ACTAPLANIN, NEW GLYCOPEPTIDE ANTIBIOTICS PRODUCED BY *ACTINOPLANES MISSOURIENSIS*

THE ISOLATION AND PRELIMINARY CHEMICAL CHARACTERIZATION OF ACTAPLANIN

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(Received for publication October 25, 1983)

Actaplanin (A4696), a new complex of broad spectrum Gram-positive antibiotics is produced by *Actinoplanes missouriensis*. High performance liquid chromatography was used to show that this complex is composed of several actaplanins. Hydrolytic experiments with actaplanins A, B_1, B_2, B_3, C_1 and G showed that these actaplanins were composed of the same peptide core, an amino sugar and varying amounts of glucose, mannose and rhamnose. The neutral sugar content was determined for each actaplanin. A bioautographic study of aglycone formation during hydrolysis of the actaplanin complex showed that within a short time a simple mixture of two antimicrobially active hydrolysis products was obtained. These substances retained the antimicrobial spectrum and a high percentage of the antibiotic activity of the parent actaplanin complex.

Methanolysis of the actaplanin complex as well as the individual actaplanins resulted in the selective loss of the neutral sugar moieties and the isolation of actaplanin Ψ(pseudo)-aglycone — the core peptide which still retained an amino sugar group. The ^{1}H NMR spectrum of this substance indicated a similarity to many features of ristocetin Ψ-aglycone. Hydrolytic studies showed that the amino sugar present in actaplanin was identical with L-ristosamine. It is concluded that the aglycone of actaplanin is a complex peptide composed of aromatic amino acids, and that the actaplanins each possess this aglycone and L-ristosamine but are differentiated by their neutral sugar composition.

Actaplanin (A4696)**, a new complex of glycopeptide antibiotics produced by *Actinoplanes missouriensis* (ATCC 23342), exhibits strong inhibitory activity against a broad spectrum of Gram-positive bacteria. The isolation of the actaplanin complex showed that actaplanin was composed of several closely related glycopeptide components (hereafter referred to as actaplanins) by their chromatographic behavior.

Several recent structural studies of glycopeptide antibiotics[1~5] have shown that these antibiotics appear to hold several chemical features in common. Typically, glycopeptides give rise to an "aglycone," neutral sugars[6~8] and an amino sugar upon mild hydrolysis[2,9~12]. Furthermore, the aglycone is a complex peptide which retains the same amino acids present in the parent after total hydrolysis and also some of the antimicrobial activity of the parent.

The purpose of the present study was to determine the general chemical characteristics of the actaplanin complex and to relate the differences and the similarities it bears to known members of its class[14]. The chemical relationship between the various actaplanins has also been determined.

** Actaplanin is the US Adapted Name (USAN) for antibiotic A4696 (US Patents 4,115,552 and 4,322,406).

Results

Actaplanin is an amorphous solid which is freely soluble in water and sparingly soluble in common organic solvents. The actaplanins can be separated by paper or thin-layer chromatographic systems and detected by bioautography against *Bacillus subtilis* or ninhydrin visualization spray. The mycelia isolated by filtration of the *Actinoplanes missouriensis* culture were extracted with dilute NaOH solution (pH 10.5) and the extract subsequently acidified. A cation exchange resin (Amberlite IR-116, Na^+ form) was used to adsorb the active basic components which were released by elution with dilute NaOH (pH 10.5). This crude preparation was further purified by desalting and decolorization to give the actaplanin complex. This procedure is summarized in Scheme 1 and in the experimental section. Silica gel TLC bioautography (see Fig. 1) showed that the actaplanin complex consisted of at least seven actaplanins designated A, B_1, B_2, B_3, C_1, C_2 and G. The relative proportions of these actaplanins could be determined by use of high performance liquid chromatography (HPLC). Fig. 2 shows a HPLC analysis of an actaplanin sample showing good resolution of the individual actaplanins. The actaplanins labeled as E (Figs. 1 and 2) may have been formed by alkaline degradation. The actaplanins were separated preparatively by polyamide chromatography using water as a mobile phase. This procedure gave actaplanins A, B_1, B_2, B_3, C_1 and G as single entities in sufficient quantities for characterization (Table 1). Bioassay of these actaplanins showed all to be highly active antibiotics having similar potency (Table 2).

The UV spectrum of actaplanin exhibited a maximum at 276 nm (ε 9,702) which shifts to 300 nm (ε 11,900) in basic solution. This spectral behavior is typical of phenolic groups. The titration curve for actaplanin showed *pKa* values of 7.09, 9.32, 10.93 and several *pKa* values at 12.0 (MW 2,037). The nature and multiplicity of titrable groups demonstrated the amphoteric nature of this antibiotic.

Scheme 1. Isolation, purification and separation of the actaplanin complex.

Fermentation broth
↓ Filtration
Mycelia
↓ pH 10.5 adjustment, Filtration
Filtrate
↓ pH 4.5 adjustment, Adsorption on Amberlite IR-116, Alkaline elution (pH 10.5), Neutralization (H_2SO_4), Concentration and freeze drying
Crude Complex
↓ Aqueous extraction, Decolorization (Duolite S761), Concentration and freeze drying
Purified actaplanin complex
↓ Chromatographic separation (Polyamide)
Actaplanins A, B_1, B_2, B_3, C_1, C_2 and G

Fig. 1. Thin-layer bioautography of actaplanin.
Letters are used to designate the individual actaplanins.
TLC system: silica gel (flexible back) Merck #5506. Solvent: MeOH - $CHCl_3$ - conc NH_4OH - 2-BuOH - H_2O (50: 25: 25: 25: 10).
Detection organism: *Bacillus subtilis*.

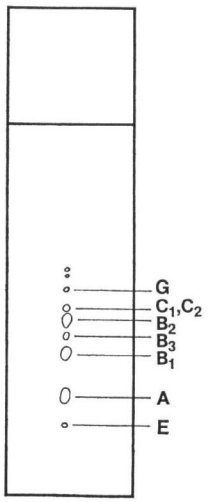


Fig. 2. HPLC analysis of actaplanin complex showing resolution of the individual factors. Letter designations correspond to the individual actaplanin factors. [Analytical HPLC analysis (Gradient: 2% aq HOAc - CH_3CN (9: 1) to 2% aq HOAc - CH_3CN (6: 4)].

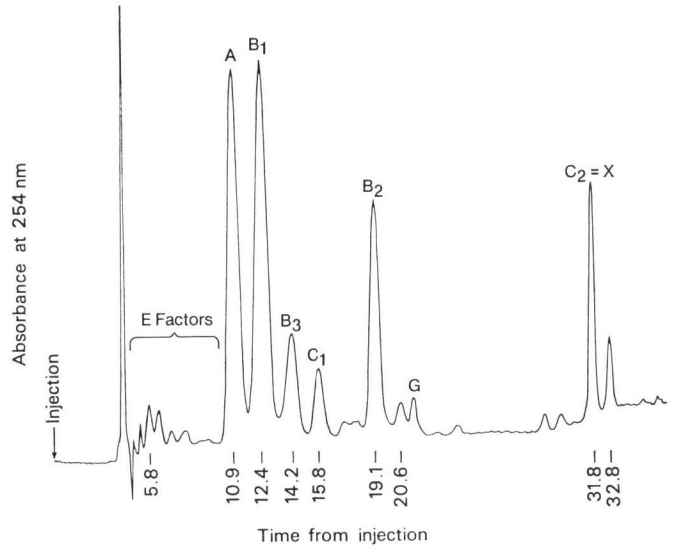


Table 1. Physico-chemical properties of the actaplanins.

Actaplanin	Molecular formula[a]	MW	Rf[b]	*K* Values[c]	$\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$)
A	$C_{90}H_{101}O_{40}N_8Cl$	1,970	0.25	1.60	280 (44.0)
B_1	$C_{90}H_{101}O_{39}N_8Cl$	1,954	0.35	1.99	280 (42.8)
B_2	$C_{84}H_{91}O_{35}N_8Cl$	1,808	0.45	3.84	280 (44.7)
B_3	$C_{84}H_{91}O_{35}N_8Cl$	1,808	0.40	2.50	280 (46.3)
C_1	$C_{84}H_{91}O_{34}N_8Cl$	1,792	0.51	2.92	280 (47.9)
G	$C_{78}H_{81}O_{30}N_8Cl$	1,686	—	4.42	280 (53.0)

[a] All actaplanins were chromatographically homogeneous; molecular formulae were calculated from the sum of the empirical formulae of the actaplanin Ψ-aglycone[22] and the known attached sugars.

[b] Silica gel plates (E. Merck #5506): Solvent MeOH - $CHCl_3$ - conc NH_4OH - 2-BuOH - H_2O, 50: 25: 25: 25: 10, Rf determined by bioautography plates vs. *Bacillus subtilis* ATCC 6633.

[c] HPLC using 2% aq HOAc - CH_3CN (90: 10) and 2% aq HOAc - CH_3CN (70: 30).

Table 2. Bioassay of purified actaplanins.

Actaplanin	Bioassay* (units/mg)
A	1,489
B_1	1,237
B_2	1,083
B_3	1,332
C_1	930

* Average of 5 assays, repeated twice daily on the same sample using a *Bacillus subtilis* agar diffusion assay.

Actaplanin Aglycone Formation and Neutral Sugar Components

Actaplanin was hydrolyzed under acidic conditions (0.15 N HCl, reflux, 2 hours) to give an amorphous solid upon concentration of the reaction mixture. The filtrate was passed over a column of Dowex 50W-X12 resin to give a solution of neutral sugars. Examination of this mixture by paper and thin-layer chromatography revealed that the actaplanin complex contained mannose, glucose and rhamnose. This hydrolysis was repeated on each individual actaplanin and the aglycone and carbohydrate fractions were similarly processed. After ion exchange treatment each neutral carbohydrate fraction was concentrated and trimethylsilylated, using trimethylsilylimidazole, and the ratios of silylated sugars were analyzed by

Table 3. Molar ratios of neutral sugars.

Actaplanin	Mannose*	Glucose	Rhamnose
A	2.80	1.0	—
B_1	2.05	1.0	1.01
B_2	1.80	1.0	—
B_3	2.37	1.0	—
C_1	1.12	1.0	1.08
G	1.25	1.0	—

* Sugars determined by gas chromatographic analysis of their trimethylsilyl derivatives. Each sugar is the sum of its α- and β-anomers.

Table 4. Selected ^{1}H NMR resonances of actaplanin Ψ-aglycone.

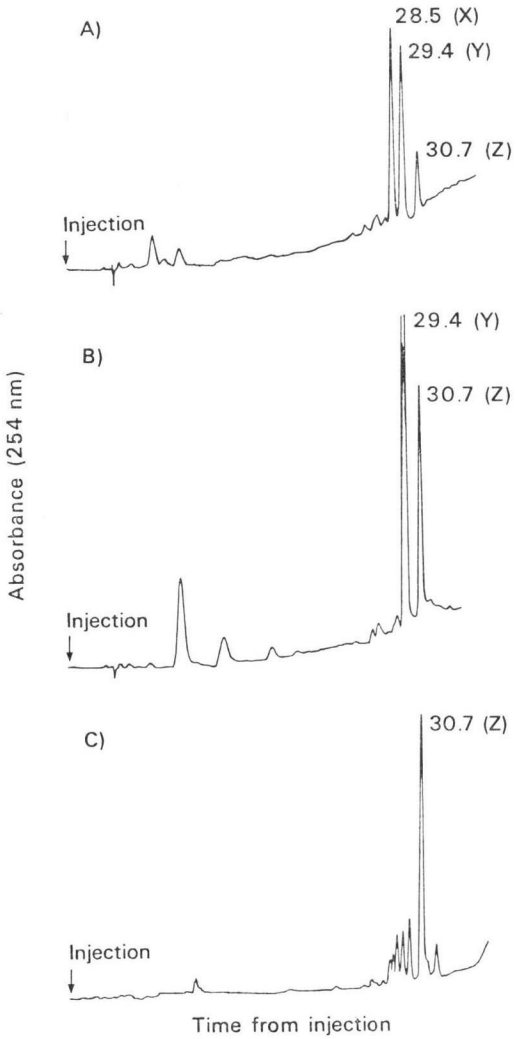
Chemical shift (δ)	No. of protons	Assignments
1.22	3	C-6-Ristosamine methyl group
2.00	3	Aromatic methyl
3.8	3	Methoxyl group
9~10	6	6 Phenolic protons (singlets)

gas-liquid chromatography[15]. The silylated sugars were analyzed as the sum of their anomers. The results of these analyses are shown in Table 3 which relates both the identity and the molar ratios of the neutral sugars found in the actaplanins. It was noteworthy that there appeared to be considerable variation in the neutral carbohydrate content of the actaplanins.

The selective removal of the neutral constituents was most conveniently accomplished by methanolysis of the actaplanin complex which gave the homogeneous product **1**. The ^{1}H NMR spectrum of **1** revealed several features which were also present in the published spectrum of ristocetin A[19]. These structural assignments are shown in Table 4.

Fig. 3. HPLC data comparing the products of mild hydrolysis of actaplanin A, B_1 and B_2 (A) and actaplanin B_3 and C_1 (B), (hydrolysis conditions: pH 1.85, 87°C, 5 hours); (C) HPLC analysis of products of hydrolysis (pH 1.8, 87°C) of actaplanin B_2 after 17 hours.

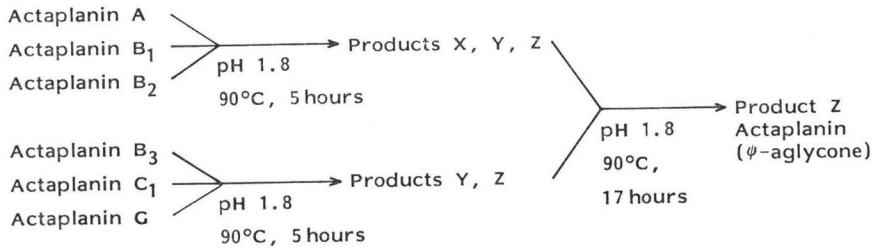
All actaplanin factors gave only Z after extended mild hydrolysis.



The ^{1}H NMR spectrum of **1** clearly showed the presence of signals which corresponded to the presence of L-ristosamine (see Table 4) and also the lack of signals attributable to the neutral sugars present in the parent antibiotic. Hence, **1** has the properties anticipated for the actaplanin Ψ-aglycone[20]. Methanolysis of the individual actaplanins gave the identical Ψ-aglycone by HPLC and ^{1}H NMR criteria. The isolation of the same Ψ-aglycone from each actaplanin confirms that besides having the identical peptide core, each actaplanin has a single molecule of L-ristosamine and that the major difference among the actaplanins lies in the neutral sugar moieties.

The individual actaplanins A, B_1, B_2, B_3, C_1 and G were subjected to mild acid hydrolysis (pH 1.8,

90°C), and the products were analyzed by HPLC (see Fig. 3). Two products: Y (retention time 29.4 minutes) and Z (retention time 30.7 minutes) were formed from each actaplanin. Actaplanins A, B_1 and B_2 also produced a third product, X (retention time 28.5 minutes), which formed rapidly during the first 30~60 minutes of the hydrolysis. As the hydrolysis of these actaplanins proceeded to 5 hours, X gave rise to increased quantities of products Y and Z. Actaplanins B_3 and C_1 produced only Y in the early stages of hydrolysis (30~60 minutes) and then gave Z upon further hydrolysis (5 hours). Prolonged hydrolysis (pH 1.8, 90°C, 17 hours) of all actaplanins resulted in the formation in each case of one and the same product, Z, which (Fig. 3C) was identified by HPLC-comparison as actaplanin Ψ-aglycone (**1**). The hydrolytic relationship of the individual actaplanins is illustrated below.

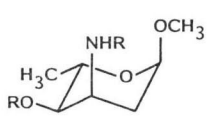


The conditions employed in these hydrolyses were equivalent to those reported by SZTARICSKAI[18] to give partial hydrolysis of the oligosaccharide side chain of ristomycin. It is therefore anticipated that a similar partial removal of the glycoside moiety from the actaplanins would also occur here resulting in the formation of X and Y. This observation and the fact that all actaplanins gave rise to the same Ψ-aglycone (**1**) confirms that all actaplanins have the same peptide core but different neutral sugar side chains. Total acid hydrolysis of the products also gave the identical amino acids by the amino acid analyses.

Bioautographic studies of the products of mild acid hydrolysis of the actaplanin complex at various time intervals showed that there was a rapid appearance of new antimicrobially active products with the concomitant disappearance of the original actaplanins (see Fig. 4). There is a convergence upon two products: **2**, major (Rf 0.72) and **3**, minor (Rf 0.88) bioactive product (aglycone). Gradually the bioactivity of these spots diminished to a low level by 24 hours. The antimicrobial spectrum of these aglycones was identical with that of the parent actaplanin complex.

The Isolation of L-Ristosamine from Actaplanin

Structural studies of the chemical composition of vancomycin[9], ristocetin[10], LL-AV 290 (avoparcin)[11], and A-35512B[2] have shown that these glycopeptides all contain an amino sugar as an integral part of their complex structures. The presence of an amino sugar in actaplanin was similarly detected. Methanolysis of actaplanin complex (3 N methanolic HCl) resulted in the formation of a mixture of neutral and basic methyl glycosides. The basic glycosides were adsorbed onto Dowex 50W-X12 ion exchange resin. Fractions eluted with 2 N NH_4OH gave a crystalline substance having the following composition: $C_7H_{15}NO_3 \cdot HCl$. The high resolution mass spectrum of this compound showed peaks at *m/z* 162 ($M^+ + 1$, $C_7H_{16}NO_3$), *m/z* 117 ($C_5H_{11}NO_2$) and a doublet at *m/z* 59 [*m/z* 59a (C_3H_7O), *m/z* 59b (C_2H_5NO)]. These fragmenta-



4 R = H
5 R = Ac

Table 5. ^{1}H NMR data for methyl *N,O*-diacetyl-α-L-ristosaminide (**5**).

Proton assignment	Chemical shift and multiplicity		
	From actaplanin (100 MHz)	From ristomycin[12] (220 MHz)	Synthetic[16] (100 MHz)
C-1 H	4.73 dd (1.0, <1)*	4.79 dd (4.0, <1)	4.71 dd
C-2 H_{eq}	1.86 d of brs (1.5, 14.0)	1.89 dd (<1, 14, 2.5)	1.90 d of brs
C-2 H_{ax}	2.07 d of t (3.5, 2.5)	2.09 d of t (4, 3.5)	2.10 d of t (4.5, 4.5)
C-3 H	4.58 (m)	4.66 (m)	4.58 (m)
C-4 H	4.52 dd (4.0, 9.0)	4.57 d of d (4.0, 9.5)	4.50 dd (4.5, 10.0)
C-5 H	3.92 m (6.0)	3.98 m	3.92 m
C-1 OCH_3	3.40 s	3.43 s	3.43 s

* Coupling constants are in Hz and shown in parentheses, chemical shifts are in ppm, letters denote multiplicity, spectra in $CDCl_3$.

tions were identical with those reported by BOGNAR *et al.*[12] for methyl α-L-ristosaminide (**4**).

This assignment was confirmed by conversion of **4** to its *N,O*-diacetyl derivative **5** which had the identical spectral properties reported by BOGNAR *et al.*[12] for methyl *N,O*-diacetyl-α-L-ristosaminide. The NMR spectrum of **5** is compared in Table 5 to the published ^{1}H NMR spectrum of methyl *N,O*-diacetyl-L-ristosaminide obtained from two sources: from ristomycin[12] and from synthetic ristosamine[16]. A signal by signal comparison showed them to have the identical spectra. The relative configurations of these derivatives must therefore also be identical.

CD studies of **4** in Cupra A show a negative chirality at 580 nm which is consistent with the assignment of the L-configuration to the actaplanin-derived ristosamine[16]. Therefore, the amino sugar derived from actaplanin is identified as L-ristosamine. The high yields of L-ristosamine obtained from actaplanin coupled with the fact that no other amino sugar was isolated or observed indicate that this amino sugar is uniformly present in each actaplanin. This proposal was tested in the case of actaplanins A and B_2. Benzoylation and methanolysis of the basic glycosides derived from actaplanins A and B_2 resulted in the isolation of methyl *N,O*-dibenzoyl-α-L-ristosaminide. Although sufficiently large quantities of all of the actaplanins were not yet available to directly analyze each factor for this amino sugar, NMR data on the Ψ-aglycone showed that L-ristosamine is present in each of the individual actaplanins.

Discussion

These studies of actaplanin have characterized this antibiotic to be a member of the glycopeptide family and related to ristocetin[1], ristomycin[5] and A-35512B[2]. Clearly, the hydrolytic formation of a peptide (aglycone) and the release of both neutral and basic sugars represents chemical behavior characteristic of this class of antibiotics. The level of antimicrobial activity of the aglycone, at least at certain stages of actaplanin hydrolysis, along with the retention of the original antimicrobial spectrum is interpreted to indicate that the neutral carbohydrate side chains do not play a decisive role in determining biological activity. The site of antimicrobial activity must reside in the peptide aglycone as it does with other glycopeptide antibiotics[2,6,14]. The carbohydrate composition is striking in the case of the actaplanins with wide variations in both the number and the type of sugars present. The major role the sugars play appears to be in altering pharmacodynamic properties such as solubility and transfer of the antibiotic to site of action[14]. In each of the actaplanins the peptide portion remains constant.

Variation of this type was also observed with ristocetins A and B[6] which differ only in their neutral sugar content, yet exhibit no major differences in their antimicrobial properties. The timed study of actaplanin aglycone formation showed several bioactive substances formed (probably partial hydrolysis products) initially but within a few hours bioactivity resided in the same few hydrolysis products. Continued hydrolysis at these temperatures showed a gradual disappearance of biological activity.

It was shown by HPLC analysis that the product obtained after mild hydrolysis (0.15 N HCl, 1.5 hours reflux) was composed of three related substances. The two major substances were isolated from this mixture on a preparative scale, and their relative biological activity has been discussed above. Their amino acid analyses were identical with actaplanin complex. Also worthy of note is that the hydrolysis products from each of the individual actaplanins were identical to each other both in their HPLC and amino acid analyses. These data imply that the aglycone obtained from the actaplanin complex or the individual actaplanins is identical. The isolation of the identical Ψ-aglycone from each individual actaplanin by methanolysis confirms the existence of a common actaplanin aglycone.

The isolation of L-ristosamine as the constituent amino sugar of actaplanin, avoparcin[4], ristocetin and ristomycin[17] and of L-vancosamine and its C-3 epimer from vancomycin[9,10] and A-35512B[2], respectively, indicates that 2,3,6-trideoxy-3-aminohexoses are characteristic of the glycopeptides discovered thus far.

In summary, this study has confirmed that actaplanin is a new complex of glycopeptide antibiotics. It has been shown that it is composed of several actaplanins which have the same peptide core (aglycone) and amino sugar but differ one from the other in the degree of glycosylation with the three constituent neutral sugars (mannose, glucose and rhamnose). The data clearly demonstrate that actaplanin is chemically related to the A-35512B-ristocetin family of glycopeptide antibiotics. These results will serve as the basis for future approaches to the structural determination of actaplanin[21,22].

Experimental

The following instruments were used in this study; ^{1}H NMR spectrometer Varian HA100; UV spectrophotometer, Cary 15; ORD and CD spectropolarimeter, Perkin Elmer 241 and Jasco J40AS; IR spectrophotometer, Beckman IR426; high resolution mass spectrometer, Varian MAT731, amino acid analyses, Beckman 120C amino acid analyzer equipped with a Bio-Cal autosample applicator and programmer and single Durrum BC-1A resin; gas chromatograph, F and M 402 equipped with a 316 cm 3% OV-101 column on Chromsorb W operated at 200°C; high pressure liquid chromatograph: Waters analytical instrument equipped with two Model 6000 pumps, a Model 660 solvent programmer, a Model UK-6 injector, a Model 440 absorbance detector (254 nm) and a μBondapak C-18 column.

Isolation and Purification of the Actaplanin Complex

The fermentation broth (3,800 liters) from *Actinoplanes missouriensis* was filtered after the addition of 5% (w/v) filter aid (Celite 545). The filter cake was resuspended in deionized water (3,600 liters) and the pH of the aqueous suspension was adjusted to pH 10.5 using aqueous sodium hydroxide. The suspended solids were separated by filtration and washed with water. The filtrate and the washings were combined and the resulting solution was acidified with 20% (w/v) aqueous sulfuric acid to pH 4.5. The acidic solution was clarified by filtration using 1% filter aid (Celite 545). The clear solution was passed through a column (55×150 cm) containing 350 liters of Amberlite IR-116 (Na^+ form) and the column washed with deionized water (1,200 liters). The Amberlite IR-116 resin was removed from the column and eluted batchwise at pH 10.5 with an aqueous solution of sodium hydroxide (total 1,000 liters). The resin eluate was neutralized (pH 7) with 20% (w/v) aqueous sulfuric acid. The resin was then washed with three portions of deionized water (150 liters total). The water washes were neutralized and combined with the neutralized eluate. The resulting solution was concentrated and subsequently freeze dried. The preparations of the crude complex varied in color from tan to dark brown and potencies were found to range between 250 and 500 units/mg.

In order to remove salts, the crude complex (1.0 kg) was slowly added with vigorous stirring to deionized water (1.5 liters). The resulting suspension was allowed to stir for twenty minutes and was subsequently neutralized (pH 7) using a 10% aqueous ammonium hydroxide solution. The insoluble actaplanin complex was separated by vacuum filtration, washed with deionized water and freeze dried. The dried, desalted complex was recovered in approximately 80% yield (based on bioactivity); potencies varied between 400 and 800 units/mg.

The dried, desalted complex (300 g) was suspended in deionized water (2 liters), and the pH of the suspension was adjusted to pH 2.7 by addition of 3 N aqueous hydrochloric acid. The acidified solution was centrifuged for 40 minutes at 2,500 rpm. The supernatant was decanted and loaded on a column (8×85 cm) containing 6 liters of decolorizing resin (Duolite S761). The activity was eluted with deionized water at a flow rate of 30 ml/minute. The elution was monitored by thin-layer chromatography. The actaplanin containing effluent was concentrated (3 mm, 35°C) to a volume of 3 liters and freeze dried. The decolorized complex was recovered as a white to tan solid in approximately 70% yield (based on bioactivity). Potencies of the dried, decolorized complex varied between 600 and 1,000 units/mg.

Separation of the Purified Actaplanin Complex by Low Pressure Liquid Chromatography

The dried, decolorized complex (5~10 g) was dissolved in deionized water (50~100 ml). The resulting aqueous solution was filtered and loaded on a chromatography column (5×100 cm) containing 2 liters of polyamide (Machery and Nagel SC6). The column was eluted with deionized water (flow rate: 5 ml/minute, inlet pressure: 1.4~2.5 kg/cm^2) and 200~300 fractions (25 ml each) were collected. The elution was monitored by UV detection (254 nm using an ISCO Model UA-5 detector) and by TLC. Fractions were combined according to TLC identity and freeze dried. For some of the separations it was necessary to double the column length (200 cm) by using two of the polyamide columns in series. Additional purification was achieved by repeated rechromatography. Potencies of the individual actaplanins A, B_1, B_2, B_3, C_1, and G varied between 950 and 1,489 units/mg.

Actaplanin Aglycone Formation

Actaplanin complex (3.0 g) was dissolved in 25 ml of H_2O, acidified with 3.75 ml of 4 N HCl and refluxed for 1.5 hours. After cooling, the mixture was evaporated to dryness (40°C, *in vacuo*), and redissolved in hot water. The aglycone was precipitated from solution by dropwise addition of 4 N HCl. The precipitate was collected by filtration after cooling and dried under reduced pressure over P_2O_5 to give 2.0 g of a tan powder. This product was examined by TLC (cellulose/BPAW) and paper chromatography and shown by bioautography in this system to consist of two active components: **2** (Rf 0.88) and **3** (Rf 0.72) [cellulose/aluminum TLC plates; butanol - pyridine - HOAc - H_2O, 15: 10: 3: 12 (BPAW)]. This product was chromatographed twice on 200 g of alumina (acidic, Grade I) which had previously been washed with methanol. Elution with methanol (500 ml) and with 10% H_2O - methanol separated these two components. Bioassay showed **1** to have an antimicrobial assay of 200 units/mg while **2** assayed 440 units/mg.

Bioautographic Study of Actaplanin Hydrolysis

A solution of 2.8 g of actaplanin complex was dissolved in 112 ml of 0.1 N HCl and heated to reflux. At various time intervals, 10.0 ml aliquots of this reaction mixture were withdrawn, the pH adjusted to 7 and the precipitate isolated by filtration after concentration to the point of maximum precipitation. The isolated hydrolysis products were weighed, bioautographed after TLC and paper chromatography and bioassayed. The results of this study are shown in Fig. 4.

Actaplanin Ψ-Aglycone (**1**)

Actaplanin complex (2.0 g) was dissolved in 50 ml of 5% methanolic HCl and refluxed for 70 minutes. The reaction mixture was evaporated to dryness at 35~40°C under reduced pressure. The residue was diluted with a small amount of water which resulted in formation of a solid which was isolated by filtration. This solid was air dried, dissolved in a small amount of methanol and reprecipitated by the addition of acetonitrile until a granular solid formed. This solid was filtered and dried. Analytical HPLC analysis (Gradient: 2% aqueous HOAc - CH_3CN (9: 1) to 2% aqueous HOAc - CH_3CN (6: 4) showed this substance to be a single homogeneous substance, **1**.

UV (MeOH) 280 nm (9,200); IR (KBr) 1720 cm^{-1}, 1650 cm^{-1}; NMR (DMSO-d_6) δ 1.22 (d, 3H, J=6 Hz), 2.01 (s, 3H), 3.58 (s, 3H), 6 phenolic protons between 9 and 10 ppm (s, 1H).

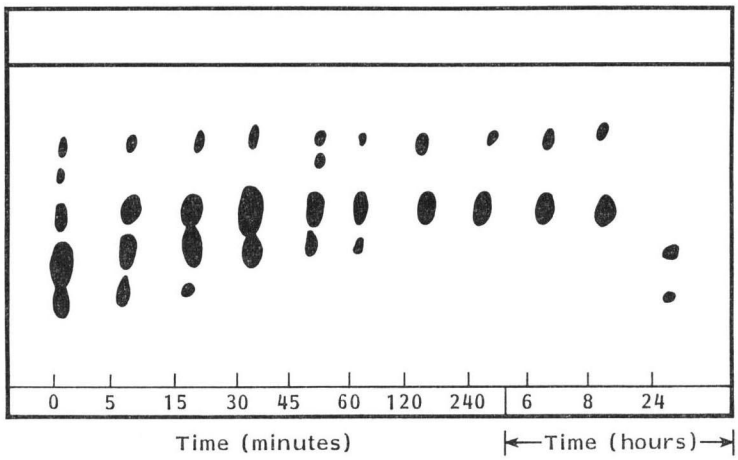
Isolation of Methyl α-L-Ristosaminide from Actaplanin Complex

A solution of actaplanin complex (10 g) in 500 ml of 3 N methanolic HCl was refluxed for 18 hours. The reaction mixture was filtered, and the filtrate was carefully concentrated to dryness (<50°C). The

Fig. 4. Bioautographic study of the products of mild hydrolysis of actaplanin complex over a 24-hour period (0.1 N HCl, reflux).

TLC system: cellulose/aluminum, BuOH - pyridine - HOAc - H_2O, 15: 10: 3: 12 (BPAW): ninhydrin spray.

Assay organism: *Bacillus subtilis*.



residual mass was dissolved in methanol (25 ml) and poured into a suspension of 80 g of BioRad AG-X4 (OH^-) resin in 170 ml of H_2O. After stirring for 10 minutes, more resin was added to raise the pH to 5.0 and the resin removed by filtration and washed twice with 40 ml of water. The pH of the clear filtrate was adjusted to 7.2 with 4 drops of conc NH_4OH and stirred for 30 minutes at room temperature. The resulting suspension was concentrated under reduced pressure and cooled to give the aglycone (4.05 g) after filtration. The filtrate was passed over a Dowex 50W-X12 (100~200 mesh, NH_4^+ form), and the column eluted with 1,500 ml of H_2O. The column was then eluted with solutions of increasing concentrations of aqueous ammonium hydroxide (0.1, 0.5, 1.0 and 2 N). All fractions were monitored by TLC (cellulose/aluminum, BPAW, ninhydrin spray). Fractions eluting with 2 N NH_4OH gave a solid (0.463 mg): Rf 0.7. This material was purified by treating a methanolic solution (2 ml) with excess ether. The mother liquors were concentrated to dryness after filtration and triturated with acetone to give a crystalline solid (**7**) (92 mg): mp 170~170.5°C (168~170°C)[12]; IR (KBr) 3270, 1620, 1505, 1080 cm^{-1}; MS *m/z* 162 (M^+), 143, 130, 117 and 111; $[\alpha]_D$ −130.3° (*c* 1, MeOH), CD (Cupra A) ΔE_{580} −0.14, ΔE_{290} +0.70; NMR (D_2O) δ 1.75 (d, 3H, *J*=6 Hz, C-5 CH_3), 2.33 (t, 2H, *J*=3 Hz, C-2 axial, C-2 equatorial), 3.83 (3H, C-1 OCH_3), 4.16 (m, 2H, C-3, and C-4 H), 4.33 (m, 1H, C-5 H), 5.32 (t, 1H, *J*=2.5, 3.0 Hz, C-1 H); (Pyridine-d_5) 1.47 (d, 1H, *J*=6 Hz, C-5 CH_3), 2.72 (q, 1H, *J*=16, 3 Hz, C-2 axial), 2.12 (dt, 1H, *J*=16, 4 Hz, C-2 equatorial).

Anal Calcd for $C_7H_{16}O_3Cl$: C 42.54, H 8.16, N 7.07, Cl 17.94
Found: C 42.25, H 8.36, N 7.12, Cl 17.73

The above data were identical with those reported for methyl α-L-ristosaminide hydrochloride (**4**)[12].

Compound (**4**) (100 mg) was converted to its *N,O*-diacetyl derivative (**5**) by treatment with 4 ml of a 1: 1 mixture of Ac_2O in pyridine (24 hours, 25°C). The solvents were evaporated under reduced pressure, and the residue was chromatographed on silica gel (benzene, methanol mixtures). The fractions eluting with 15% MeOH in benzene gave an oil (93 mg) which crystallized from petroleum ether (30~60°C) as needles (67 mg): mp 45~46°C (ref. 51~52°C)[12].

NMR ($CDCl_3$) δ 1.20 (d, 3H, *J*=6 Hz, C-5 CH_3), 1.86 (dbs, 1H, *J*=14, 1.5 Hz, C-2 equatorial), 1.98 (s, 3H, C-3 NHAc), 1.99 (s, 3H, C-4 OAc), 2.07 (d t, 1H, *J*=14, 3.5 Hz, C-2 axial), 3.40 (s, 3H, C-1 OCH_3), 3.92 (m, *J*=6.0 Hz, C-5 H), 4.52 (dd, 1H, *J*=4.0, 9.0 Hz, C-4 H), 4.58 (m, C-3 H), 4.73 (dd, 1H, *J*=4.0, 1 Hz, C-1 H); MS (electron impact) *m/z* 214 (M^+−31), 185, 170, 153, 142, 128, 117, 114, 101; IR ($CHCl_3$) 1740, 1680, 1510 cm^{-1}.

Anal Calcd for $C_{11}H_{19}NO_5$: C 53.87, H 7.81, N 5.71
Found: C 53.58, H 7.90, N 5.91

Chemical Degradation of the Individual Actaplanins

Neutral Sugar Determination: Actaplanin A (50 mg) was dissolved in 15 ml of boiling 2 N HCl and refluxed for 2 hours. The reaction mixture was reduced to 25% of its original volume, and the precipitated aglycone removed by filtration. The filtrate was evaporated to dryness and 4 ml of H_2O added. This solution was passed through a small Dowex 50W-X12 (H^+ form, 100~200 mesh) ion exchange column and eluted with water. The early fractions were pooled and evaporated to a syrup which was treated with 0.5 ml of trimethylsilylimidazole. The silylation mixture was analyzed by gas liquid chromatography using the conditions described in the list of instruments above[15]. The analytical results are summarized in Table 2.

Ψ-Aglycone Formation: Each individual actaplanin (A, B_1, B_2, B_3, C_1 and G) was methanolized according to the procedure used above to produce **1** from the actaplanin complex. Each actaplanin gave a product identical in all respects with **1**.

L-Ristosamine Determination in Actaplanins A and B_2: Actaplanin B_2 (50 mg) was dissolved in 2 ml of pyridine and 0.5 ml of benzoyl chloride. After stirring for 24 hours at room temperature, the reaction mixture was evaporated to dryness. The residual material was dissolved in 3 N methanolic HCl and refluxed for 8 hours, cooled and evaporated to dryness. The resulting gummy solid was extracted with a 3:1 mixture of ethyl acetate and water, and the aqueous layer extracted two additional times with EtOAc. After drying over Na_2SO_4, the organic layer was evaporated to dryness. The residue was extracted directly with Et_2O. The ether extract was compared to authentic methyl *N,O*-dibenzoyl-α-L-ristosaminide by TLC (silica gel: 20% EtOAc - toluene). The ether extract contained a compound which had the same Rf (0.56) as the authentic material. Field desorption mass spectral analysis showed (M+1) peaks at *m/z* 370 and *m/z* 356 corresponding to the molecular ions for methyl *N,O*-dibenzoyl-α-L-ristosaminide[18] and *N,O*-dibenzoyl-L-ristosamine, respectively.

This procedure was repeated for actaplanin A. The TLC and mass spectral results indicated the presence of L-ristosamine.

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