GLIDOBACTINS A, B AND C, NEW ANTITUMOR ANTIBIOTICS

II. STRUCTURE ELUCIDATION

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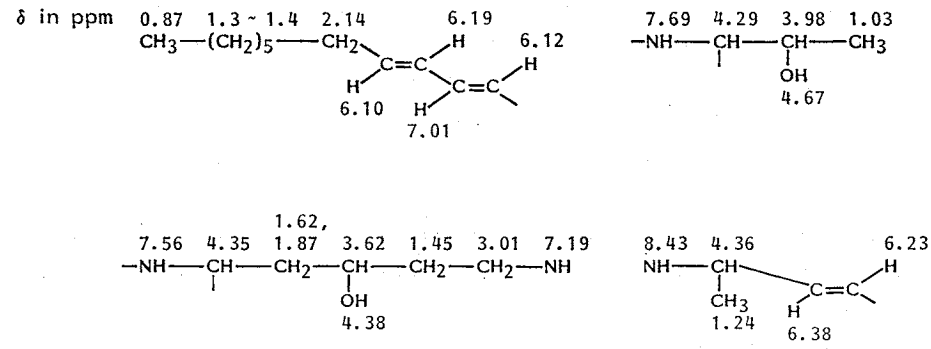
The structures of new antitumor antibiotics, glidobactins A (**Ia**), B (**Ib**) and C (**Ic**) were elucidated by a combination of chemical and enzymatic degradations and spectral analyses. They have in common a cyclized tripeptide nucleus composed of L-threonine, 4(*S*)-amino-2(*E*)-pentenoic acid and *erythro*-4-hydroxy-L-lysine, and differ from each other in the unsaturated fatty acid moiety attached to the peptide.

In the course of continuing search for novel antitumor antibiotics in the microbial metabolites, *Polyangium brachysporum* sp. nov. No. K481-B101 (ATCC 53080) collected in Greece was found to produce novel antibiotic complex with antifungal and antitumor activity[1~3]. The antibiotic complex named glidobactin was extracted and separated into three active components, glidobactins A (**Ia**), B (**Ib**) and C (**Ic**). In addition to their broad antifungal activity, all the components exhibited potent antitumor activity against P388 leukemia implanted in mice with the T/C values in the range of 200 to 250%. In this report, we present structural studies of **Ia**, **Ib** and **Ic**, which have shown unique acylated 12 membered cyclic peptide structures.

Spectral Characteristics

Glidobactins A (**Ia**), B (**Ib**) and C (**Ic**) were isolated from the fermentation broth of strain K481-B101 by butanol extraction followed by column chromatographies on silica gel and reversed phase silica gel. **Ia** and **Ic** were obtained as colorless needles from aqueous methanol and methanol, respectively, while **Ib** was isolated as crystalline powder. **Ia**: $C_{27}H_{44}N_4O_6$; *m/z* 520 (M^+); mp 259~261°C; $[\alpha]_D^{24}$ −111° (*c* 0.5, MeOH). **Ib**: $C_{29}H_{46}N_4O_6$; *m/z* 546 (M^+); mp 232~234°C; $[\alpha]_D^{24}$ −92° (*c* 0.5, MeOH). **Ic**: $C_{26}H_{48}N_4O_6$; *m/z* 548 (M^+); mp 273~275°C; $[\alpha]_D^{24}$ −104° (*c* 0.5, MeOH). The UV spectra of the three components exhibited the same absorption maximum at 261 nm in methanol suggesting the presence of an $\alpha,\beta,\gamma,\delta$-unsaturated carbonyl functionality. The IR absorption at around 1630 and 1540 cm^{-1} indicated amide group in their molecules, as described in the preceding paper[3].

The ^{13}C NMR spectrum of **Ia** displayed 26 carbon signals with one of them being double intensity (δ 28.0). The off-resonance experiments allowed to assign these signals as three methyl (δ 13.4, 18.2 and 19.4), nine methylene (δ 21.6, 28.0, 28.1, 30.8, 31.8, 39.3, 39.4 and 41.7), five methine (δ 44.7, 51.1, 57.9, 66.2 and 66.4), six doublet sp^2 (δ 122.8, 123.0, 128.6, 139.4, 141.5 and 142.6) and four singlet sp^2 assignable to amide (δ 165.5, 167.4, 169.0 and 170.8) carbons. This was ascertained from the ^1H NMR spectrum which showed the presence of three methyl (δ 0.87 (t), 1.03 (d) and 1.24 (d)), six vinyl (d, all of them being splitted by *trans* coupling), four amide (δ 7.19, 7.56, 7.69



and 8.43) and two hydroxyl protons (δ 4.38 and 4.67) in addition to 23 other methylene or methine protons in around δ 1.3~4.7.

The detail ^{1}H NMR analyses (Table 1) assisted by the ^{1}H-^{1}H 2D correlated spectroscopy (COSY) carried out for **Ia** provided the above 4 structural fragments in the molecule.

The ^{1}H and ^{13}C NMR spectra of **Ib** and **Ic** were very similar to those of **Ia** differing by the presence of an additional –CH=CH– and $-CH_2CH_2-$ unit, respectively, to **Ia**. The spectral differences reflected the differences in the molecular formulae among **Ia**, **Ib** and **Ic**.

The presence of two hydroxyl groups in **Ia** was revealed by the fact that acetylation in pyridine afforded di-*O*-acetyl derivative (**IIa**: *m/z* 604 (M^+)). When hydrogenated over palladium on charcoal, **Ia** yielded hexahydroglidobactin A (**III**), whose ^{1}H NMR indicated the absence of the six *trans*-double bond protons observed in the spectrum of **Ia** and increased protons at around δ 1.3~3.0.

Constituent Amino Acids and Fatty Acid

Ia was heated with 6 N HCl under reflux for 16 hours. After removal of the lipophilic product (**VIII**) by ethyl acetate extraction, the hydrolysate was concentrated to an oily residue which was chromatographed on Dowex 50W-X4 to obtain L-threonine (**IV**: $[\alpha]_D^{25}$ −12.7°) and three unusual amino acids (**V**, **VI** and **VII**). Amino acid **V** was identified as a mixture of diastereoisomers of 4-amino-3-hydroxy-*n*-valeric acid by its ^{1}H NMR, electron impact (EI)-MS (*m/z* 134 $(M+H)^+$) and a direct comparison with the authentic sample[4]. The molecular formula of **VI** was assigned to be $C_5H_9NO_2$ by elemental analysis and EI-MS (*m/z* 115 (M^+)). Its ^{1}H NMR showed the presence of one methyl δ 1.50 (d, J=6.2 Hz), one methine δ 4.0~4.2 (m) and two *trans* olefinic protons δ 6.05 (d, J=15.3 Hz) and 6.57 (dd, J=6.2 and 15.3 Hz). These spectral data combined with the specific rotation ($[\alpha]_D^{22.5}$ −6° in 5 N HCl) indicated **VI** to be 4-(*S*)-amino-2(*E*)-pentenoic acid[5]. Dehydration of compound **V** with concentrated sulfuric acid afforded compound **VI**[5]. Amino acid **VII** was determined to be 4-hydroxylysine based on its elemental analysis ($C_6H_{14}N_2O_3$), EI-MS (*m/z* 163 $(M+H)^+$) and ^{1}H NMR spectrum (δ 1.8~2.1, 4H, m, 3.18, 2H, t and 3.6~4.3, 2H, m) and also by formation of a γ-lactone compound ($\nu_{C=O}$ 1770 cm^{-1}) upon treatment with 6 N HCl. The amino acid was deoxygenated by hydroiodic acid and red phosphorus, and the product was treated with 2,4-dinitrofluorobenzene. The di-DNP derivative obtained was identical with authentic di-DNP-L-lysine by TLC and IR and CD spectra[6]. IZUMIYA *et al.* reported mutarotation of 4-hydroxy-lysines[7] and the shift observed for **VII** (Δ −37° from the lactone form to the free amino acid form) clearly indicated *erythro*-L-configuration.

The lipophilic acidic fraction (**VIII**) obtained in the above acid hydrolysis showed the UV maximum at 258 nm (ε 24,000) and a carboxyl absorption (1680 cm^{-1}) in the IR spectrum. Upon treatment with diazomethane, **VIII** yielded a monomethyl derivative **VIIIe** (*m/z* 210 (M^+), λ_{max}^{MeOH} nm 260). These data coupled with their ^1H and ^{13}C NMR spectra indicated 2,4-dodecadienoic acid for **VIII**. The observed coupling constants (J=16.0 Hz) made it obvious that both double bonds are *trans* configuration[8].

Ia has been given a molecular formula of $C_{27}H_{44}N_4O_6$ and exhibited six olefinic carbons and four amide carbons in the ^{13}C NMR. In addition, the ^1H NMR of **Ia** did not exhibit the structural fragment assignable to **V**. Thus, amino acid **V** was considered as an artifact produced by hydration of the natural amino acid **VI** during the acid hydrolysis.

IV: L-Threonine; $CH_3\underset{\displaystyle OH}{\underset{|}{C}}H\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH$

V: 4-Amino-3-hydroxy-*n*-valeric acid; $CH_3CH\underset{\displaystyle NH_2}{\underset{|}{C}}H\underset{\displaystyle OH}{\underset{|}{C}}HCH_2COOH$

VI: 4(*S*)-Amino-2(*E*)-pentenoic acid; $CH_3\underset{\displaystyle NH_2}{\underset{|}{C}}H{=}CHCOOH$

VII: *Erythro*-4-hydroxy-L-lysine; $NH_2CH_2CH_2\underset{\displaystyle OH}{\underset{|}{C}}HCH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH$

VIII: 2(*E*),4(*E*)-Dodecadienoic acid; $CH_3(CH_2)_6CH{=}CHCH{=}CHCOOH$

Mild Acid Hydrolysis

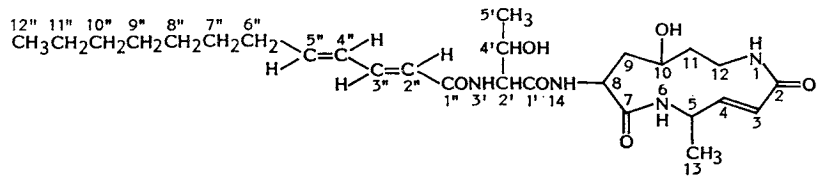
Upon hydrolysis with 6 N HCl at 35°C for 3 hours, **Ia** was degraded to a bioinactive compound (**IX**, isolated as sodium salt: $C_{27}H_{45}N_4O_7Na$, secondary ion (SI)-MS, (*m/z* 561 ($M+H)^+$) which produced the same amino acids **IV**, **V**, **VI** and **VII** and fatty acid (**VIII**) as those of **Ia** upon complete acid hydrolysis. When treated with acetic anhydride and pyridine, **IX** afforded an acetyl-γ-lactone compound ($\nu_{C=O}$ 1770 cm^{-1}). Reaction of **IX** with 2,4-dinitrofluorobenzene followed by acid hydrolysis of the resulting DNP-derivative yielded 4-DNP-amino-2-pentenoic acid (**DNP-VI**) together with free amino acids **IV** and **VII**. These results evidenced that the mild hydrolysis cleaved only one of the peptide bonds of **Ia** producing a straight chain peptide with amino acid **VI** at the *N*-terminal.

Enzymatic Hydrolysis

Glidobactin A was cleaved by papain to yield an acidic, lipophilic compound (**X**) and a basic water-soluble compound (**XI**). Compound **X** was shown to be 2,4-dodecadienoylthreonine (**VIII**~**IV**) by its IR ($\nu_{C=O}$ 1720 and 1650 cm^{-1}), chemical ionization (CI)-MS (*m/z* 298 ($M+H)^+$ and *m/z* 179 (M^+ −**IV**)) and ^1H NMR spectrum (Table 1). The structure was further substantiated by the fact that **X** yielded **IV** and **VIII** on hydrolysis in 6 N HCl.

When refluxed in 6 N HCl, compound **XI** afforded amino acid **V**, **VI** and **VII** as revealed by TLC. In the EI-MS of **XI**, the molecular ion was observed at *m/z* 241 indicating a cyclic peptide composed of **VI** and **VII**. In accordance with this assumption, the ^{13}C NMR measured at pD 8.0 (Table 2) exhibited two carbonyl, two vinyl, three methine, three methylene and one methyl carbon signals. When the spectrum was measured at pD 2.0, the protonation shifts were observed on a carbonyl (δ 175.3, Δ 6.1 ppm) and a methylene carbons (δ 44.6, Δ 4.8 ppm) indicating the twelve membered cyclic peptide

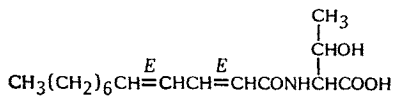
Table 1. The ^1H NMR of glidobactin A (**Ia**) and enzymatic hydrolysis products (**X** and **XI**) in DMSO-d_6.



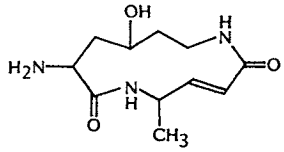
Position No.	Ia	X	XI
1	7.19 (dd, J=5.7, 7 Hz)		7.37 (dd, J=5.3, 6.9 Hz)
3	6.23 (d, J=15.9 Hz)		6.17 (dd, J=1, 15.9 Hz)
4	6.38 (dd, J=5.9, 15.9 Hz)		6.45 (m)
5	4.36 (m)		4.3 (m)
6	8.43 (d, J=7.5 Hz)		8.35 (d, J=7.2 Hz)
8	4.35 (m)		3.26 (m)
9	1.87 (ddd, J=6.6, 7, 13.5 Hz),		1.74 (m),
	1.62 (ddd, J=3.7, 3.8, 13.5 Hz)		1.53 (m)
10	3.62 (m)		3.48 (m)
10-OH	4.38 (br s)		4.5 (br s)
11	1.45 (m)		1.35 (m)
12	3.01 (m)		3.0 (m)
13	1.24 (d, J=7 Hz)		1.24 (m)
14	7.56 (d, J=7.6 Hz)		—
2′	4.29 (dd, J=4.1, 8.7 Hz)	4.15 (dd, J=3, 7.4 Hz)	
3′	7.69 (d, J=8.6 Hz)	7.55 (d, J=8.1 Hz)	
4′	3.98 (m)	3.92 (m)	
4′-OH	4.67 (br s)	—	
5′	1.03 (d, J=6.2 Hz)	0.92 (d, J=6.6 Hz)	
2″	6.12 (d, J=15.2 Hz)	6.16 (d, J=15 Hz)	
3″	7.01 (dd, J=10.4, 15.2 Hz)	6.96 (dd, J=10, 15 Hz)	
4″	6.19 (dd, J=10, 15.2 Hz)	6.17 (dd, J=10, 15 Hz)	
5″	6.1 (dt, J=15.2, 6.6 Hz)	6.04 (dt, J=15, 6.6 Hz)	
6″	2.14 (dt, J=6.6, 6.8 Hz)	2.11 (dt, J=5, 6.6 Hz)	
7″	1.4 (m)	1.37 (m)	
8″-11″	1.3 (m)	1.25 (m)	
12″	0.87 (t, J=7 Hz)	0.86 (t, J=7.3 Hz)	

Ia (400 MHz, at 70°C), **XI** (400 MHz, at 23°C) and **X** (270 MHz, at 23°C).

structure shown below. The ^1H NMR (Table 1) exhibited two amide NH protons at δ 7.37 (dd) and 8.35 (d) supporting the assigned structure. Further support was obtained by the mono-*N*-acetyl-**XI** (m/z 283 (M^+)) which exhibited two doublet amide (δ 7.98 and 8.57) and one triplet amide protons (δ 7.34).



Compound **X**

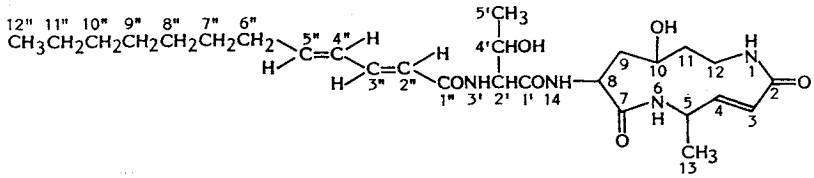


Compound **XI**

Structure of Glidobactin A (**Ia**)

As discussed in the preceding paper[3], glidobactin A (**Ia**) is negative to ninhydrin reaction and did not exhibit ester carbonyl absorption

Table 2. The ^{13}C NMR of glidobactin A (**Ia**) and enzymatic hydrolysis products (**X** and **XI**).

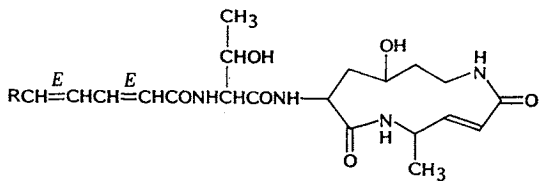


Position No.	Ia	X	XI	Position No.	Ia	X	XI
2	167.4 (s)		172.0 (s)	5′	19.4 (q)	20.0 (q)	
3	123.0 (d)		122.9 (d)	1″	165.5 (s)	165.6 (s)	
4	142.6 (d)		146.6 (d)	2″	122.8 (d)	123.5 (d)	
5	44.7 (d)		46.7 (d)	3″	139.4 (d)	139.5 (d)	
7	170.8 (s)		176.7 (s)	4″	128.6 (d)	128.7 (d)	
8	51.1 (d)		54.0 (d)	5″	141.5 (d)	141.5 (d)	
9	41.7 (t)		44.6 (t)	6″	31.8 (t)	32.3 (t)	
10	66.2 (d)		68.9 (d)	7″	28.0[a](t)	28.6[c](t)	
11	39.3[b](t)		39.0 (t)	8″	28.0[a](t)	28.6[c](t)	
12	39.4[b](t)		41.0 (t)	9″	28.1[a](t)	28.5[c](t)	
13	18.2 (q)		18.5 (q)	10″	30.8 (t)	31.3 (t)	
1′	169.0 (s)	173.3 (s)		11″	21.6 (t)	22.1 (t)	
2′	57.9 (d)	58.2 (d)		12″	13.4 (q)	13.8 (q)	
4′	66.4 (d)	66.8 (d)					

[a~c] Assignment may be interchanged.

Ia (100 MHz, DMSO-d_6, at 70°C), **X** (20 MHz, DMSO-d_6, at 23°C), **XI** (20 MHz, D_2O at pH 8.0, at 23°C).

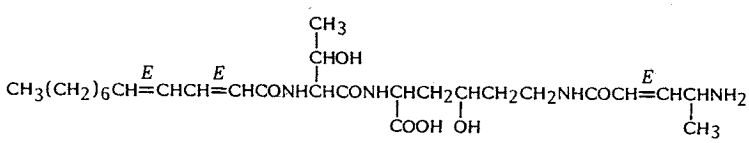
Fig. 1. Structures of glidobactins A (**Ia**), B (**Ib**) and C (**Ic**) and **IX**.



Glidobactin A (**Ia**) R = $CH_3(CH_2)_6$-

Glidobactin B (**Ib**) R = $CH_3(CH_2)_4CH$=^{Z}$CH(CH_2)_2$-

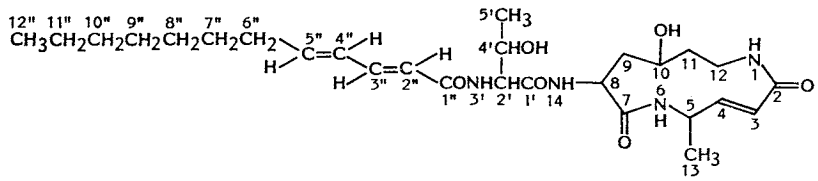
Glidobactin C (**Ic**) R = $CH_3(CH_2)_8$-



Peptide **IX**

in the IR spectrum. Thus, joining **X** and **XI** by a peptide bond naturally provides the structure of glidobactin A (Fig. 1). Compound **X** was coupled with 1-hydroxy-1,2,3-benzotriazole (HOBT) in the presence of *N,N′*-dicyclohexylcarbodiimide (DCC) to give HOBT ester which was reacted with **XI** in dimethylformamide. The product, after purification, was identical in all respects with glidobactin A (**Ia**).

Table 3. Long range ^{1}H-^{13}C correlation of glidobactin A (**Ia**)[a].



Chemical shift of C=O carbon (ppm)	C-H proton correlated (ppm)	N-H proton correlated (ppm)	Assignment of C=O carbon
165.5	7.01, 6.19	7.69	1″
167.4	6.23	—	2
169.0	4.29	7.56	1′
170.8	1.62	8.43	7

[a] In DMSO-d_6, 400 MHz for proton and 100 MHz for ^{13}C at 70°C.
—: No connectivity was observed.

The data obtained for **IX** are consistent with the linear peptide structure (Fig. 1) which was produced by cleavage of the peptide between **VI** and **VII**. As described latter part, **IX** was cyclized to yield the original antibiotic **Ia** by treatment with HOBT and DCC.

The ^{1}H and ^{13}C NMR spectra of **Ia** were analyzed as shown in Tables 1 and 2. The assignments were made on the basis of ^{1}H-^{1}H 2D and ^{1}H-^{13}C 2D COSY experiments. The ^{1}H-^{13}C COSY *via* small coupling optimized for J=8.0 Hz made the assignments of four amide carbonyl carbons possible as they gave correlation peaks to a neiboring C-H as well as an amide NH proton in a different spin system (Table 3). The assignments are consistent with the proposed structure of **Ia**.

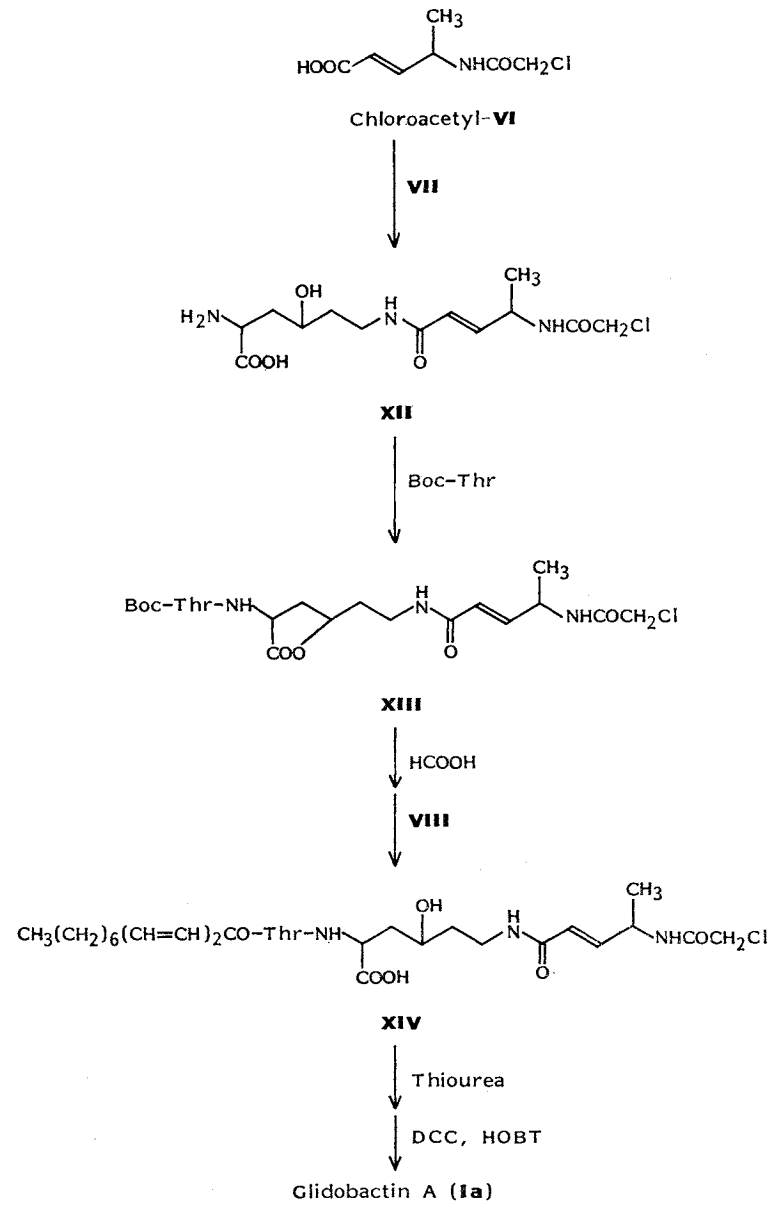
Synthesis of Glidobactin A (**Ia**)

Treatment of 4-amino-2(*E*)-pentenoic acid (**VI**) with chloroacetyl chloride in dimethylformamide gave *N*-chloroacetyl-**VI** which was coupled with *erythro*-4-hydroxy-L-lysine (**VII**) using the HOBT-methodology. The structure of the product (**XII**) was established to be $^{\varepsilon}N$-acyl derivative based on the ^{1}H NMR which exhibited a doublet (8.42 ppm) and a double-doublet amide (8.05 ppm) protons. Acylation of **XII** with the active ester of BOC-L-threonine afforded fully protected tripeptide lactone (**XIII**). Removal of the BOC group of **XIII** followed by acylation with 2(*E*),4(*E*)-dodecadienoic acid (**VIII**) using HOBT-active ester method gave **XIV** in 50% yield. The chloroacetyl group of **XIV** was removed by thiourea to yield the straight chain peptide which was identical with **IX** in their physicochemical and spectral properties. Compound **IX** was treated with HOBT and DCC in dimethylformamide and the products were purified by reversed phase silica gel chromatography. One of the products isolated was bioactive and identified as glidobactin A by a direct comparison with the natural antibiotic (Scheme 1).

Structures of Glidobactins B (**Ib**) and C (**Ic**)

On heating with 6 N HCl, **Ib** and **Ic** afforded the same amino acid complex as that obtained from **Ia**. The fatty acids of the antibiotics (**XV** from **Ib** and **XVI** from **Ic**) were extracted from the hydrolysates and converted to methyl esters (**XVe**: *m/z* 236 (M^+) and **XVIe**: *m/z* 238 (M^+)). The UV (λ_{max} 260 nm) and EI-MS of **XVe** and **XVIe** were similar to those of **VIIIe** (methyl 2,4-dodecadienoate), and the differences observed in their spectral data indicated that **XVe** and **XVIe** had an additional

Scheme 1. Synthesis of glidobactin A.



CH=CH and CH_2CH_2 moiety to **VIIIe**, respectively, reflecting the differences of molecular formulae between glidobactins B (**Ib**) and C (**Ic**) and glidobactin A (**Ia**). Ozonolysis of **Ib** followed by reductive degradation of the ozonide yielded *n*-hexanal which was identified as 2,4-dinitrophenylhydrazone (*m/z* 280 (M^+)). The extensive decoupling studies enabled the assignment of *Z* geometry for the additional double bond of **Ib** (J=11.0 Hz). Thus, **XVe** and **XVIe** are methyl 2(*E*),4(*E*),8(*Z*)-tetradecatrienoate and methyl 2(*E*),4(*E*)-tetradecadienoate, respectively and **XV** and **XVI** are the corresponding free acids. Hence, the structures of **Ib** and **Ic** are represented as shown in Fig. 1.

Discussion

The structures of glidobactins A, B and C were established to be quite unique acylpeptides having an unusual twelve membered cyclic ring (Fig. 1). The twelve membered ring peptide is composed of two amino acids, 4(*S*)-amino-2(*E*)-pentenoic acid and *erythro*-4-hydroxy-L-lysine, both of which have never been found in the natural source. The three components differ only in the length and/or unsaturation of the fatty acid attached to their common peptide core. As described in the preceding paper[3], glidobactins A, B and C exhibit strong antifungal and antitumor activities with the potency being slightly different among the three components. Both hexahydroglidobactin A and the linear peptide compound **IX** are completely bio-inactive indicating the importance of the double bonds and the cyclic structure of the antibiotics. On the other hand, diacetylglidobactin A retains antifungal and antitumor activity. The structure-activity relationship observed above suggested that the chemical or biological modifications of this rather simple molecule may yield derivatives with improved activity. Manuscripts detailing the modification studies are in preparation and will be submitted to this journal.

Experimental

TLC was carried out on precoated silica gel plates (Kieselgel $60F_{254}$, Merck, layer thickness 0.25 mm). The IR spectra were determined on a Jasco IRA-1 spectrometer and the UV spectra on a Shimadzu UV-200. The ^1H NMR and ^{13}C NMR spectra were recorded on a Jeol C-60HL, a Varian Model FT 80A, a Jeol FX 270, a Bruker WM-360 or a Jeol GX400 using tetramethylsilane as a standard. The ordinary mass spectra were obtained with a Hitachi RMU-6MG mass spectrometer modified with an in-beam/electron impact system, the high-resolution (HR) spectra on a Hitachi-M80 mass spectrometer, the SI-MS on a Hitachi M-80B (Xenon, 8 KeV) and CI-MS on a Jeol JMS-DX 300 with NH_3 as reagent gas. Optical rotations were determined with a Jasco model DIP 140.

Acetylation of Glidobactins A (Ia), B (Ib) and C (Ic)

A mixture of glidobactin A (**Ia**, 100 mg), acetic anhydride (4 ml) and pyridine (4 ml) was stirred overnight at room temperature. The mixture was poured into ice water (15 ml) and the solution was extracted with EtOAc (3×15 ml). The combined extracts were washed with water (10 ml), dried over sodium sulfate and evaporated to give crude solid (97 mg). This was chromatographed on a silica gel column (Wakogel C-200, 60 ml), developing with $CHCl_3$ and then with MeOH - $CHCl_3$ (5:95). The eluate was monitored by TLC ($CHCl_3$ - MeOH, 9:1), and the fractions containing the major product (Rf 0.29) were combined and evaporated to give 48 mg of **IIa** as colorless powder. **IIa**: MP 197~198°C; $[\alpha]_D^{24}$ −117° (*c* 0.5, MeOH); EI-MS *m/z* 604 (M^+); UV λ_{max}^{MeOH} nm (ε) 261 (42,000); IR ν_{max}^{KBr} cm^{-1} 3300, 1745, 1650, 1540.

Anal Calcd for $C_{31}H_{48}N_4O_8 \cdot H_2O$: C 59.78, H 8.09, N 9.00.
Found: C 59.78, H 7.93, N 8.89.

Similarly, acetylation of **Ib** (20 mg) and **Ic** (35 mg) yielded **IIb** (11 mg) and **IIc** (28 mg), respectively. **IIb**: MP 176~178°C; $[\alpha]_D^{23}$ −99° (*c* 0.5, MeOH); EI-MS *m/z* 630 (M^+); UV λ_{max}^{MeOH} nm (ε) 261 (48,000); IR ν_{max}^{KBr} cm^{-1} 3300, 1740, 1650, 1540.

Anal Calcd for $C_{33}H_{50}N_4O_8 \cdot H_2O$: C 61.09, H 8.08, N 8.64.
Found: C 60.64, H 8.18, N 8.43.

IIc: MP 199~201°C; $[\alpha]_D^{24}$ −107° (*c* 0.5, MeOH); EI-MS *m/z* 632 (M^+); UV λ_{max}^{MeOH} nm (ε) 261 (47,000), IR ν_{max}^{KBr} cm^{-1} 3300, 1740, 1645, 1540.

Anal Calcd for $C_{33}H_{52}N_4O_8 \cdot \frac{1}{2}H_2O$: C 61.76, H 8.32, N 8.73.
Found: C 61.45, H 8.28, N 8.48.

Hydrogenation of Ia

Ia (200 mg) in BuOH (20 ml), EtOH (10 ml) and water (10 ml) mixture was hydrogenated at 3 kg/cm^2 over 10% palladium on charcoal in a Parr equipment for 6 hours. After the catalyst was removed by filtration, the filtrate was evaporated *in vacuo* to a residue which was triturated with hot 50% aqueous EtOH (10 ml). The insolubles were collected by filtration and dried *in vacuo* to give

177 mg of white powder of **III**: MP 271°C; HREI-MS *m/z* 526.3757 (calcd for $C_{27}H_{50}N_4O_6$: 526.3727); UV end absorption.

Acid Hydrolysis of **Ia**

A suspension of **Ia** (200 mg) in 6 N HCl (9 ml) was refluxed for 16 hours and the resulting solution was concentrated *in vacuo* to dryness. The residue was vigorously shaken with a mixture of water (40 ml) and EtOAc (30 ml). Evaporation of the EtOAc layer yielded brown oil (74 mg) containing **VIII**. The aqueous layer was concentrated *in vacuo* to an oil (210 mg) which was chromatographed on a column of Dowex 50W-X4 (70 ml). The column was developed with 0.1 M pyridine - formic acid (pH 3.1), 0.2 M pyridine - formic acid (pH 3.1) and 0.2 M pyridine - acetic acid (pH 4.4), successively, and the eluate was monitored by ninhydrin test and TLC (MeOH - 10% $AcONH_4$ - 10% NH_4OH, 10 : 9 : 1). **IV** was eluted by 0.1 M pyridine - formic acid (pH 3.1), **V** and **VI** were by 0.2 M pyridine - formic acid (pH 3.1) in that order and **VII** was by 0.2 M pyridine - AcOH (pH 4.4). Evaporation of the appropriate fractions gave nearly pure solids of **IV**, **V**, **VI** and **VII**. **VI** was desalted with Sephadex LH-20 chromatography eluting with 50% aqueous MeOH and **IV**, **V** and **VII** were again purified by Dowex 50W-X4 and then desalted. **IV**: Colorless rods from aqueous EtOH (38 mg); mp 244°C; $[\alpha]_D^{25.5}$ −12.7° (*c* 0.7, 5 N HCl); ^1H NMR (60 MHz, D_2O) δ 1.46 (3H, d, J=7.0 Hz), 4.07 (1H, J=5.4 Hz), 4.4 (1H, m). **V**: White amorphous powder (20 mg); $[\alpha]_D^{26}$ −3.3° (*c* 0.3, 2 N HCl); EI-MS *m/z* 134 $(M+H)^+$; IR ν_{max}^{KBr} cm^{-1} 3400, 1720, 1610; ^1H NMR (60 MHz, D_2O) δ 1.34 (*ca.* 1.5H, d, J=7.5 Hz), 1.38 (*ca.* 1.5H, d, J=7.5 Hz), 2.4~2.6 (2H, m), 3.1~3.7 (1H, m), 3.8~4.4 (1H, m). **VI**: Colorless needles from aqueous EtOH (8 mg); mp 223~224°C (literature[5] 221.5~223.5°C); $[\alpha]_D^{24.5}$ −6° (*c* 1.0, 5 N HCl) (literature $[\alpha]_D^{22.5}$ −5.8°); EI-MS *m/z* 115 (M^+); IR ν_{max}^{KBr} cm^{-1} 1630, 1580; ^1H NMR (60 MHz, D_2O) δ 1.50 (3H, d, J=6.2 Hz), 4.0~4.2 (1H, m), 6.05 (1H, d, J=15.3 Hz), 6.57 (1H, dd, J=6.2 and 15.3 Hz).

Anal Calcd for $C_6H_9NO_2$: C 52.16, H 7.88, N 12.17.
Found: C 51.99, H 8.12, N 12.06.

VII: Colorless needles from aqueous EtOH containing conc HCl (16 mg); mp 223~225°C (literature[7] 206~207°C); $[\alpha]_D^{26}$ +23° (*c* 0.79, 6 N HCl) (literature $[\alpha]_D^{20}$ +23.6°); EI-MS *m/z* 163 $(M+H)^+$; IR ν_{max}^{KBr} cm^{-1} 3300, 1640, 1590, 1560; ^1H NMR (60 MHz, D_2O) δ 1.8~2.1 (4H, m), 3.18 (2H, t, J= 8.2 Hz), 3.6~4.3 (2H, m).

Anal Calcd for $C_6H_{14}N_2O_3 \cdot HCl$: C 36.28, H 7.61, N 14.10, Cl 17.85.
Found: C 36.13, H 7.62, N 13.74, Cl 18.07.

The oily residue (74 mg) obtained from the EtOAc extract was chromatographed on a silica gel column (100 ml) developed with $CHCl_3$ - MeOH (99 : 1). The UV-absorbing fractions were collected and evaporated to afford 29 mg of oil, which was further purified by preparative TLC developed by $CHCl_3$ - MeOH (9 : 1). The appropriate band (Rf 0.49) was collected and eluted with the developing solvent mixture. Evaporation of the eluate gave 16 mg of oil which was chromatographed on Sephadex LH-20 and crystallized to give colorless needles of **VIII** (10 mg): MP 48~49°C (literature[8] 49~51°C); UV λ_{max}^{MeOH} nm (ε) 258 (24,000); IR ν_{max}^{KBr} cm^{-1} 1680, 1630, 1605; ^1H NMR (80 MHz, DMSO-d_6) δ 0.89 (3H, t, J=6.2 Hz), 1.3 (10H, m), 2.23 (2H, m), 5.73 (1H, d, J=15.0 Hz), 6.18 (2H, m), 7.14 (1H, m); ^{13}C NMR (20 MHz, DMSO-d_6) δ 14.0 (q), 22.3 (t), 28.5 (t), 28.7 (t), 28.8 (t), 31.5 (t), 32.5 (t), 120.4 (d), 128.5 (d), 143.8 (d), 144.5 (d), 167.8 (s). **VIII** (7 mg) in ether (5 ml) was treated with excess amount of diazomethane at room temperature. After 3 hours, the reaction mixture was washed with aqueous $NaHCO_3$ (1 ml), water (1 ml), 10% HCl (1 ml) and water (1 ml), successively. Evaporation of the dried ether extract gave 5 mg of oil (**VIIIe**); EI-MS *m/z* 210 (M^+), 179 $(M^+ - OCH_3)$; UV λ_{max}^{MeOH} nm (ε) 260 (22,000).

Acid Hydrolysis of **Ib** and **Ic**

Ib and **Ic** (20 mg each) were hydrolyzed with 6 N HCl (0.5 ml) under a similar condition as used in the hydrolysis of **Ia**. The reaction mixture was separated into a lipophilic acidic substance and water-soluble amino acid complex. The acidic product was treated with excess of diazomethane and the residual ester was purified by preparative TLC ($CHCl_3$). **XVe** (1.0 mg from **Ib**): EI-MS *m/z* 236 (M^+); UV λ_{max}^{MeOH} nm (ε) 260 (20,000). **XVIe** (1.5 mg from **Ic**): EI-MS *m/z* 238 (M^+); UV λ_{max}^{MeOH} nm

(ε) 260 (19,000). The amino acid complex from **Ib** and **Ic** was identical with that from **Ia** by TLC (**IV**, **V**, **VI** and **VII**).

Mild Hydrolysis of **Ia**

A suspension of **Ia** (432 mg) in 6 N HCl (43 ml) was stirred at 35°C for 3 hours. The solution was adjusted to pH 7 and extracted with BuOH (2×40 ml). The combined extracts were evaporated to dryness (pale yellow solid, 500 mg) which was chromatographed on a column of Sephadex LH-20 (800 ml) eluting with 50% aqueous MeOH. The eluate was monitored by TLC (BuOH - AcOH - H_2O, 3 : 1 : 1; Rf 0.38) and the fractions containing the major product were combined and evaporated to give white amorphous powder (85 mg). This solid (50 mg) was dissolved in water (3 ml, pH 9) and chromatographed on a reversed phase silica gel (Merck Silica gel 60 silanised, 50 ml) column eluting with aqueous MeOH (50 and 70%). The ninhydrin-positive fractions were combined, evaporated *in vacuo* and lyophilized to afford 45 mg of **IX**·Na salt: MP >155°C (dec); $[\alpha]_D^{24}$ −16° (*c* 0.5, H_2O); SI-MS *m/z* 561 $(M+H)^+$; UV λ_{max}^{MeOH} nm (ε) 260 (32,000); IR ν_{max}^{KBr} cm^{-1} 3300, 1630, 1610, 1530.

Anal Calcd for $C_{27}H_{45}N_4O_7Na \cdot H_2O$: C 56.04, H 8.19, N 9.68.
Found: C 55.75, H 8.32, N 9.44.

Determination of the terminal amino acid was carried out by the following proceure: A mixture of **IX** (2.5 mg), 2,4-dinitrofluorobenzene (2.5 mg) and $NaHCO_3$ (5 mg) in 50% aqueous EtOH (0.5 mg) was stirred for 1 hour and then concentrated to dryness. The residue was partitioned between water (1 ml) and ether (1 ml). The aqueous layer was acidified to pH 2 and extracted with $CHCl_3$ (2×2 ml). After evaporation of the extracts, the residue was purified by preparative TLC ($CHCl_3$ - MeOH, 9 : 1). The major yellow band (Rf 0.5) was extracted with $CHCl_3$ and the extract was concentrated to give 2 mg of DNP-**IX**. The DNP-**IX** was hydrolyzed with 6 N HCl (1 ml) at 100°C overnight. EtOAc extraction of the hydrolysate yielded yellow solid of DNP-**VI** (1 mg) which was identified with an authentic sample prepared by dinitrophenylation of **VI**. DNP-**VI**: ^1H NMR (360 MHz, $CDCl_3$) δ 1.63 (3H, d, *J*=5.5 Hz), 4.48 (1H, m), 5.98 (1H, dd, *J*=1.5 and 16.2 Hz), 6.80 (1H, d, *J*=6.5 Hz), 7.04 (1H, dd, *J*=16.2 and 5.5 Hz), 8.28 (1H, dd, *J*=2.1 and 6.5 Hz), 8.57 (1H, br d, *J*=6.5 Hz), 9.19 (1H, d, *J*=2.1 Hz).

Hydrolysis of **Ia** with Papain

A mixture of **Ia** (4 g) and papain (Sigma P-3375, 50 g) in 20 liters of 10% aqueous MeOH was stirred at 28°C for 22 hours. The mixture was acidified to pH 3.3 by AcOH and extracted with EtOAc (10 liters). Evaporation of the extract afforded an oil (6.3 g) which was chromatographed on silica gel (250 ml) with a mixture of CH_2Cl_2 and MeOH (9 : 1) to give a semi-pure oily compound **X** (3.3 g). Further chromatographic purification of the oil on Sephadex LH-20 followed by crystallization gave colorless needles of **X**, 1.15 g (yield 50%): MP 90~91°C; $[\alpha]_D^{27}$ +17.4° (*c* 0.5, MeOH); EI-MS *m/z* 279 $(M^+ - H_2O)$; UV λ_{max}^{MeOH} nm (ε) 260 (28,000); IR ν_{max}^{KBr} cm^{-1} 3300, 1720, 1690, 1650, 1630; ^1H NMR (Table 1); ^{13}C NMR (Table 2).

Anal Calcd for $C_{16}H_{27}NO_4 \cdot H_2O$: C 60.94, H 9.27, N 4.44.
Found: C 61.34, H 9.03, N 4.20.

The acidic aqueous solution of above enzymatic degradation was concentrated to dryness. The residue (36 g) was dissolved in 50 ml of water, adjusted to pH 9.0 and applied on a column of reversed phase silica gel (1.6 liters) which was developed with water. The fractions containing compound **XI** were detected by TLC, pooled and concentrated *in vacuo*. The residue was chromatographed on Sephadex LH-20 (250 ml) with 50% aqueous MeOH and then on reversed phase silica gel (250 ml) with acidic water (pH 3.0) to afford pure **XI** hydrochloride, 747 mg (yield 35%). MP 190°C (dec); $[\alpha]_D^{26}$ −113° (*c* 0.5, H_2O); EI-MS *m/z* 241 (M^+); IR ν_{max}^{KBr} cm^{-1} 3400, 1660, 1620, 1530; ^1H NMR (Table 1); ^{13}C NMR (Table 2).

Anal Calcd for $C_{11}H_{19}N_3O_3 \cdot HCl \cdot H_2O$: C 44.67, H 7.50, N 14.21, Cl 11.99.
Found: C 45.04, H 7.82, N 13.81, Cl 12.55.

Acetylation of **XI** (10 mg) by the conventional way (Ac_2O - MeOH) gave mono-*N*-acetyl-**XI** (7 mg): EI-MS *m/z* 283 (M^+); IR ν_{max}^{KBr} cm^{-1} 3400, 1650, 1630, 1540; ^1H NMR (80 MHz, DMSO-d_3) δ 1.23 (3H, d, *J*=7.5 Hz), 1.35~1.6 (4H, m), 1.81 (3H, s), 3.00 (2H, m), 3.52 (1H, m), 4.31 (2H, m), 4.66

(1H, m), 6.11 (1H, d, J=16.3 Hz), 6.46 (1H, dd, J=6.2 and 16.3 Hz), 7.34 (1H, t), 7.98 (1H, d, J=8.0 Hz), 8.57 (1H, d, J=7.2 Hz).

Chemical Synthesis of **Ia**

(1) Preparation by the Coupling **X** and **XI**

A mixture of **X** (15 mg), DCC (10 mg) and HOBT (8 mg) in 2 ml of DMF was stirred for 2 hours at room temperature. **XI** (10 mg) was then added to the mixture and stirring continued overnight. The solution was concentrated to a residue which was chromatographed on reversed phase silica gel (40 ml) with 80% MeOH elution. The bioactive fractions were evaporated and the residue was purified by preparative HPLC (column: SSC-ODS-842, mobile phase: 90% aqueous MeOH). Evaporation of the appropriate fractions gave **Ia** (7.4 mg, yield 28%) which was identical in all respects with the natural antibiotic. TLC (silanized, EtOH - H_2O, 55: 45) Rf 0.45. HPLC (Lichrosorb RP-18, EtOH - H_2O, 65: 35) retention time (Rt): 6.43 minutes.

(2) Total Synthesis

4(*S*)-Chloroacetylamino-2(*E*)-pentenoic Acid (Chloroacetyl-**VI**)

To a suspension of 4(*S*)-amino-2(*E*)-pentenoic acid (**VI**, 115 mg, 1 mM) in DMF (3 ml) was added *N,O*-bis(trimethylsilyl)acetamide (406 mg, 2 mM) and the mixture was stirred for 0.5 hour at room temperature. Chloroacetyl chloride (113 mg, 1 mM) was added to the solution at 5°C and the mixture was stirred overnight. After dilution with H_2O (20 ml), the mixture was extracted three times with EtOAc (20 ml each). The combined extracts were concentrated to an oil which was triturated with a mixture of EtOAc (10 ml) and *n*-hexane (20 ml). The resulting solid was crystallized from the same solvent system to give 120 mg (63%) of chloroacetyl-**VI** as colorless needles: MP 126~127°C; IR ν_{max}^{KBr} cm^{-1} 3250, 1710, 1650, 1540.

Anal Calcd for $C_7H_{10}NO_3Cl$: C 43.88, H 5.26, N 7.31, Cl 18.50.
Found: C 43.90, H 5.08, N 7.29, Cl 18.41.

$^{\varepsilon}$*N*-[4(*S*)-Chloroacetylamino-2(*E*)-pentenoyl]-*erythro*-4-hydroxy-L-lysine (**XII**)

A mixture of chloroacetyl-**VI** (110 mg, 0.57 mM), DCC (127 mg, 0.62 mM) and HOBT (95 mg, 0.62 mM) in THF (3 ml) was stirred overnight at room temperature. After removing the precipitate by filtration, the filtrate was concentrated and the residue was triturated with *n*-hexane (10 ml) to yield white precipitate of active ester. The solid was added to 50% aqueous DMF (3 ml) solution of *erythro*-4-hydroxy-L-lysine hydrochloride (**VII**, 113 mg, 0.57 mM) and Et_3N (0.22 ml, 1.7 mM) at room temperature and the mixture was stirred overnight. The mixture was concentrated and residue was chromatographed on a column of Sephadex LH-20 (140 ml). Upon developing with 50% aqueous MeOH, the eluate was examined by TLC (BuOH - AcOH - H_2O, 3 : 1 : 1, ninhydrin detection). The fractions containing the major product were concentrated to a residue which was further purified by a reversed phase silica gel column (20 ml) with 10% aqueous MeOH elution. Evaporation of appropriate fractions afforded 76 mg (39%) of **XII** as white amorphous solid: MP >189°C (dec); EI-MS m/z 335 (M^+): IR ν_{max}^{KBr} cm^{-1} 3275, 1660, 1625, 1550; ^1H NMR (360 MHz, DMSO-d_6) δ 1.19 (3H, d, J=6.3 Hz), 1.5 (3H, m), 1.85 (1H, m), 3.17 (2H, m), 3.72 (1H, m), 4.05 (2H, s), 4.46 (1H, m), 5.92 (1H, dd, J=16.1 and 1.5 Hz), 6.52 (1H, dd, J=16.1 and 6.3 Hz), 7.60 (1H, br s), 8.05 (1H, dd, J=3.1 and 1.5 Hz), 8.42 (1H, d, J=7.2 Hz).

$^{\varepsilon}$*N*-[4(*S*)-Chloroacetylamino-2(*E*)-pentenoyl]-$^{\alpha}$*N*-[*N*-(*tert*-butoxycarbonyl)-L-threonyl]-*erythro*-4-hydroxy-L-lysine-γ-lactone (**XIII**)

A mixture of *N*-(*tert*-butoxycarbonyl)-L-threonine (46 mg, 0.21 mM), DCC (44 mg, 0.21 mM) and HOBT (32 mg, 0.21 mM) in THF (3 ml) was stirred for 1 hour at room temperature. After removing precipitate by filtration, the filtrate was evaporated to an oil which was dissolved in DMF (0.1 ml) and added to a mixture of **XII** (70 mg, 0.21 mM), Et_3N (55 μl, 0.42 mM) in 50% aqueous DMF (0.5 ml) with vigorous stirring. The resulting solution was diluted with H_2O (5 ml) and washed with ether (5 ml). The aqueous layer was acidified to pH 2, washed with EtOAc (2×5 ml) and then extracted with BuOH (3×5 ml). The extracts were concentrated and the residue was chromatographed

on a reversed phase silica gel (20 ml) column with a stepwise elution of 10, 30 and 50% aqueous MeOH. The eluate containing **XIII** was combined, evaporated and lyophilized to give colorless amorphous powder of **XIII** (93 mg, 83%): MP >92° (dec); SI-MS *m/z* 541 $(M+Na)^+$; IR ν_{max}^{KBr} cm^{-1} 3300, 1780, 1705, 1670, 1540; ^1H NMR (80 MHz, DMSO-d_6) δ 1.14 (3H, d, J=7.8 Hz), 1.17 (3H, d, J=5.5 Hz), 1.4 (9H, s), 1.75 (2H, m), 3.82 (2H, m), 4.03 (2H, s), 4.5 (4H, m, changed to 2H, m, by D_2O addition), 5.87 (2H, d, J=16.3 Hz), 6.5 (1H, dd, J=5.5 and 16.3 Hz), 8.05 (1H, br s, lost with D_2O), 8.36 (1H, d, J=6.0 Hz, lost with D_2O).

$^{\varepsilon}$*N*-[4(*S*)-Chloroacetylamino-2(*E*)-pentenoyl]-$^{\alpha}$*N*-[*N*-2(*E*),4(*E*)-dodecadienoyl-L-threonyl]-*erythro*-4-hydroxy-L-lysine (**XIV**)

A mixture of **XIII** (87 mg, 0.16 mM) and formic acid (1.5 ml) was stirred at room temperature for 1 hour. The solution was concentrated, added with H_2O (1.5 ml) and Et_3N (25 μl), and then filtered. The filtrate was chromatographed on a Sephadex LH-20 column (140 ml) with 50% aqueous MeOH elution. The ninhydrin positive fractions were evaporated *in vacuo* to yield highly hygroscopic solid of BOC-free **XIII** (64 mg). 2(*E*),4(*E*)-Dodecadienoic acid (**VIII**, 20 mg, 0.1 mM), was converted to the active ester by reaction with DCC (21 mg, 0.1 mM) and HOBT (15 mg, 0.1 mM) in THF (1 ml). The active ester in DMF (1 ml) was added to a solution of the above deblocked **XIII** (58 mg) in H_2O (1.5 ml) at room temperature. The mixture was stirred for 3 hours and then diluted with H_2O (3 ml), adjusted to pH 2, washed with ether and extracted with BuOH (2×4 ml). The combined extracts were evaporated to a residue which was applied on a column of Sephadex LH-20 (140 ml). Upon elution with 50% aqueous MeOH, the appropriate fractions were pooled, evaporated and lyophilized to give colorless amorphous powder of **XIV** (45 mg, 50% yield from **XIII**): MP >96°C (dec); SI-MS *m/z* 637 $(M+Na)^+$, 615 $(M+H)^+$; UV λ_{max}^{MeOH} nm (ε) 260 (34,000); IR ν_{max}^{KBr} cm^{-1} 3300, 1710, 1660, 1620, 1540; ^1H NMR (80 MHz, DMSO-d_6) δ 0.86 (3H, t, J=6.2 Hz), 1.05 (3H, d, J=7.8 Hz), 1.2~1.8 (17H, m), 2.12 (2H, m), 3.16 (2H, m), 3.9 (1H, m), 4.05 (2H, s), 4.2 (3H, m), 5.86 (1H, d, J=15.8 Hz), 6.10 (3H, m), 6.52 (1H, dd, J=5.1 and 15.8 Hz), 6.98 (1H, dd, J=8.1 and 15.8 Hz), 7.91 (3H, m, lost with D_2O), 8.36 (1H, d, J=8.1 Hz, lost with D_2O).

$^{\varepsilon}$*N*-[4(*S*)-Amino-2(*E*)-pentenoyl]-$^{\alpha}$*N*-[*N*-2(*E*),4(*E*)-dodecadienoyl-L-threonyl]-*erythro*-4-hydroxy-L-lysine (**IX**)

A mixture of **XIV** (39 mg, 0.06 mM), $NaHCO_3$ (11 mg, 0.13 mM) and thiourea (46 mg, 0.6 mM) in 50% aqueous THF (2 ml) was stirred overnight at room temperature. The mixture was diluted with H_2O (5 ml), adjusted to pH 7 and extracted with BuOH (2×5 ml). The extracts were evaporated to a residue which was chromatographed on reversed phase silica gel (40 ml) with 50 and 70% aqueous MeOH elution. Upon monitoring by ninhydrin test, the ninhydrin-positive fractions were pooled concentrated to give white solid of **IX** (17 mg, 50%). MP 157°C (dec). The physico-chemical properties of the synthesized **IX** was identical with those of **IX** obtained by mild hydrolysis of **Ia**.

Cyclization of **IX** to **Ia**

IX (13 mg, 0.024 mM) was stirred with HOBT (3.7 mg, 0.024 mM) and DCC (5 mg, 0.024 mM) in DMF (26 ml) for 18 hours at room temperature. The mixture was evaporated to a residue which was dissolved in H_2O (5 ml) and extracted with two 5 ml-portions of BuOH. The extracts were, after concentration, chromatographed on a reversed phase silica gel column (20 ml) with MeOH - H_2O mixture (3:2 to 7:3). The eluate was examined by HPLC (Lichrosorb RP-18, MeOH - H_2O, 8:2, Rt: 9.8 minutes). The appropriate fractions were concentrated and the residue was crystallized from aqueous MeOH to give 0.3 mg (2.3%) of **Ia**. The identity of the synthetic **Ia** with the natural glidobactin A was confirmed by a direct comparison of their biological properties, spectral data and HPLC.

Ozonolysis of Glidobactin B (**Ib**)

To a solution of **Ib** (40 mg) in MeOH (10 ml) was introduced ozone at −70°C until the solution became blue. The solution was warmed up to room temperature, concentrated and diluted with water (4 ml). The aqueous solution was refluxed for 20 minutes with zinc dust (200 mg) and then steam-distilled. Treatment of the distillate with 2,4-dinitrophenylhydrazine (106 mg) and conc H_2SO_4

(0.54 ml) yielded yellow precipitate (9.5 mg), which was purified by preparative TLC (benzene - EtOAc, 20 : 1). The yellow major band (Rf 0.73) was collected and extracted with benzene - EtOAc mixture (1 : 1) to give pale yellow amorphous powder (1 mg) which was identified as *n*-hexanal 2,4-dinitrophenylhydrazone: EI-MS *m/z* 280 (M^+); ^1H NMR (80 MHz, $CDCl_3$) δ 0.94 (3H, t-like), 1.2~1.7 (6H, m), 2.36 (2H, dt, J=5.2 and 7.5 Hz), 7.49 (1H, t, J=7.5 Hz), 7.89 (1H, d, J=10.0 Hz), 8.27 (1H, dd, J= 1.5 and 10.0 Hz), 9.06 (1H, d, J=1.5 Hz).

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