the 2-amidinoethylamino moiety of **1** was modified (**8**~**16**), to our regret, **8** showed only weak activity and the others showed none. A similar result has been reported for negamycin, [2-[(3*R*,5*R*)-3,6-diamino-5-hydroxyhexanoyl]-1-methylhydrazino]acetic acid, that is, modification of the (1-methylhydrazino)acetic acid part also resulted in a decrease of the antibacterial activity[6]. The amidino group in **1** may play an important role in the electrostatic binding of **1** to the bacterial cell membrane[1].

Table 2. Protective effects of **1** and **29** in experimentally infected mice.

Microorganism	Compound	ED_{50}[a] (mg/kg)
Staphylococcus aureus 308A-1	**1**	1.39
	29	4.82
S. aureus N133A[b]	**1**	4.50
	29	3.13
Escherichia coli O-111	**1**	67.2
	29	3.72
Klebsiella pneumoniae DT-S	**1**	>100
	29	4.06
Pseudomonas aeruginosa P9	**1**	45.0
	29	17.7

[a] Mice were infected intraperitoneally with 0.5 ml of a suspension of bacteria (10^8 cfu/ml): Groups of five mice at each dosage level were subcutaneously given 0.2 ml of an antibiotic solution immediately after infection: The ED_{50} was calculated from the survival rate at 5 days after infection.
[b] Methicillin-resistant strain.

Sperabillin A (**1**) and B (**2**) which have the 4″*Z*-form showed somewhat stronger activity than sperabillin C (**3**) and D (**4**) which have the 4″*E*-form, respectively[1]. This prompted us to modify the hexadienoyl moiety. Among the compounds synthesized for this purpose (**18**, **25**~**28**), the palmitoyl derivative (**28**) afforded a broad spectrum *in vitro* as shown in Table 1, but the other acyl groups caused loss of activity.

The most striking aspect of the *in vitro* experiments was that **29** showed stronger antibacterial activity than **1** and **2** against both Gram-positive and Gram-negative bacteria except for *Alcaligenes faecalis* (Table 1). The C-2 methyl (**32**) and C-2,5 dimethyl (**34**) groups of the (*E*,*E*)-muconic acid moiety had little influence of activity. However, the C-3 methyl group resulted in loss of activity (**33**), and the

(*E,Z*)-2,5-dimethyl muconic acid derivative (**35**) showed no activity.

The importance of the stereochemistry of the 3,6-diamino-5-hydroxyhexanoic (or -heptanoic) acid moiety was described in a previous paper[7].

The protective effects of the derivatives which inhibited bacterial growth were evaluated in experimentally infected mice. Although the ethylenediamino and palmitoyl derivatives (**8** and **29**) were not effective, **29** showed good protective effects. When **1** and **29** were administered subcutaneously, **29** was more effective than **1** against Gram-negative bacteria and as effective as **1** against Gram-positive bacteria (Table 2).

The acute toxicities (LD_{50}) of **29** in mice were 400~800 mg/kg (sc, ip) and 50~100 mg/kg (iv), and these values were similar to those of **1**.

The study of the detailed mechanism of action for these antibiotics is in progress, including examination of their immunostimulating activities. Polymerization of these antibiotics led to the expression of anti-tumor activity. The chemistry and biological activity of the polymers obtained will be described in a forthcoming paper[8].

Experimental

UV spectra in water were taken on a Hitachi 320 spectrophotometer. Optical rotations in water were obtained with a JASCO DIP-181 digital polarimeter at 20~26°C. IR spectra were measured with a Hitachi 285 grating IR spectrophotometer using KBr pellets. ^{1}H NMR spectra were recorded on a Varian EM-390 (90 MHz), Bruker AC-300 (300 MHz) or JEOL JNM GX-400FT (400 MHz) instrument in D_2O, unless otherwise stated. Chemical shifts (δ) are reported in ppm downfield from tetramethylsilane or sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d_4. SI-MS spectra were measured with a Hitachi M-80 A mass spectrometer with xenon ion beam source. EI-MS spectra were measured with a JEOL JMS-DX303 mass spectrometer.

Lactone **6**

To a solution of **5** (7.40 g, 21 mmol) in acetone (240 ml) were added acetic anhydride (2.35 ml, 24 mmol) and TFA (1.60 ml, 21 mmol). The mixture was stirred for 4 hours at room temperature, and concentrated. The residue obtained was suspended in 5% aq $NaHCO_3$ (150 ml) and extracted twice with EtOAc (150 ml, 100 ml). The combined organic layers were washed with 2% aq $NaHCO_3$ and water successively and then evaporated to give an oily residue. Trituration with ether gave **6** (5.71 g, 81%) as a white powder: $[\alpha]_D$ −10.5° (*c* 0.49, EtOH); EI-MS *m/z* 338 (M^+); UV λ_{max}^{EtOH} nm (ε) 260 (27,800); IR ν cm^{-1} 1730, 1710, 1680, 1660, 1620; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43 (9H, s), 1.48 (m), 1.87 (3H, dd, J=1.7 and 7.3 Hz), 2.20 (dddd, J=1.5, 2.7, 4.9 and 13.4 Hz), 2.34 (dd, J=9.4 and 17.5 Hz), 2.87 (ddd, J=1.4, 6.3 and 17.5 Hz), 3.51 (dd, J=5.9 and 13.9 Hz), 3.54 (dd, J=4.2 and 13.9 Hz), 3.97 (m), 4.46 (m), 5.90 (dq, J=10.7 and 7.3 Hz), 6.02 (d, J=15.1 Hz), 6.16 (m), 7.57 (ddd, J=1.0, 11.5 and 14.9 Hz).

Anal Calcd for $C_{17}H_{26}N_2O_5$: C 60.34, H 7.74, N 8.28.
Found: C 60.30, H 7.93, N 8.17.

Condensation of **6** with Amines

To a solution of **6** (800 mg, 2.4 mmol) in CH_2Cl_2 (40 ml) was added 1,2-diaminoethane (1.6 ml, 24 mmol) and the mixture was stirred for 1 hour at room temperature. After evaporation of the solvent, the residue was diluted with water (50 ml) and washed with EtOAc (25 ml) at pH 2. The aqueous layer adjusted to pH 5.5 was concentrated and chromatographed on a column of Diaion HP-20 (50~100 mesh, 60 ml) with elutions of water (240 ml), 50% aq MeOH (180 ml) and 50% MeOH-5 mM HCl (180 ml). The pure fraction was concentrated and freeze-dried to give **7** as a white powder (898 mg). A solution of the powder (200 mg) in TFA (2.0 ml) was allowed to stand for 30 minutes at room temperature, and then concentrated. The residue dissolved in water (20 ml) was passed through a column of Amberlite IRA-402 (Cl^-, 20 ml) and

the column was washed with water (20 ml). The effluent was concentrated and freeze-dried to give a white powder of **8** (177 mg, 87% from **6**): $[\alpha]_D$ −13.1° (*c* 0.51); SI-MS *m/z* 299 $(M+H)^+$; UV λ_{max} nm (ε) 265 (23,900); IR ν cm^{-1} 1640, 1540; ^1H NMR (90 MHz) δ 2.03 (2H, m), 2.08 (3H, d, *J*=6 Hz), 2.98 (2H, d, *J*=6 Hz), 3.37 (2H, t, *J*=6 Hz), 3.57 (2H, d, *J*=6 Hz), 3.77 (2H, t, *J*=6 Hz), 4.15 (2H, m), 6.1~6.7 (3H, m), 7.78 (1H, dd, *J*=11 and 15 Hz).

Anal Calcd for $C_{14}H_{26}N_4O_3 \cdot 2HCl \cdot 0.5H_2O$: C 44.21, H 7.69, N 14.73, Cl 18.64.
Found: C 44.47, H 7.57, N 14.99, Cl 18.63.

When 1,3-diaminopropane, 1,4-diaminobutane, 4-(2-aminoethyl)morpholine, (*R*)-3-amino-2-*tert*-butoxycarbonylaminopropionic acid, (*S*)-3-amino-2-*tert*-butoxycarbonylaminopropionic acid or ethylamine was added instead of 1,2-diaminoethane, the corresponding compound was afforded (**9~14**).

9: Yield 84%, $[\alpha]_D$ −8.9° (*c* 0.49); SI-MS *m/z* 313 $(M+H)^+$; UV λ_{max} nm (ε) 265 (24,000); IR ν cm^{-1} 1645, 1550 cm^{-1}; ^1H NMR (90 MHz) δ 2.10 (3H, d, *J*=6 Hz), 2.10 (4H, m), 2.95 (2H, d, *J*=6 Hz), 3.27 (2H, t, *J*=7 Hz), 3.53 (2H, t, *J*=7 Hz), 3.58 (2H, d, *J*=7 Hz), 3.9~4.4 (2H, m), 6.1~6.7 (3H, m), 7.82 (1H, dd, *J*=10 and 15 Hz).

Anal Calcd for $C_{15}H_{28}N_4O_3 \cdot 2HCl \cdot 0.5H_2O$: C 45.69, H 7.92, N 14.21, Cl 17.98.
Found: C 46.18, H 8.07, N 14.39, Cl 18.17.

10: Yield 86%; $[\alpha]_D$ −6.9° (*c* 0.54); SI-MS *m/z* 327 $(M+H)^+$; UV λ_{max} nm (ε) 265 (25,000); IR ν cm^{-1} 1640, 1540; ^1H NMR (90 MHz) δ 1.87 (4H, m), 2.05 (2H, m), 2.10 (3H, d, *J*=6 Hz), 2.93 (2H, d, *J*=7 Hz), 3.25 (2H, t, *J*=7 Hz), 3.43 (2H, t, *J*=6 Hz), 3.57 (2H, d, *J*=6 Hz), 3.9~4.4 (2H, m), 6.0~6.7 (3H, m), 7.80 (1H, dd, *J*=10 and 15 Hz).

Anal Calcd for $C_{16}H_{30}N_4O_3 \cdot 2HCl \cdot 0.5H_2O$: C 47.06, H 8.15, N 13.72, Cl 17.36.
Found: C 47.37, H 8.19, N 13.71, Cl 17.31.

11: Yield 90%; $[\alpha]_D$ −10.9° (*c* 0.56); SI-MS *m/z* 369 $(M+H)^+$; IR ν cm^{-1} 1650, 1535 cm; ^1H NMR (90 MHz) δ 2.07 (2H, m), 2.10 (3H, d, *J*=6 Hz), 2.98 (2H, d, *J*=6 Hz), 3.4~4.0 (10H, m), 4.0~4.4 (6H, m), 6.0~6.7 (3H, m), 7.78 (1H, dd, *J*=10 and 15 Hz).

Anal Calcd for $C_{18}H_{32}N_4O_4 \cdot 2HCl \cdot 0.5H_2O$: C 48.00, H 7.83, N 12.44, Cl 15.74.
Found: C 48.19, H 8.01, N 12.35, Cl 15.62.

12: Yield 45%; SI-MS *m/z* 343 $(M+H)^+$; ^1H NMR (300 MHz) δ 1.75~2.00 (2H, m), 1.89 (3H, dd, *J*=2 and 7 Hz), 2.74 (1H, dd, *J*=8 and 16 Hz), 2.83 (1H, dd, *J*=5 and 16 Hz), 3.34 (1H, dd, *J*=7 and 14 Hz), 3.43 (1H, dd, *J*=5 and 14 Hz), 3.66 (1H, dd, *J*=6 and 15 Hz), 3.83 (1H, dd, *J*=4 and 15 Hz), 3.90 (1H, m), 3.96 (1H, dd, *J*=4 and 6 Hz), 4.01 (1H, m), 6.04 (1H, m), 6.10 (1H, d, *J*=15 Hz), 6.25 (1H, m), 7.59 (1H, dd, *J*=12 and 15 Hz).

Anal Calcd for $C_{15}H_{26}N_4O_5 \cdot HCl \cdot H_2O$: C 45.40, H 7.37, N 14.12.
Found: C 45.34, H 7.48, N 14.03.

13: Yield 52%; SI-MS *m/z* 343 $(M+H)^+$; IR ν cm^{-1} 1660, 1540; ^1H NMR (300 MHz) δ 1.70~1.98 (2H, m), 1.86 (3H, dd, *J*=2 and 7Hz), 2.71 (1H, dd, *J*=8 and 16 Hz), 2.79 (1H, dd, *J*=6 and 16 Hz), 3.30 (1H, dd, *J*=7 and 14 Hz), 3.40 (1H, dd, *J*=5 and 14 Hz), 3.67 (1H, dd, *J*=6 and 15 Hz), 3.81 (1H, dd, *J*=4 and 15 Hz), 3.87 (1H, m), 3.98 (1H, m), 4.02 (1H, dd, *J*=4 and 6 Hz), 6.01 (1H, m), 6.06 (1H, d, *J*=15 Hz), 6.24 (1H, m), 7.55 (1H, dd, *J*=12 and 15 Hz).

Anal Calcd for $C_{15}H_{26}N_4O_5 \cdot HCl \cdot 2H_2O$: C 43.43, H 7.53, N 13.50.
Found: C 43.26, H 7.16, N 13.28.

14: Yield 49%; IR ν cm^{-1} 1735, 1650, 1540; ^1H NMR (300 MHz) δ 1.11 (3H, d, *J*=7 Hz), 1.70~1.95 (2H, m), 1.88 (3H, dd, *J*=2 and 7 Hz), 2.68 (2H, d, *J*=7 Hz), 3.21 (2H, q, *J*=7 Hz), 3.33 (1H, dd, *J*=6 and 14 Hz), 3.40 (1H, dd, *J*=4 and 14 Hz), 3.85 (1H, m), 4.00 (1H, m), 6.03 (1H, m), 6.07 (1H, d, *J*=15 Hz), 6.25 (1H, m), 7.56 (1H, dd, *J*=12 and 15 Hz).

Anal Calcd for $C_{14}H_{25}N_3O_3 \cdot HCl \cdot H_2O$: C 49.77, H 8.35, N 12.44.
Found: C 50.28, H 8.16, N 11.85.

Guanidino Derivative (**15**)

To a solution of **7** (500 mg, 1.2 mmol) in water (1.5 ml) were added *S*-methylisothiourea ($\frac{1}{2}H_2SO_4$

salt, SMIT, 180 mg, 1.3 mmol) and $Ba(OH)_2 \cdot 8H_2O$ (205 mg, 0.65 mmol) and the mixture was stirred for 1.5 hours at 80°C. Additional SMIT (90 mg, 0.65 mmol) and $Ba(OH)_2 \cdot 8H_2O$ (103 mg, 0.33 mmol) were added to the reaction mixture and stirred for 1.5 hours and the procedure was repeated twice. The reaction mixture was adjusted to pH 5.3 and filtered. The filtrate was applied to a column of CM-Sephadex C-25 (Na^+, 50 ml), eluting with 50 mM NaCl. The eluate was concentrated, adjusted to pH 5.3 and chromatographed on a column of Diaion HP-20 (50~100 mesh, 20 ml), eluting with 50% aq MeOH and 50% MeOH - 5 mM HCl. The pure fraction was concentrated to give a white powder (291 mg). The powder (275 mg) was deprotected with TFA by the method described above to give **15** (241 mg, 53% from **7**) as a white powder: $[\alpha]_D$ −6.9° (*c* 0.50); SI-MS *m/z* 341 $(M+H)^+$; UV λ_{max} nm (ε) 265 (25,000); IR ν cm^{-1} 1645, 1530; ^1H NMR (90 MHz) δ 2.10 (3H, d, *J*=6 Hz), 2.10 (2H, m), 2.95 (2H, d, *J*=6 Hz), 3.4~3.7 (6H, m), 3.9~4.4 (2H, m), 6.0~6.7 (3H, m), 7.80 (1H, dd, *J*=10 and 15 Hz).

Anal Calcd for $C_{15}H_{28}N_6O_3 \cdot 2HCl \cdot 0.5H_2O$: C 42.66, H 7.40, N 19.90, Cl 16.79.
Found: C 42.79, H 7.56, N 19.78, Cl 16.75.

Carbamoyl Derivative (**16**)

A solution of **1** (170 mg, 0.41 mmol) in water (30 ml) was passed through a column of Dowex 1×2 (OH^-, 50~100 mesh, 15 ml) and the column was washed with water (45 ml). The effluent was concentrated and lyophilized to give a crude powder (141 mg). The powder dissolved in water (30 ml) was chromatographed on Amberlite CG-50 (H^+, 100~200 mesh, 15 ml), eluting with 2% aq NH_3 (45 ml). The eluate was concentrated and freeze-dried to afford a white powder of **16** (100 mg, 71%): SI-MS *m/z* 327 $(M+H)^+$; UV λ_{max} nm (ε) 265 (29,600); IR ν cm^{-1} 1660, 1550; ^1H NMR (90 MHz) δ 1.87 (2H, t, *J*=6 Hz), 2.08 (3H, d, *J*=6 Hz), 2.70 (4H, m), 3.63 (5H, m), 4.22 (m), 6.37 (3H, m), 6.77 (dd, *J*=9 and 15 Hz).

Anal Calcd for $C_{15}H_{26}N_4O_4 \cdot H_2O$: C 52.31, H 8.19, N 16.27.
Found: C 52.62, H 7.36, N 15.60.

Tetrahydro Derivatives (**17**, **19**, **20**)

A solution of **1** (20 g, 49 mmol) in water (500 ml) was hydrogenated over 10% Pd-C (2.0 g) for 4 hours at room temperature. After filtration, the filtrate was concentrated and freeze-dried to give **17** as a white powder (19 g, 97%): $[\alpha]_D$ −5.2° (*c* 0.60); SI-MS *m/z* 330 $(M+H)^+$; ^1H NMR (90 MHz) δ 1.10 (3H, t, *J*=6 Hz), 1.40~2.20 (8H, m), 2.50 (2H, t, *J*=7 Hz), 2.93 (2H, t, *J*=6 Hz), 2.97 (2H, d, *J*=6 Hz), 3.50 (2H, d, *J*=6 Hz), 3.82 (2H, t, *J*=6 Hz), 4.17 (2H, m).

Anal Calcd for $C_{15}H_{31}N_5O_3 \cdot 2HCl \cdot H_2O$: C 42.86, H 8.39, N 16.66, Cl 16.87.
Found: C 42.88, H 8.84, N 16.75, Cl 17.26.

N-tert-Butoxycarbonylation of **17** was carried out by the same method described in a previous paper,[2] affording **19** (76%) as a white powder: $[\alpha]_D$ −13.3° (*c* 0.67); SI-MS *m/z* 430 $(M+H)^+$; IR ν cm^{-1} 1640, 1520; ^1H NMR (90 MHz) δ 1.08 (3H, t, *J*=6 Hz), 1.4~2.0 (8H, m), 1.65 (9H, s), 2.48 (2H, t, *J*=7 Hz), 2.63 (2H, m), 2.90 (2H, t, *J*=6 Hz), 3.43 (2H, m), 3.77 (2H, t, *J*=6 Hz), 3.98 (1H, m), 4.28 (1H, m).

Anal Calcd for $C_{20}H_{39}N_5O_5 \cdot HCl \cdot 0.5H_2O$: C 50.57, H 8.70, N 14.74, Cl 7.46.
Found: C 51.05, H 9.06, N 14.82, Cl 7.64.

By the same method, **2** was treated to yield **20** as a white powder (46%): $[\alpha]_D$ +23.1° (*c* 0.38); SI-MS *m/z* 444 $(M+H)^+$; IR ν cm^{-1} 1680, 1660, 1640; ^1H NMR (90 MHz) δ 1.10 (3H, t, *J*=6 Hz), 1.35 (3H, d, *J*=7 Hz), 1.63 (9H, s), 1.4~1.9 (8H, m), 2.47 (2H, t, *J*=7 Hz), 2.62 (1H, d, *J*=6.5 Hz), 2.62 (1H, d, *J*=7 Hz), 2.88 (2H, t, *J*=7 Hz), 3.75 (2H, t, *J*=7 Hz), 3.9~4.4 (3H, m).

Anal Calcd for $C_{21}H_{41}N_5O_5 \cdot HCl \cdot 0.8H_2O$: C 51.01, H 8.89, N 14.17, Cl 7.17.
Found: C 51.01, H 8.94, N 13.87, Cl 7.21.

Cultivation of Microorganism

P. acidovorans IFO 13582 was cultivated using the following medium; meat extract 1%, polypeptone (Nihon Pharmaceutical Co.) 1%, yeast extract 0.1%, and NaCl 0.5% (pH 6.8). The cultivation was carried out for 3 days at 28°C with shaking. Cells were harvested by centrifugation.

Enzymatic Deacylation Procedure

To a solution of **17** (2HCl salt, 202 mg, 0.48 mmol) in 30 mM phosphate buffer (pH 7.0, 100 ml) were

added the cells (10 g) and the mixture was shaken for 15 hours at 37°C. The reaction mixture was centrifuged and the supernatant was adjusted to pH 7.0, and then chromatographed on Amberlite CG-50 (H^+, 100~200 mesh, 40 ml), eluting with 20 mM HCl. The fraction containing **18** was concentrated and freeze-dried to give a powder (147 mg). The powder was chromatographed on Diaion HP-20 (50~100 mesh, 40 ml), eluting with water. Pure fraction was freeze-dried to give **18** (3HCl salt, 118 mg, 65%) as a white powder: $[\alpha]_D$ −7.6° (*c* 0.45); SI-MS *m/z* 232 $(M+H)^+$; IR νcm^{-1} 1640, 1550; ^1H NMR (90 MHz) δ 2.13 (2H, m), 3.00 (4H, m), 3.30 (2H, m), 3.80 (2H, t, J=6 Hz), 4.10 (m), 4.37 (m).

Anal Calcd for $C_9H_{21}N_5O_2 \cdot 3HCl \cdot 2H_2O$: C 28.70, H 7.49, N 18.59, Cl 28.23.
Found: C 28.58, H 7.19, N 18.32, Cl 28.41.

Compound **19** (2HCl salt, 10 g, 22 mmol) was treated as described above. The reaction filtrate was purified by Amberlite IRC-50 (Na^+, 1.0 liter) chromatography, eluting with water (4 liters), 0.5 M and 1.0 M aq NaCl (8 and 5 liters, respectively). The pure fraction was desalted by carbon chromatography (800 ml) to give **22** (2HCl salt, 6.82 g, 79%) as a white powder: $[\alpha]_D$ −1.6° (*c* 0.66); SI-MS *m/z* 332 $(M+H)^+$; ^1H NMR (90 MHz) δ 1.67 (9H, s), 1.83 (2H, m), 2.67 (2H, d, J=7 Hz), 2.90 (2H, t, J=6 Hz), 3.30 (2H, m), 3.78 (2H, m), 4.27 (2H, m).

Anal Calcd for $C_{14}H_{29}N_5O_4 \cdot 2HCl \cdot 0.5H_2O$: C 40.68, H 7.80, N 16.94, Cl 17.15.
Found: C 40.79, H 8.19, N 16.91, Cl 17.76.

By the same method, **23** was obtained as a white powder (74%): $[\alpha]_D$ +4.0° (*c* 0.55); SI-MS *m/z* 346 $(M+H)^+$; IR νcm^{-1} 1670; ^1H NMR (90 MHz) δ 1.50 (3H, d, J=6.5 Hz), 1.65 (9H, s), 1.90 (2H, m), 2.70 (2H, m), 2.90 (2H, t, J=7 Hz), 3.47 (1H, m), 3.80 (1H, m), 4.34 (1H, m).

Anal Calcd for $C_{15}H_{31}N_5O_4 \cdot 2HCl \cdot 0.4H_2O$: C 42.33, H 8.00, N 16.46, Cl 16.66.
Found: C 42.39, H 8.65, N 16.60, Cl 16.73.

Hydrogenation of **7** followed by protection of the primary amine gave **21**, and subsequent enzymatic deacylation afforded **24** as a white powder: ^1H NMR (300 MHz) δ 1.44 (9H, s), 1.45 (9H, s), 1.67 (2H, m), 2.39 (1H, dd, J=9 and 14 Hz), 2.47 (1H, dd, J=5 and 14 Hz), 2.94 (1H, dd, J=10 and 13 Hz), 3.13 (1H, dd, J=3 and 13 Hz), 3.17~3.35 (4H, m), 3.93 (1H, m), 4.12 (1H, m).

Acylation of **22**

To a suspension of **22** (2HCl salt, 964 mg, 2.3 mmol), (2*E*,4*E*)-2,4-hexadienoic acid (307 mg, 2.7 mmol) and Et_3N (0.48 ml, 3.5 mmol) in DMF (10 ml) were added HOBT (369 mg, 2.7 mmol) and DCC (563 mg, 2.7 mmol). The reaction mixture was stirred for 1 hour at 0°C and then at room temperature for 8 hours. The mixture was filtered and the filtrate was concentrated. The residue was diluted with water (200 ml), adjusted to pH 2.5 and washed with EtOAc (100 ml). The aqueous layer adjusted to pH 5.5 was concentrated and loaded on a column of Diaion HP-20 (50~100 mesh, 50 ml), eluting with 50% MeOH-5 mM HCl (200 ml). The pure fraction was concentrated and freeze-dried to give a powder (999 mg). A solution of the powder (853 mg) in TFA (5 ml) was allowed to stand at room temperature for 30 minutes. Work up in a similar manner described above gave **3** (725 mg, 91%) as a white powder.

Anal Calcd for $C_{15}H_{27}N_5O_3 \cdot 2HCl \cdot 0.5H_2O$: C 44.23, H 7.42, N 17.19, Cl 17.41.
Found: C 44.35, H 7.42, N 17.28, Cl 17.59.

The physico-chemical data of the product were identical to those of **3** isolated from the fermentation broth.

Compound **24** was treated in a similar manner described above to give **4** as a white powder.

Anal Calcd for $C_{16}H_{29}N_5O_3 \cdot HCl \cdot 0.5H_2O$: C 45.61, H 7.65, N 16.62, Cl 16.83.
Found: C 45.15, H 7.98, N 16.44, Cl 16.59.

The physico-chemical data of the product were identical to those of **4** isolated from the fermentation broth.

When cinnamic acid, crotonic acid, (*E*,*E*)-muconic acid or palmitic acid was added instead of (2*E*,4*E*)-2,4-hexadienoic acid, the corresponding compound was afforded.

25: Yield 81%; $[\alpha]_D$ −11.9° (*c* 0.48); SI-MS *m/z* 362 $(M+H)^+$; IR νcm^{-1} 1670, 1620, 1550; ^1H NMR (90 MHz) δ 2.07 (2H, m), 2.87 (2H, t, J=7 Hz), 2.97 (2H, d, J=7 Hz), 3.65 (2H, d, J=6 Hz), 3.77 (2H, t,

J=7 Hz), 4.0~4.3 (2H, m), 6.87 (1H, d, J=16 Hz), 7.77 (1H, d, J=16 Hz), 7.6~7.9 (5H, m).

Anal Calcd for $C_{18}H_{27}N_5O_3 \cdot 2HCl \cdot 0.7H_2O$: C 48.37, H 6.85, N 15.67, Cl 15.86.
Found: C 48.26, H 7.02, N 15.62, Cl 16.52.

26: Yield 85%; $[\alpha]_D$ −9.7° (c 0.51); SI-MS m/z 300 $(M+H)^+$; ^{1}H NMR (90 MHz) δ 2.00 (2H, m), 2.10 (3H, dd, J=1 and 6 Hz), 2.93 (2H, t, J=6 Hz), 2.97 (2H, d, J=6 Hz), 3.53 (2H, d, J=6 Hz), 3.80 (2H, t, J=6 Hz), 4.13 (2H, m), 6.23 (1H, dd, J=1 and 15 Hz), 7.05 (1H, dq, J=15 and 6 Hz).

Anal Calcd for $C_{13}H_{25}N_5O_3 \cdot 2HCl \cdot 0.5H_2O$: C 40.95, H 7.40, N 18.37, Cl 18.60.
Found: C 41.24, H 7.52, N 18.36, Cl 18.60.

27: Yield 48%; UV λ_{max} nm (ε) 267 (24,600); ^{1}H NMR (300 MHz) δ 1.7~2.0 (2H, m), 2.70 (4H, m), 3.36 (1H, dd, J=7 and 12), 3.42 (1H, dd, J=5 and 12 Hz), 3.59 (2H, dt, J=3 and 7 Hz), 3.88 (1H, m), 4.04 (1H, m), 6.31 (1H, d, J=15 Hz), 6.47 (1H, d, J=15 Hz), 7.30 (2H, m).

Anal Calcd for $C_{15}H_{25}N_5O_5 \cdot 2HCl \cdot 1.5H_2O$: C 39.57, H 6.64, N 15.38.
Found: C 39.87, H 6.76, N 15.41.

28: Yield 64%; $[\alpha]_D$ −5.2° (c 0.46); SI-MS m/z 470 $(M+H)^+$; IR ν cm^{-1} 1640, 1540; ^{1}H NMR (90 MHz) δ 1.05 (3H, br t, J=6 Hz), 1.45 (24H, br s), 1.80 (2H, m), 2.10 (2H, m), 2.48 (2H, m), 2.97 (4H, m), 3.50 (2H, m), 3.80 (2H, t, J=6 Hz), 4.17 (2H, m).

Anal Calcd for $C_{25}H_{51}N_5O_3 \cdot 2HCl \cdot 0.7H_2O$: C 54.08, H 9.87, N 12.61, Cl 12.77.
Found: C 54.11, H 9.70, N 12.31, Cl 11.83.

Condensation of Muconic Acid with Two Molar Amounts of **22**

To a solution of **22** (3.0 g, 7.1 mmol) in DMF (45 ml) were added Et_3N (1.48 ml, 11 mmol), (*E,E*)-muconic acid (495 mg, 3.5 mmol), HOBT (1.15 g, 8.5 mmol) and DCC (1.76 g, 8.5 mmol) and the mixture was stirred for 41 hours at room temperature, and then filtered. The filtrate was diluted with water (300 ml), washed with EtOAc (4×150 ml), adjusted to pH 5 and concentrated to a small volume. The residue was chromatographed on Amberlite CG-50 (Na^+, 300 ml), eluting with 1 M aq NaCl. The eluate was applied to a column of Diaion HP-20 (50~100 mesh, 200 ml), eluting with 30% aq MeOH and 30% MeOH-5 mM HCl. Removal of the solvent afforded a white powder (2.0 g). The powder was dissolved in TFA (15 ml) and the solution was allowed to stand for 40 minutes at room temperature. The mixture was concentrated and the residue was triturated with ether to give a powder. The powder was dissolved in water and passed through Amberlite IRA-402 (SO_4^{2-}). The effluent was concentrated and freeze-dried to give **29** (1.84 g, 34%) as a white powder: UV λ_{max} nm (ε) 272 (34,800); ^{1}H NMR (300 MHz) δ 1.77 (2H, ddd, J=5, 10 and 15 Hz), 1.91 (2H, ddd, J=3, 8 and 15 Hz), 2.70 (4H, t, J=7 Hz), 2.74 (4H, m), 3.35 (2H, dd, J=7 and 14 Hz), 3.43 (2H, dd, J=5 and 14 Hz), 3.59 (4H, t, J=7 Hz), 3.87 (2H, m), 4.02 (2H, m), 6.46 (2H, m), 7.25 (2H, m).

Anal Calcd for $C_{24}H_{44}N_{10}O_6 \cdot 2H_2SO_4 \cdot 2H_2O$: C 35.99, H 6.54, N 17.49, S 8.01.
Found: C 35.96, H 6.72, N 17.58, S 8.22.

In a similar manner, (*E,E*)-2-methylmuconic acid, (*E,E*)-3-methylmuconic acid, (*E,E*)-2,5-dimethylmuconic acid and (*Z,Z*)-2,5-dimethylmuconic acid were coupled with **22** to yield **32**~**35**, respectively.

32: Yield 70%; IR ν cm^{-1} 1690, 1650, 1540; ^{1}H NMR (90 MHz) δ 1.8~2.0 (4H, m), 2.17 (3H, s), 2.7~2.9 (8H, m), 3.47 (4H, m), 3.67 (4H, t, J=6 Hz), 3.96 (2H, m), 4.07 (2H, m), 6.44 (1H, d, J=15 Hz), 7.00 (1H, d, J=11 Hz), 7.59 (1H, dd, J=11 and 15 Hz).

Anal Calcd for $C_{25}H_{46}N_{10}O_6 \cdot 4HCl \cdot 2H_2O$: C 39.27, H 7.12, N 18.32.
Found: C 39.01, H 7.42, N 18.01.

33: Yield 36%; IR ν cm^{-1} 1690, 1650, 1545; ^{1}H NMR (90 MHz) δ 1.8~2.0 (4H, m), 2.21 (3H, s), 2.7~2.9 (8H, m), 3.47 (4H, m), 3.67 (4H, t, J=6 Hz), 4.0 (4H, m), 6.29 (1H, s), 6.44 (1H, d, J=15 Hz), 7.26 (1H, d, J=15 Hz).

Anal Calcd for $C_{25}H_{46}N_{10}O_6 \cdot 4HCl \cdot 2H_2O$: C 39.27, H 7.12, N 18.32.
Found: C 38.98, H 7.40, N 18.02.

34: Yield 54%; IR ν cm^{-1} 1690, 1640, 1530; ^{1}H NMR (90 MHz) δ 1.8~2.0 (4H, m), 2.17 (6H, s), 2.7~2.9 (8H, m), 3.47 (4H, m), 3.67 (4H, t, J=6 Hz), 4.0 (4H, m), 7.20 (2H, s).

Anal Calcd for $C_{26}H_{48}N_{10}O_6 \cdot 4HCl \cdot 2H_2O$: C 40.11, H 7.25, N 17.99.
Found: C 40.30, H 7.10, N 17.88.

35: Yield 36%; IR ν cm^{-1} 1690, 1650, 1540; ^{1}H NMR (90 MHz) δ 1.8~2.0 (4H, m), 2.07 (3H, s), 2.14 (3H, s), 2.7~2.9 (8H, m), 3.47 (4H, m), 3.67 (4H, t, J=6 Hz), 4.0 (4H, m), 6.55 (1H, d, J=12 Hz), 7.01 (1H, d, J=12 Hz).

Anal Calcd for $C_{26}H_{48}N_{10}O_6 \cdot 4HCl \cdot 2H_2O$: C 40.11, H 7.25, N 17.99.
Found: C 40.01, H 7.35, N 18.10.

In a similar manner described above, **24** and (*E,E*)-muconic acid afforded **30** (96%) as a white powder: UV: λ_{max} nm (ε) 272 (35,500); ^{1}H NMR (300 MHz) δ 1.80 (2H, ddd, J=5, 10 and 15 Hz), 1.92 (2H, ddd, J=3, 8 and 15 Hz), 2.75 (2H, dd, J=8 and 16 Hz), 2.82 (2H, d, J=6 and 16 Hz), 3.17 (4H, t, J=6 Hz), 3.34 (2H, dd, J=7 and 14 Hz), 3.44 (2H, dd, J=5 and 14 Hz), 3.54 (4H, t, J=6 Hz), 3.88 (2H, m), 4.02 (2H, m), 6.46 (2H, m), 7.25 (2H, m).

Anal Calcd for $C_{22}H_{42}N_8O_6 \cdot 2H_2SO_4 \cdot 3.5H_2O$: C 34.15, H 6.90, N 14.48, S 8.29.
Found: C 33.81, H 6.88, N 14.27, S 8.07.

By stepwise condensation of (*E,E*)-muconic acid with **24** and **22**, compound **31** was obtained as a white powder (74% from **24**): UV λ_{max} nm (ε) 272 (35,200); ^{1}H NMR (300 MHz) δ 1.70~2.00 (4H, m), 2.65~2.85 (6H, m), 3.18 (2H, t, J=6 Hz), 3.40 (4H, m), 3.56 (4H, m), 3.88 (2H, m), 4.03 (2H, m), 6.46 (2H, m), 7.25 (2H, m).

Anal Calcd for $C_{23}H_{43}N_9O_6 \cdot 2H_2SO_4 \cdot 3.5H_2O$: C 34.49, H 6.80, N 15.74, S 8.01.
Found: C 34.31, H 6.37, N 15.45, S 7.72.

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