# ACTINOIDIN $A_2$, A NOVEL GLYCOPEPTIDE: PRODUCTION, PREPARATIVE HPLC SEPARATION AND CHARACTERIZATION

J. J. DINGERDISSEN, R. D. SITRIN, P. A. DEPHILLIPS, A. J. GIOVENELLA,
S. F. GRAPPEL, R. J. MEHTA, Y. K. OH, C. H. PAN,
G. D. ROBERTS, M. C. SHEARER and L. J. NISBET

Research and Development Division, Smith Kline and French Laboratories,
709 Swedeland Road, Swedeland, Pennsylvania 19479, U.S.A.

(Received for publication September 8, 1986)

An unidentified *Nocardia* sp. (SK&F-AAJ-193) was isolated and found to produce actinoidin A and a novel analog which we have named actinoidin $A_2$. This new glycopeptide antibiotic differs from actinoidin A by the presence of rhamnose instead of acosamine. This analog was isolated using Dianion HP-20 resin followed by a specific glycopeptide affinity column (Affigel-10-D-Ala-D-Ala). The purification was accomplished using preparative ion-pairing chromatography. Actinoidin $A_2$ is active against *Staphylococcus aureus* and coagulase-negative Staphylococci although it is less potent than actinoidin A.

During the course of screening for novel glycopeptide antibiotics,[1] an organism belonging to the genus *Nocardia* (SK&F-AAJ-193) was isolated and found to produce actinoidin A (I)[2~4] and a novel analog which we have named actinoidin $A_2$ (II). The new glycopeptide appears to contain the same aglycone structure as actinoidin A but based on carbohydrate analysis and fast atom bombardment (FAB)-MS data differs in structure by the presence of rhamnose instead of acosamine (Fig. 1). The difficult separation of the chemically similar glycopeptides I and II at the gram-scale was achieved by preparative ion-pair chromatography taking advantage of their charge differences. This manuscript describes the isolation and fermentation of the producing organism, the isolation, separation and characterization of the antibiotics and their biological evaluation.

## Materials and Methods

### Reference Antibiotics

Actinoidins A and B were supplied by G. F. GAUSE, Institute for New Antibiotics, Moscow.

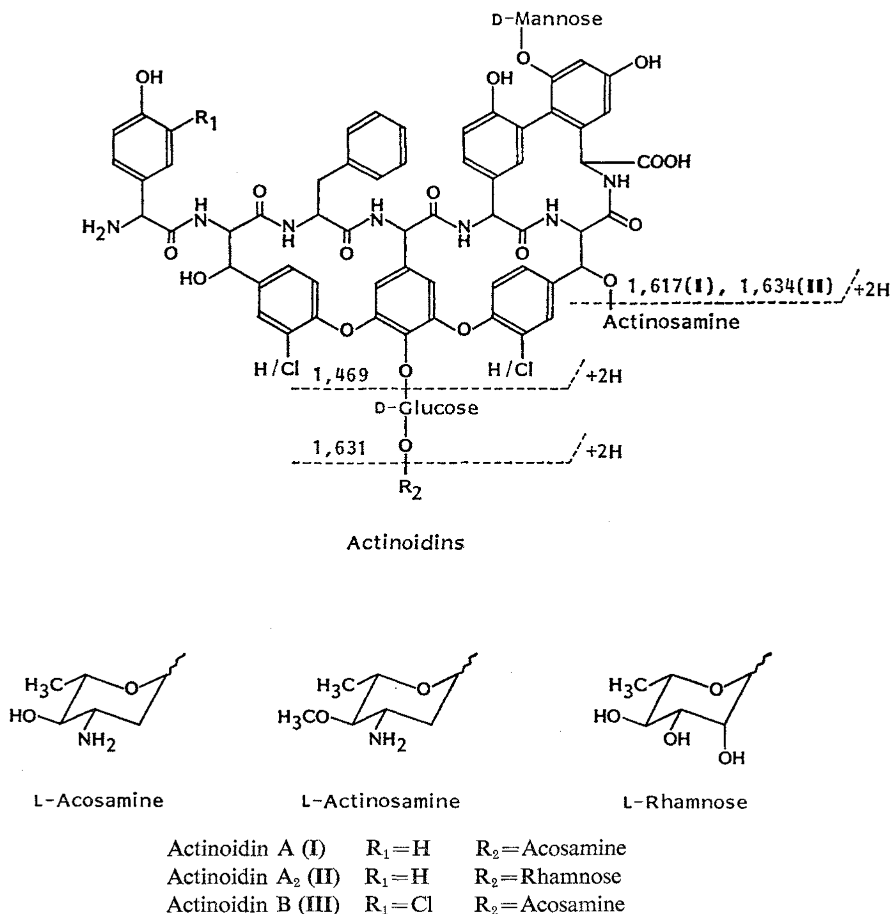
### Determination of Minimum Inhibitory Concentrations

The minimum inhibitory concentrations (MICs, $\mu$g/ml) for aerobic bacteria were determined by microtiter broth-dilution tests using Dynatech MIC-2000 equipment. The growth medium was Trypticase soy broth, pH 7.0, and the inoculum size was approximately $10^5$ cfu/ml. The microtiter plates were incubated at 37°C overnight.

### Determination of Activity in *In Vivo* Mouse Protection Studies

In mouse protection studies, the growth from an 18-hour Trypticase soy agar slant culture of *Staphylococcus aureus* HH 127 was diluted in hog gastric mucin (5%) to a level of $3.0\times10^7$ cfu/ml. This inoculum, 0.5 ml/mouse, was injected intraperitoneally to produce a uniformly lethal mouse infection in 18~21 g Webster-derived CD1 male mice (Charles River Laboratories). The test antibiotics were administered subcutaneously at 1 and 5 hours after infection. The final percentages of

Fig. 1. Proposed structures of actinoidins A (I), $A_2$ (II), B (III), acosamine, actinosamine and rhamnose.




survival for groups of 10 mice each, obtained after 3 days of observation, were used to estimate the 50% effective dose ($ED_{50}$, mg/kg) and the 50% lethal dose ($LD_{50}$, cfu/mouse) values. Studies with other strains of bacteria were undertaken in a similar manner.

The $ED_{50}$ and the $LD_{50}$ values were determined by the logit transformation analysis. Control compounds were commercial preparations.

Analytical Procedures

Analytical HPLC was performed on a Beckman 345 gradient HPLC system monitored at 220 nm with a Beckman Model 155 detector and an Altex CR1B integrator. Chromatography was run on a Beckman Ultrasphere ODS column (4.6×15 cm) equipped with a Brownlee guard cartridge containing C-18 packing (Brownlee 18-GU). Phosphoric acid buffer (0.1 M) was prepared from Fisher HPLC grade phosphoric acid, 85% adjusted to pH 3.2 with KOH. Acetonitrile was HPLC grade, (UV, Burdick and Jackson).

Amino acid analyses were run following 6 N HCl hydrolysis as previously described.[5] Actinoidinic acid and *p*-hydroxyphenylglycine were monitored by HPLC using a solvent consisting of 0.1% trifluoroacetic acid in water on a Beckman Ultrasphere ODS column (4.6×250 mm) as previously described.[5] *p*-Hydroxyphenylglycine was obtained from Sigma. IR spectra were obtained using a Perkin Elmer 299B Spectrophotometer. UV spectra were determined using a Beckman DU-7. Isoelectric focusing was carried out as described previously.[6] FAB-MS were obtained using aVG-ZAB 1F-HF mass spectrometer equipped with a standard FAB ion source.[7] Thermogravimetric

Table 1. Composition of media 13H and E-1.

| Seed Medium (13H) | | Production Medium (E-1) | |
|---|---|---|---|
| Component | g/liter | Component | g/liter |
| Starch | 15.0 | Glucose | 20 |
| Sucrose | 5.0 | Yeast extract | 1 |
| Glucose | 5.0 | $CaCO_3$ | 1 |
| Corn steep liquor | 5.0 | $CoCl_2$ | 0.001 |
| Hy-Soy | 7.5 | HySoy | 10 |
| $K_2HPO_4$ | 1.5 | pH 7.0 | |
| NaCl | 0.5 | | |
| $CaCO_3$ | 1.5 | | |
| Mineral supplement | 5.0 (ml/liter)[a] | | |

[a] Mineral supplement (g/liter): $ZnSO_4 \cdot 7H_2O$ (2.8), ferric ammonium citrate (2.7), $CuSO_4 \cdot 5H_2O$ (0.125), $MnSO_4 \cdot H_2O$ (1.0), $CoCl_2 \cdot 6H_2O$ (0.1), $NaB_4O_7 \cdot H_2O$ (0.09), $Na_2MoO_4 \cdot 2H_2O$ (0.05).

analyses (TGA) were carried out on a Perkin Elmer TGS 2 instrument. Carbohydrates were analyzed as described previously.[5] Preparative chromatography was accomplished using a Water's Prep 500A Liquid Chromatography System equipped with a Whatman Magnum 40 column (4.8×50 cm) dry packed with Whatman Partisil 40 ODS-3 (37~60 μm) as described previously.[5,8]

## Results and Discussion

### Isolation of the Producing Microorganism

The producing organism, an unidentified *Nocardia* sp. (SK&F-AAJ-193), was isolated from a soil sample collected in Pima County, Arizona. Whole cell hydrolysates of SK&F-AAJ-193 contain *meso*-diaminopimelic acid, glutamic acid, alanine, muramic acid, glucosamine, galactose and arabinose. Activity was originally detected in a receptor-based screen for glycopeptides involving antagonism of antimicrobial activity by the cell wall peptide mimetic di-*N*-acetyl-L-Lys-D-Ala-D-Ala.[1]

### Production of Actinoidin $A_2$

The entire contents of a 14-day agar slant of SK&F-AAJ-193 was suspended in 5 ml of sterile distilled water and transferred into 500 ml of seed medium 13H (Table 1) in a 4-liter aspirator bottle. The aspirator bottle was incubated at 28°C on a rotary shaker at 250 rpm with a 5 cm-throw for 3 days.

For scaling up to 550 liters, the first seed was transferred to 10 liters of seed medium 13H in a 14-liter fermentor which was maintained at 28°C, aerated at 4 liters/minute and agitated at 250 rpm. On the third day, 5 liters of the second seed culture was transferred to 50 liters of medium 13H in a 75 liters fermentor (Chemapec Inc.). The third seed was incubated at 28°C, aerated at 25 liters/minute and agitated at 250 rpm. After 3 days incubation, 50-liter was inoculated into 500 liters of the production medium (Table 1) in a 750-liter fermentor (ABEC Corp.). The production stage was conducted at 28°C, aerated at 150 liters/minute and agitated at 120 rpm.

Actinoidin A production peaked at 124 μg/ml, as measured by analytical HPLC, by 20 hours (see Fig. 2). Production of actinoidin $A_2$ occurred during a decline in the actinoidin A level and plateaued around 18 μg/ml (Fig. 2) on the fourth day, by which time it was the major component.

### Isolation of the Actinoidin Complex

The fermentation broth (550 liters) was clarified by rotary drum filtration (Komline-Sanderson,

Fig. 2. Fermentation profile of actinoidins A and $A_2$.
● Actinoidin A concentrations, ○ actinoidin $A_2$ concentrations, ▲ dissolved oxygen, △ pH.

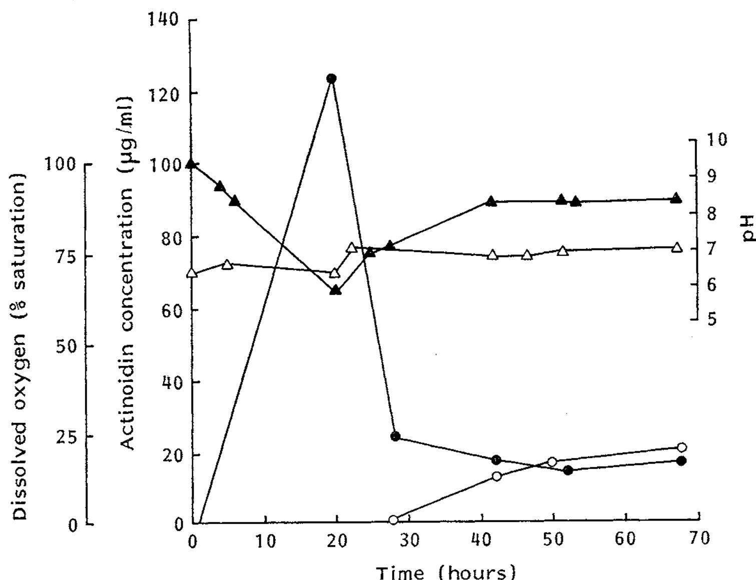
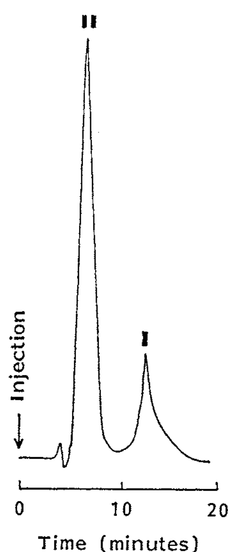



Fig. 3. Analytical chromatogram of the crude mixture of I and II in ion-pairing system.

Whatman Partisil ODS-3, 4.6×250 mm, 25% acetonitrile in 0.3% HFBA, 2 ml/minute, UV at 254 nm.




Laboratory Scale Model) using precoat filter aid (Hyflo Supercel, Johns-Manville Products Corp.) at the existing broth pH (pH 7.3). The broth filtrate (380 liters) was chilled to 4°C, and applied to three Dianion HP-20 (Mitsubishi Chem. Inc.) resin columns (8.5×110 cm) at a flow rate of 0.5 column-volume per hour. After washing with 8 column-volume of deionized water, the desired complex was recovered by elution using acetonitrile - water (20 : 80). The eluate was concentrated at 35°C in a rising film evaporator to yield a crude extract. The extract was applied in three batches onto a 0.5-liter affinity column of Affigel-10-D-Ala-D-Ala specific for glycopeptide antibiotics.[9,10] The column was washed in each run with 1 liter of water, 1 liter of 10% acetonitrile and eluted with 2 liters of 50% acetonitrile containing 0.1 N ammonium hydroxide. This procedure yielded 10.2 g of a mixture of **I** and **II**.

## Chromatographic Purification of Actinoidin $A_2$

Separation of the mixture was effected by preparative reversed-phase high performance liquid chromatography (RP-HPLC) using an ion-pairing mode. An analytical HPLC chromatogram of the crude extract is shown in Fig. 3. The separation was readily scaled up to 12 g for a single injection

Fig. 4. Preparative chromatogram of crude mixture of I and II.
Whatman Partisil Prep 40 ODS-3 in Whatman Magnum Prep 40 column (4.8×50 cm), 17~25% acetonitrile in 0.3% HFBA, 85 ml/minute, UV at 310 nm.

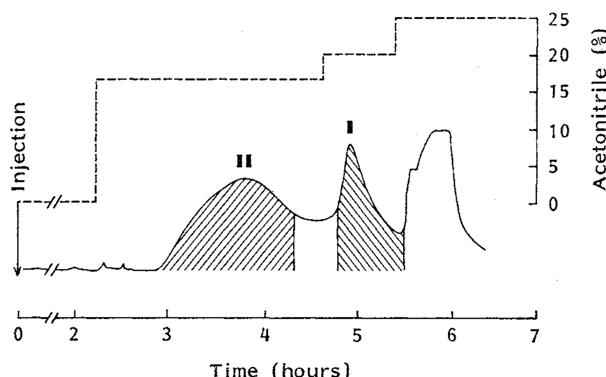



Table 2. Physico-chemical data on actinoidins A and $A_2$ isolated from AAJ-193.

| | A | $A_2$ |
|---|---|---|
| Elemental analysis | | |
| Calcd for: | $C_{84}H_{94}N_9O_{31}Cl \cdot 10H_2O$: C 51.97, H 5.92, N 6.49, Cl 1.83, TGA 9.5% | $C_{84}H_{93}N_8O_{33}Cl \cdot 10H_2O$: C 51.52, H 5.82, N 5.72, Cl 1.81, TGA 9.2% |
| Found: | C 50.06, H 5.11, N 5.67, Cl 2.49, TGA 9.5% | C 48.61, H 5.04, N 5.13, Cl 1.31, TGA 8.7% |
| FAB-MS $(M+H)^+$ | | |
| Fragments | 1,760, 1,631, 1,617, 1,469 | 1,777, 1,631, 1,634, 1,469 |
| UV $\lambda_{max}^{CH_3CN\text{-}H_2O}$ nm ($E_{1cm}^{1\%}$) | | |
| pH 2 | 280 (35) | 280 (35) |
| pH 10 | 300 (40) | 300 (40) |
| $[\alpha]_D^{25}$ ($c$ 0.1, $H_2O$) | 15.9° | 3.3° |
| pI | 8.5 | 6.9 |
| MP | >300°C (dec) | >300°C (dec) |
| IR $\nu_{max}^{KBr}$ ($cm^{-1}$) | 3400, 2910, 1650, 1600, 1580, 1500, 1380, 1320, 1250, 1205, 1100, 1050, 1010 | 3400, 2910, 1650, 1580, 1500, 1380, 1320, 1250, 1205, 1100, 1050, 1010 |

on a large particle low efficiency column (Fig. 4), thus producing the desired amounts of pure antibiotics in a single run in a matter of hours. Removal of excess heptafluorobutyric acid (HFBA) from the pooled fractions from this preparation was accomplished by re-adsorption onto the same column, washing with 0.1 N phosphate buffer, pH 3.2, elution with 20% acetonitrile - 0.1 N phosphate buffer followed by desalting on Dianion HP-20. A detailed description of the development of this ion-pairing chromatographic system will be described elsewhere.

## Chemical Characterization

The two major antibiotics displayed IR and UV spectra (Table 2) consistent with the glycopeptide class of antibiotics, as exemplified by vancomycin,[11,12] ristocetin,[13,14] teicoplanin[15] and the aridicins.[5] Component **I** was presumptively shown to be actinoidin A based on co-elution on HPLC, while the second component appeared to be novel in that it eluted on HPLC at a retention time distinct from the

Table 3. Activity of actinoidins A and $A_2$ against Gram-positive bacteria.

| Glycopeptide antibiotic | $MIC_{50}$ (range)[a] | | | | |
|---|---|---|---|---|---|
| | *Staphylococcus aureus* | | *Staphylococcus epidermidis*[b] | | *Streptococcus faecalis* |
| | MS[c] (N=13) | MR[d] (N=15) | MS (N=13) | MR (N=9) | MIC |
| Actinoidin A | 1.6 (1.6~3.1) | 3.1 (1.6~6.3) | 3.1 (0.8~6.3) | 12.5 (6.3~12.5) | 1.6 |
| Actinoidin $A_2$ | 3.1 (1.6~6.3) | 6.3 (3.1~12.5) | 25 (3.1~>50) | 50 (12.5~>50) | 3.1 |
| Vancomycin | 1.6 (1.6~3.1) | 1.6 (0.8~6.3) | 3.1 (0.8~3.1) | 3.1 (3.1~6.3) | 3.1 |
| Teicoplanin | 1.6 (0.8~1.6) | 3.1 (0.8~3.1) | 3.1 (0.4~12.5) | 12.5 (3.1~25) | ≤0.1 |

[a] $MIC_{50}$ is the minimum inhibitory concentration that inhibited 50% of strains tested. The MIC range is given in parentheses.
[b] *S. epidermidis* includes a number of species of coagulase-negative Staphylococci.
[c] MS indicates methicillin-susceptible strains.
[d] MR indicates methicillin-resistant strains.

Table 4. Activity of actinoidins A and $A_2$, against *Staphylococcus aureus* HH 127 in mouse protection tests.

| Compound[a] | MIC (μg/ml) | $ED_{50}$ (mg/kg) |
|---|---|---|
| Actinoidin A | 1.6 | 1.6 |
| Actinoidin $A_2$ | 1.6 | 2.6 |
| Vancomycin | 1.6 | 1.4 |
| Teicoplanin | 1.6 | 1.4 |

[a] Compounds administered subcutaneously 1 and 5 hours post infection. Challenge dose: 28× $LD_{50}$.

other glycopeptide antibiotics in our collection, including actinoidin B. The actinoidin glycopeptides were first described by SHORIN *et al.*[2] as products of *Proactinomyces actinoides*. Subsequent structural studies indicated that the two components A and B, differed in the number of chlorine atoms present. Based on extensive degradation studies as well as analogy to other glycopeptide structures reported in the literature the tentative structures shown in Fig. 1 have been proposed for actinoidins A and B.[2~4]

The identity of **I** as actinoidin A was further supported by isoelectric focusing data, (8.5 pH units for both) and FAB-MS ($M+H^+$ clusters for both at *m/z* 1,760 with major fragments at *m/z* 1,631, 1,617 and 1,469). The FAB-MS peaks were attributable to losses of acosamine, actinosamine, and acosaminyl-glucose as shown in Fig. 1. Component **II** had a pI of 6.9, a FAB-MS cluster of *m/z* 1,777 and fragments at 1,631, 1,634 and 1,469. The presence of pseudo-aglycone fragment with the same mass (*m/z* 1,469) and the identical nature of the cluster profile (isotope ratios) suggested that the two antibiotics (**I** and **II**) had the same or isomeric nuclei but differed in carbohydrate content.

The antibiotics and an authentic sample of actinoidin A were hydrolyzed in dilute acid and the released carbohydrates were analyzed, as alditol acetates, using gas chromatography mass spectrometry.[5] The chromatograms of component **I** and actinoidin A both showed the presence of the alditol acetates of actinosamine (289 amu), acosamine (317 amu), mannose (434 amu) and glucose (434 amu). Component **II**, in the same analysis, lacked the peak corresponding to acosamine, but showed a new peak at 376 amu corresponding to the alditol acetate of a deoxyhexose, rhamnose, confirmed by comparison with an authentic sample. The substitution of rhamnose ($C_6H_{12}O_5$) for acosamine ($C_6H_{13}NO_3$) accounts for the 17 mass unit difference between the antibiotics and is consistent with the observed FAB-MS fragmentation pattern of **II**, *m/z* at 1,631 (loss of rhamnose), 1,634 (loss of actinosamine)

and 1,469 (loss of rhamnose and glucose). This last fragment suggests that the rhamnose is on the same location as the acosamine in actinoidin A, but does not exclude the possibility that the rhamnose is attached elsewhere in the molecule.

Further confirmation of the chemical similarity of these antibiotics to the actinoidins comes from the amino acid analyses obtained using a recently described HPLC procedure.[5,16] Acid hydrolysis of **I**, **II** and actinoidin A each yielded actinoidinic acid[4] and *p*-hydroxyphenylglycine (**IV**). The presence of phenylalanine in the hydrolysates was more difficult to discern on HPLC due to the low extinction coefficient of this amino acid. However, phenylalanine and *p*-hydroxyphenylglycine could both be readily detected in all three hydrolysates using a Beckman Autoanalyzer ion-exchange system and detection by ninhydrin.

The physico-chemical data is consistent with component **I** being actinoidin A and component **II** being a novel glycopeptide which differs from actinoidin A in the substitution of rhamnose for the amino sugar acosamine. Because of this similarity we have named the major component **II** "actinoidin $A_2$" and propose a structure for this antibiotic as shown in Fig. 1. Because certain structural features for actinoidin A, such as location of the chlorines and sugar substituents, have not as yet been completely resolved in the literature, samples of actinoidins A and $A_2$ are currently being studied by 2D NMR spectroscopy. The results of the studies will be published elsewhere.[17]

### Biological Evaluation

Studies were carried out to determine the antimicrobial potency and spectrum of activity of actinoidin $A_2$ in comparison with actinoidin A, vancomycin and teicoplanin. In common with other members of this class, actinoidin $A_2$ was active against Gram-positive bacteria and was inactive against Gram-negative strains. Actinoidin $A_2$ was consistently 2-fold less active than actinoidin A against *S. aureus* strains. This difference was more pronounced with coagulase-negative Staphylococci (Table 3).

In mouse protection tests, actinoidin $A_2$ was effective against strains of *Staphylococcus* sp. which were susceptible *in vitro*. Thus, the $ED_{50}$ against *S. aureus* HH 127 was 2.6 mg/kg compared to 1.6 mg/kg for actinoidin A, 1.4 mg/kg for vancomycin and 1.4 mg/kg for teicoplanin (Table 4).

## Conclusions

The new glycopeptide antibiotic, actinoidin $A_2$, is structurally related to actinoidin A, but differs in having the neutral sugar rhamnose rather than the amino sugar acosamine. Although similar in polarity, this resulted in a lower net positive-charge for actinoidin $A_2$. This necessitated the development of an ion-pair preparative chromatographic separation system using HFBA to effectively separate the two antibiotics. Actinoidin $A_2$ has somewhat lower potency than actinoidin A against *S. aureus* and coagulase-negative Staphylococci, potentially due to its decreased positive-charge.

### Acknowledgments

We gratefully acknowledge the technical assistance of P. Coleman and R. Ferrin for taxonomic work, C. Phelen for screening studies, J. Hobdell and G. Udowenko for the isolation work, R. Gerber and S. Vogt-Speth for the fermentation supplies, L. Phillips and R. Reid for *in vivo* studies, E. Reich for analytical data and P. Jeffs and S. Christensen for helpful discussions and suggestions in preparing the manuscript.

## References

1) Rake, J. B.; R. Gerber, R. J. Mehta, D. J. Newman, Y. K. Oh, C. Phelen, M. C. Shearer, R. D. Sitrin

& L. J. Nisbet: Glycopeptide antibiotics: A mechanism-based screen employing a bacterial cell wall receptor mimetic. J. Antibiotics 39: 58~67, 1986

2) Shorin, V. A.; S. D. Yudinstsev, I. A. Kunrat, L. E. Goldberg, N. S. Pevzner, M. G. Braszhnikova, N. N. Lomakina & E. F. Oparysheva: New antibiotics, actinoidin. Antibiotica 2: 44~49, 1957

3) Sztaricskai, F.; C. M. Harris & T. M. Harris: Structural investigation of the antibiotic actinoidin: Identification of the tris (amino acid). Tetrahedron Lett. 1979: 2861~2864, 1979

4) Williams, D. H.; V. Rajananda, M. P. Williamson & G. Bojessen: The vancomycin and ristocetin group of antibiotics. Top Antibiotic Chem. 5: 119~158, 1980

5) Sitrin, R. D.; G. W. Chan, J. J. Dingerdissen, W. Holl, J. R. E. Hoover, J. R. Valenta, L. Webb & K. M. Snader: Aridicins, novel glycopeptide antibiotics. II. Isolation and characterization. J. Antibiotics 38: 561~571, 1985

6) Henner, J. & R. D. Sitrin: Isoelectric focusing and electronphoretic titration of antibiotics using bioautographic detection. J. Antibiotics 37: 1475~1478, 1984

7) Roberts, G. D.; S. A. Carr, S. Rottschaefer & P. W. Jeffs: Structural characterization of glycopeptide antibiotics related to vancomycin by fast atom bombardment mass spectrometry. J. Antibiotics 38: 713~720, 1985

8) Sitrin, R. D.; G. Chan, P. DePhillips, J. Dingerdissen, J. Valenta & K. Snader: Preparative reversed phase HPLC as a recovery and purification process for nonextractable polar antibiotics. *In* Purification of Fermentation Products. ACS Symposium Series, No. 271. *Ed.*, D. LeRoith, *et al.*, pp. 71~89, Amer. Chem. Soc., Washington, D.C., 1985

9) Folena-Wasserman, G.; R. Sitrin & K. Snader: Affinity purification of glycopeptide antibiotics. Program and Abstracts of the 25th Intersci. Conf. on Antimicrob. Agents Chemother., No. 792, p. 238, Minneapolis, Sept. 29~Oct. 2, 1985

10) Folena-Wasserman, G.; R. D. Sitrin, K. Snader & F. Chapin: Affinity purification of glycopeptide antibiotics. J. Chromatogr., in press

11) Williams, D. H.; M. P. Williamson, D. W. Butcher & S. J. Hammond: Detailed binding sites of the antibiotics vancomycin and ristocetin A: Determination of intermolecular distances in antibiotic/substrate complexes by use of the time-dependent NOE. J. Am. Chem. Soc. 105: 1332~1339, 1983

12) Harris, C. M.; H. Kopecka & T. M. Harris: Vancomycin: Structure and transformation to CDP-I. J. Am. Chem. Soc. 105: 6915~6922, 1983

13) Williams, D. H.; V. Rajananda, G. Bojessen & M. P. Williamson: Structure of the antibiotic ristocetin A. J. Chem. Soc. Chem. Commun. 1979: 906~908, 1979

14) Kalman, J. R. & D. H. Williams: An NMR study of the structure of the antibiotic ristocetin A. The negative nuclear Overhauser effect in structure elucidation. J. Am. Chem. Soc. 102: 897~905, 1980

15) Bardone, M. R.; M. Paternoster & C. Coronelli: Teichomycins, new antibiotics from *Actinoplanes teichomyceticus* nov. sp. II. Extraction and chemical characterization. J. Antibiotics 31: 170~177, 1978

16) Jeffs, P. W.; G. Chan, L. Mueller, C. DeBrosse, L. Webb & R. D. Sitrin: Aridicin aglycone: Characterization of the amino-acid components. J. Org. Chem. 51: 4272~4278, 1986

17) Heald, S.; L. Mueller & P. W. Jeffs: Actinoidin A and $A_2$: Structure determination by 2D NMR. in preparation