# HELVECARDINS A AND B, NOVEL GLYCOPEPTIDE ANTIBIOTICS

## II. STRUCTURAL ELUCIDATION

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Helvecardins A and B, which are produced by *Pseudonocardia compacta* subsp. *helvetica*, are new members of glycopeptide antibiotics. They contain the same pseudoaglycone as β-avoparcin and the same compositions of neutral sugar, amino sugar, and amino acid except that 2′-*O*-methylrhamnose was detected in helvecardins instead of rhamnose in β-avoparcin, and mannose was not detected in helvecardin B. From these results and $^1$H NMR and mass spectral analyses, helvecardins A and B were determined β-avoparcin 2′-*O*-methylated on rhamnose and demannosylhelvecardin A, respectively.

In a previous paper[1] we reported the taxonomy of the producing organism, and the production, isolation, and physico-chemical properties of new glycopeptide antibiotics, helvecardins A and B, which were isolated from a culture filtrate of *Pseudonocardia compacta* subsp. *helvetica* SANK 65185. In this paper we report the structural elucidation of these antibiotics based on spectral analyses and chemical degradation studies. Their biological properties will be reported in a subsequent paper[2].

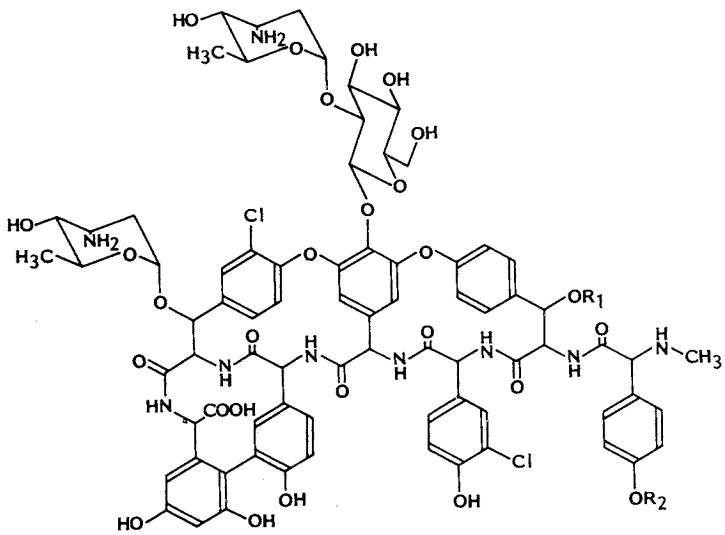
## Results and Discussion

### Acid Hydrolysis of Helvecardins A and B

To analyze the components of helvecardins A (**1**) and B (**2**), they were reductively hydrolyzed with hydroiodic acid in the presence of red phosphorus[3], and their products were compared with those of β-avoparcin (**3**)[4].

Monodechlorodideoxyvancomycinic acid, actinoidic acid, 3-chloro-4-hydroxyphenylglycine, and *N*-methyl-*p*-hydroxyphenylglycine were commonly detected in **1**, **2**, and **3**, as mentioned in the Experimental section. It is well-known that the allylic positions of the vancomycinic acid moiety, which is one of the characteristic components of glycopeptide antibiotics, are easily deoxygenated under the reductive condition[4,5].

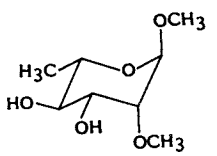
By methanolysis of **1**, **2**, and **3** with hydrochloric acid in methanol followed by trimethylsilylation, ristosamine (2 mol) and glucose (1 mol) were detected as common constituents, but mannose (1 mol) was only detected in **1** and **3**, rhamnose (1 mol) only in **3**, and an unknown substance only in both **1** and **2** by GC-MS analysis. These results suggest that the aglycone parts of helvecardins are the same as that of **3**, and that they differ only in their sugar components. The results of acid hydrolysis of **1** and **2**, are summarized in Table 1.



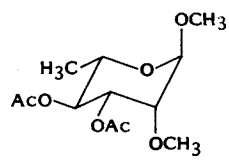
Helvecardin A (**1**) $R_1$=Mannose $R_2$=2-$OCH_3$-Rhamnose
Helvecardin B (**2**) $R_1$=H $R_2$=2-$OCH_3$-Rhamnose
$\beta$-Avoparcin (**3**) $R_1$=Mannose $R_2$=Rhamnose

Table 1. Hydrolysis products of helvecardins A and B.

| | A | B |
|---|---|---|
| Sugar | Ristosamine (2 mol), glucose (1 mol), 2′-*O*-methylrhamnose (1 mol), mannose (1 mol) | Ristosamine (2 mol), glucose (1 mol), 2′-*O*-methylrhamnose (1 mol) |
| Amino acid | Monodechlorodideoxyvancomycinic acid, actinoidic acid, 3-chloro-4-hydroxyphenylglycine, *N*-methyl-*p*-hydroxyphenylglycine | Monodechlorodideoxyvancomycinic acid, actinoidic acid, 3-chloro-*p*-hydroxyphenylglycine, *N*-methyl-*p*-hydroxyphenylglycine |



**4**



**5**

Structural Determination of the Unknown Sugar as 2′-*O*-Methylrhamnose

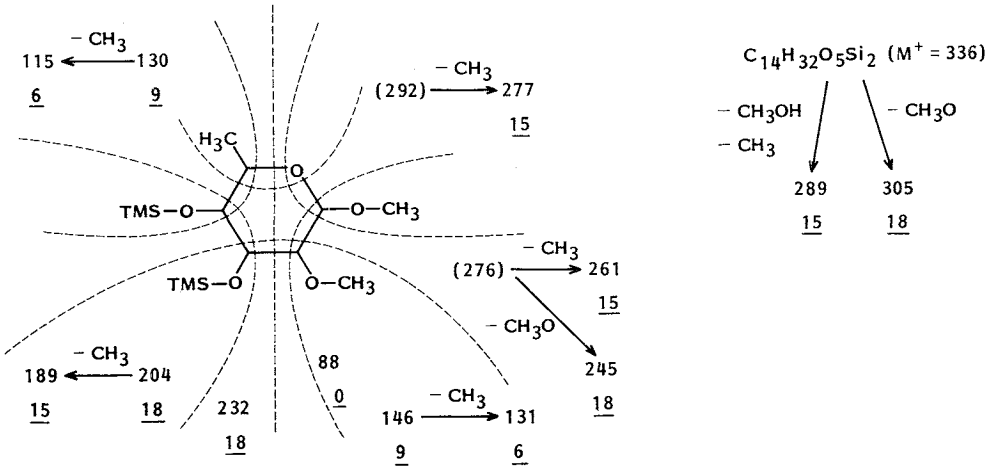
$^1$H NMR spectra of **1** and **2** showed the presence of an *O*-methyl group at 3.27 and 3.30 ppm, respectively. Methanolysis of **2** followed by chromatography on MCI gel (Mitsubishi Chemical Industries Ltd.) gave neutral sugar derivative (**4**).

The CI mass spectrum ($NH_3$) of the trimethylsilyl (TMS) derivative of **4** showed a molecular ion peak at *m/z* 354 $(M+NH_4)^+$ corresponding to the di-trimethylsilylate of di-*O*-methyldeoxyhexose ($C_{14}H_{32}O_5Si_2$). In addition, acetylation of **4** afforded diacetate (**5**), *m/z* 277 $(M+H)^+$.

From the fragmentation analysis of di-trimethylsilylate of **4** in EI mass (Fig. 1) and $^1$H NMR spectral analyses of **4** and **5** (Table 2), **4** was assigned to be 1′,2′-di-*O*-methylrhamnoside. Therefore, the unknown sugar was deduced to be 2′-*O*-methylrhamnose, which was bound at the C-1-position to the aglycone (*vide infra*).

Fig. 1. EI mass fragmentation analysis of TMS derivative and TMS-$d_9$ derivative of **4**.

Underlined figures and figures in parenthesis represent $m/z$ of the TMS derivative and mass shifts in the TMS-$d_9$ derivative, respectively.




## Analysis of Pseudoaglycone

The pseudoaglycones of **1** and **2**, obtained by mild acid hydrolysis in methanol, were directly compared with that of **3** and were confirmed to be identical with that of **3**. Comparison of these pseudoaglycone with ristosaminyl-aglycone $C_{65}H_{60}N_8O_{20}Cl_2$, $QM^+ = 1{,}343$ as Cl=35, obtained from demannosyl-$\beta$-avoparcin strongly indicated that one of the constituents of helvecardins was not dechlorodideoxyvancomycinic acid, but dechlorovancomycinic acid as the native form, and that **1** is the $\beta$-avoparcin 2′-*O*-methylated on rhamnose and **2** is a demannosyl derivative of **1** as indicated by their MW's determined by FAB-MS analyses, and their molecular formulae were confirmed to be $C_{90}H_{103}N_9O_{36}Cl_2$ (M=1,955, as Cl=35) and $C_{84}H_{93}N_9O_{31}Cl_2$ (M=1,793, as Cl=35), respectively.

Table 2. $^1$H NMR data of **4** and **5** in $CDCl_3$ (ppm).

| Proton | Sugar (**4**) | Diacetate (**5**) |
|---|---|---|
| 1′ | 4.86 | 4.72, $J_{1,2}$=1.8 Hz |
| 2′ | | 3.61, $J_{2,3}$=2.5 Hz |
| 3′ | 3.5~3.8 | 5.20, $J_{3,4}$=9.0 Hz |
| 4′ | (4H, m) | 5.10, $J_{4,5}$=9.0 Hz |
| 5′ | | 3.8 |
| 6′ | 1.27 | 1.2 |
| $OCH_3 \times 2$ | 3.40, 3.45 | 3.40, 3.48 |
| OAc×2 | | 2.05, 2.10 |

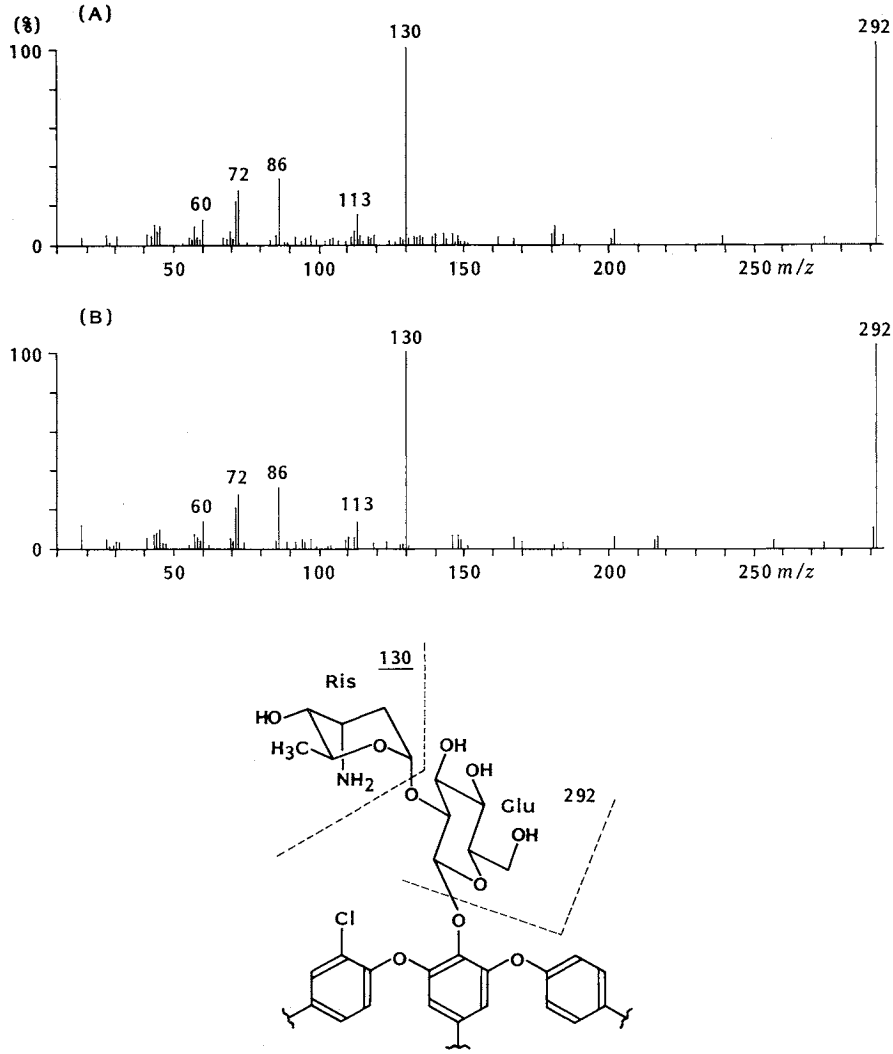
## Conversion of 1 to 2 by α-Mannosidase Treatment

To confirm that **2** is the demannosyl derivative of **1**, **1** was treated with α-mannosidase[6] and the product was shown to be identical with **2** in mass, NMR, and HPLC analyses.

## Total Structure

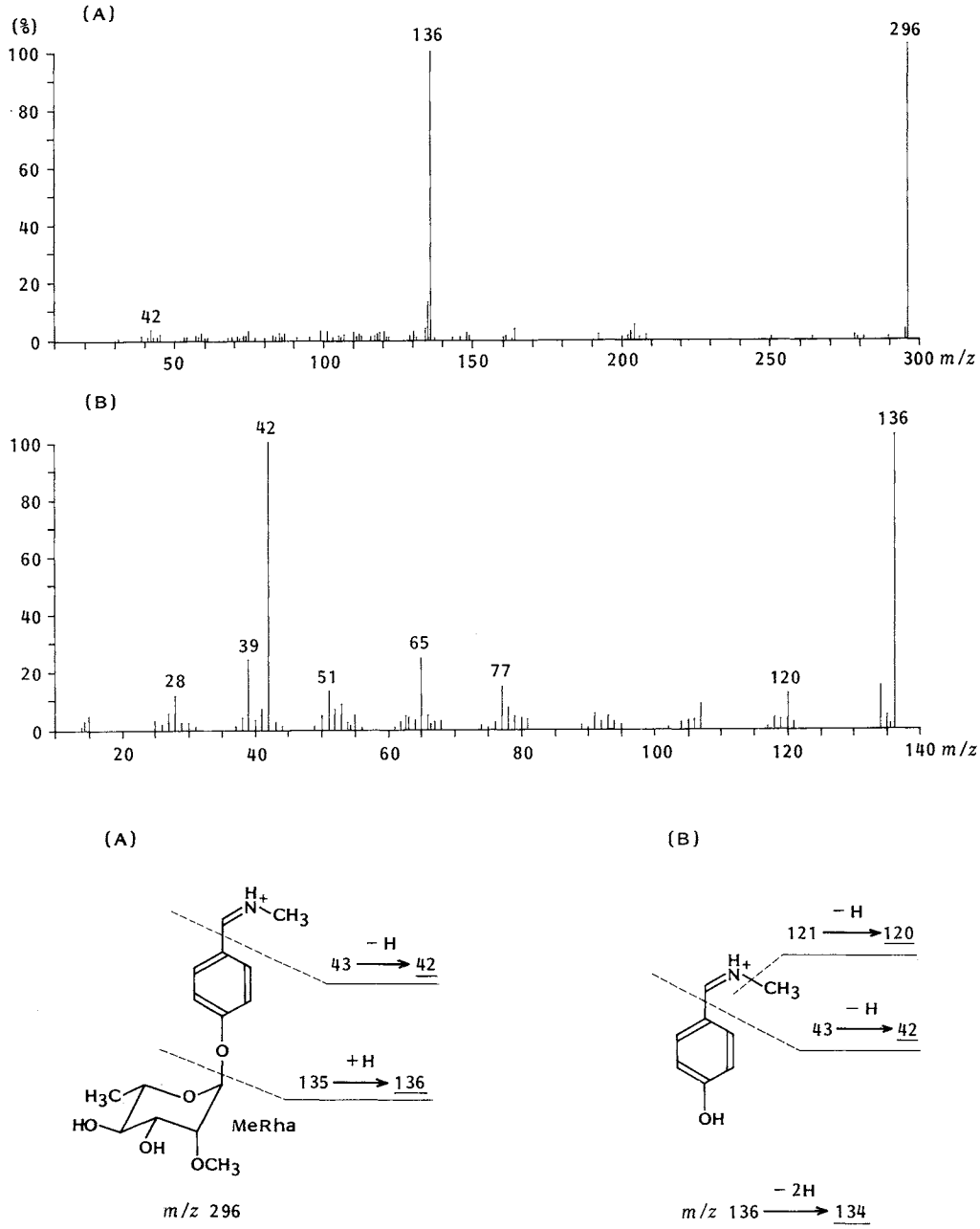
A comparison of pseudoaglycones derived from **1** and **3** or **2** and demannosyl-$\beta$-avoparcin by NMR and mass analyses showed that they were identical to each other indicating that the positions of ristosamine and mannose are the same as those of **3**. By a direct comparison of the FAB mass and MS/MS spectra between **1** and **3**, the existence of 2′-ristosaminylglucose in **1** was confirmed from the fragment ion peaks at $m/z$ 292 ($C_{12}H_{22}NO_7$) which gives daughter ion at $m/z$ 130 ($C_6H_{12}NO_2$) (Fig. 2). To determine the exact position of this di-saccharide in the molecule, **1** and **3** were hydrolyzed under very mild conditions (1 N HCl, 37°C, 1~2 days) to obtain derhamnosyl derivatives. Hydrolysates of **1** and **3**, contained common products, which had different retention times from the pseudoaglycone mentioned before. Based on NMR

Fig. 2. MS/MS fragmentation analysis of ristosaminylglucose moiety of **1** and **3**.




and HPLC analyses, they were determined to be identical with each other and on further hydrolysis, followed by GC-MS analysis, they were shown to contain glucose and mannose but not rhamnose or 2′-*O*-methylrhamnose. **1** or **3** has three *C*-methyl signals around 0.8～1.3 ppm which belong to two ristosamines and one 2′-*O*-methylrhamnose of **1** or rhamnose of **3**. From the lack of two *C*-methyl signals one of the two ristosamines was also lost from **1** or **3** and the remaining ristosamine should be at the same position as in the pseudoaglycone. From these results it was determined that the hydrolysis products contained glucosyl pseudoaglycone and that the position of glucose in **1** was the same position as in **3**. This was also determined from the detailed analysis of NMR of **1** in comparison with that of **3** (in preparation). Finally, the position of 2′-*O*-methylrhamnose was determined to be phenolic OH of *N*-methyl-*p*-hydroxyphenylglycine by Edman degradation[3]. The TPH derivative of the *N*-terminal amino acid in **1** showed a different Rt from those of **3** and derhamnosyl-*β*-avoparcin, indicating that 2′-*O*-

Fig. 3. MS/MS fragmentation analysis of *N*-methyl-*p*-*O*-(2′-*O*-methylrhamnosyl)phenylglycine moiety and its de-2′-*O*-methylrhamnosyl derivative.




methylrhamnose is bound to *N*-methyl-*p*-hydroxyphenylglycine, which is located at the *N*-terminal. In addition, both the HRFAB mass spectra of **1** and **2** showed peaks at *m/z* 296.150 ($C_{15}H_{22}NO_5$) and 136.076 ($C_8H_{10}NO$). As shown in Fig. 3, MS/MS analysis of the ions showed that they were derived from *N*-methyl-*O*-(2′-*O*-methylrhamnosyl)-*p*-hydroxyphenylglycine moiety and the deglycosylated derivative, respectively.

## Experimental

Reductive Hydrolysis of **1**, **2**, and **3**

A small amount (1~2 mg) of each sample was hydrolyzed with 1 ml of 55% hydroiodic acid containing a trace of red phosphorus in a sealed ampoule for 20 hours at 105°C. After drying *in vacuo*, the residue was analyzed by HPLC. Actinoidic acid, *N*-methyl-*p*-hydroxyphenylglycine, and 3-chloro-4-hydroxyphenylglycine were eluted at 4.0, 5.6, and 8.0 minutes (ODS-H-2151, 2 ml/minute, 5% aqueous $CH_3CN$ containing 0.2% TFA), respectively, and monodechlorodideoxyvancomycinic acid was eluted at 10.0 minutes (ODS-H-2151, 1.5 ml/minute, 16% aqueous $CH_3CN$ containing 0.2% TFA).

Methanolysis of **1**, **2** and **3**

A 12.6-mg sample of each of **1**, **2** and **3** was hydrolyzed with 1 ml of 5% HCl-MeOH for 20 hours at 90°C in a sealed ampoule. After each reaction mixture was concentrated *in vacuo* and dried, 1 mg of hydrolysate was converted to its TMS derivatives with *N*-*O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA, 30 $\mu$l) in pyridine (50 $\mu$l) at 60°C overnight. *N*-*O*-Bis-(trimethyl-$d_9$-silyl)acetamide was used instead of BSTFA for the preparation of deuterated TMS(TMS-$d_9$) derivatives. The TMS derivatives of the sugar parts were assigned to those of glucose (6.9 minutes), mannose (5.7 minutes), ristosamine (2.2 minutes) and unknown *O*-methyl-6-deoxyhexose (3.9 minutes) by GC (2% OV-17, 3 mm × 50 cm column, 100~230°C, 10°C/minute temperature programming, He gas 60 ml/minute). GC-MS analysis of the sugar derivatives was also performed under similar conditions to those above.

Separation and Acetylation of 2′-Di-*O*-methylrhamnoside from **2**

The remaining hydrolysates obtained by methanolysis of **2** mentioned above were applied to MCI gel and eluted with 50% aqueous MeOH. Each eluate was concentrated *in vacuo* to yield 1′,2′-di-*O*-methylrhamnoside (**4**). The residue was acetylated with acetic anhydride (0.3 ml) in pyridine (1 ml) to give 1′,2′-di-*O*-methyl-3′,4′-diacetylrhamnoside (**5**). MS *m/z* 277 $(M+H)^+$, 245 $(M-31)$.

Mild Hydrolysis of **1**, **2**, **3** and Demannosyl-$\beta$-avoparcin

To obtain pseudoaglycone of **1**, **2**, **3** and demannosyl-$\beta$-avoparcin, 1-mg solution of each in 0.5 ml of 5% HCl-MeOH was kept for 2 hours at 60°C in a sealed ampoule. After drying of the reaction mixture, the residue was separated by preparative HPLC, in which ODS-H-2151 (Senshu Kagaku Co.) was washed with 18% or 24% aqueous $CH_3CN$ containing 0.2% TFA. Pseudoaglycone of **1** was found to be identical with that of **3**, and ristosaminyl-aglycone of **2** was also found to be identical with that of demannosyl-$\beta$-avoparcin by HPLC and NMR spectra.

Isolation of Glucosyl-pseudoaglycone from **1** and **3**

10 mg of **1** and 12 mg of **3** were hydrolyzed with 1 ml of 1 N HCl at 37°C for 2~3 days. After neutralization, they were analyzed by HPLC, and the portions of a common peak in **1** and **3** were isolated (Senshu, ODS-H-4251, 15% $CH_3CN$ and 0.02 M phosphate, pH 6.8). They showed the following same Rt's in HPLC (Senshu, ODS-H-2151), 10.04 minutes by 12% $CH_3CN$ in phosphate buffer, pH 6.8, and 6.72 minutes by 16% $CH_3CN$ in 0.2% TFA.

$\alpha$-Mannosidase Treatment of **1**

1 mg of **1** was dissolved in 1 ml of 0.2 M phosphate buffer, pH 6.8, and 200 $\mu$l of $\alpha$-mannosidase (*ca.* 50 units/ml, Boehringer Mannheim) was added, and the solution was incubated for 3 days at 37°C with shaking. Then the reaction mixture was applied to a SEP-PAK $C_{18}$ cartridge (Waters Associates), followed by washing with deionized water and elution with 80% aqueous acetone. The eluate was concentrated *in vacuo* and lyophilized to yield demannosylhelvecardin A, which was found to be identical with **2** by comparison of HPLC and NMR spectra.

Edman Degradation of **1**, **2**, **3** and Derhamnosyl-$\beta$-avoparcin

Edman degradation and analysis of the products were performed according to the method described previously[3].

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