PREPARATION AND CHARACTERIZATION OF SOME BROMINE ANALOGS OF THE GLYCOPEPTIDE ANTIBIOTIC ACTAPLANIN

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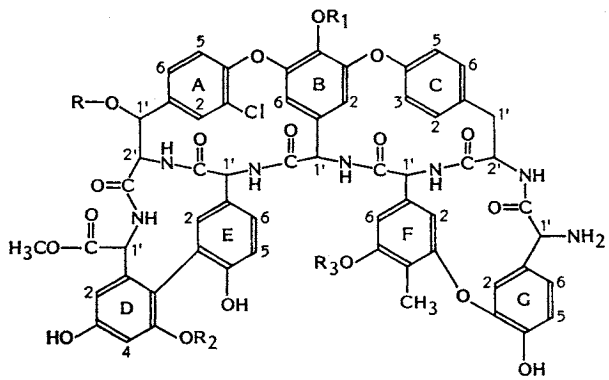
Actaplanin (Fig. 1) also known as A4696, is an antibiotic complex of chlorinated compounds containing amino acids, hexoses, and L-ristosamine[1~3]. In order to alter the biological activities of various chlorinated compounds, numerous investigators have supplied organically bound fluorine and bromine to cultures producing such antibiotics[4~6]. Bromine of inorganic origin has been found to be incorporated into microbial metabolites by others[7]. During our studies on the biosynthesis of actaplanins it was decided to determine if *Actinoplanes missouriensis* (ATCC 23342) could use inorganic bromine to form the bromo analogs of the actaplanins. This report documents our progress in examining that phenomenon.

Fermentation

A natural selectant of *A. missouriensis* on an agar slant was used to inoculate 800 ml of seed medium in a 2-liter Erlenmeyer flask. The seed medium contained (w/v); glucose 0.5%, potato dextrin 2.0%, Nutrisoy flour 1.5%, yeast extract 0.25%, $CaCO_3$ 0.1% and tap water. The culture was incubated on a rotary shaker (250 rpm, 5-cm throw) at 28°C for 48 hours. Four hundred ml of the primary seed culture were used to inoculate 950 liters of secondary seed medium in a stirred stainless steel reactor. The secondary seed medium contained (w/v); glucose 0.75%, $CaCO_3$ 0.15%, yeast 0.28%, Nutrisoy flour 2.25%, corn starch 3.0% and tap water. The culture was incubated at 30°C for 48 hours with sufficient aeration and agitation to maintain the dissolved oxygen at greater than 20%. The secondary seed culture (5 ml) was used to in-

Fig. 1. Structures of the actaplanin complex.



R=Ristosamine

Actaplanins	R_1	R_2	R_3	MW
A	MG	M	M	1,968
B_1	RG	M	M	1,952
B_2	G	M	M	1,806
B_3	MG	M	H	1,806
C_1	RG	M	H	1,790
G	G	M	H	1,644
ϕ	H	H	H	1,320

MG: Mannosylglucose, RG: rhamnosylglucose, G: glucose, M: mannose, ϕ: actaplanin pseudoaglycon, MW: integer molecular weight.

oculate 80 ml of the production medium contained in 500-ml Erlenmeyer flasks. The production medium contained (w/v); glucose 1.43%, beet molasses 1.13%, glycerol 2.88%, K_2HPO_4 0.04%, $(NH_4)_2SO_4$ 0.01%, yeast 1.5%, $CaCO_3$ 0.38%, potato dextrin 0.75% and tap water. Prior to sterilization the pH of the medium was adjusted to 7.0 with NaOH. The flasks were incubated at 30°C on a rotary shaker (250 rpm, 5-cm throw) for 48 hours. To each flask was then added 80 mg of NaBr in 5 ml of deionized water. After an additional 72 hours of incubation, the fluids from 50 flasks were pooled and 46 ml of 12 N HCl added to the mixture. The acidified broth was centrifuged at $10,000 \times g$ for 20 minutes and decanted. The supernatant was neutralized with NaOH and frozen until processed further.

Isolation

Eight liters of supernatant were applied to a glass column (5.7×19 cm) packed with Diaion HP-20 (Mitsubishi Chemical Industries Limited). The effluent was discarded and the column developed using H_2O, H_2O - CH_3OH, H_2O - CH_3OH - CH_3COOH mixtures. Each fraction was analyzed using HPLC (Table 1) and the active fractions combined, concentrated and lyophilized to give preparations I (8.6 g) and II (6.0 g). One g of II was dissolved in 20 ml of H_2O, adjusted to pH 3.5 with HCl and applied to a glass column (5.1×42 cm) packed with 10~20 μm silica gel based C18 reversed phase resin[8]. The column was developed at a flow-rate of 10 ml/minute with a linear gradient of (A) 2 liters of aqueous 0.5 M $NH_4H_2PO_4$ - CH_3CN (95 : 5) and (B) 2 liters of aqueous 0.5 M $NH_4H_2PO_4$ - CH_3CN (80 : 85). The eluate was collected in 25-ml fractions. Fraction 67 was concentrated, desalted and lyophilized to yield 17 mg of A4696-Brβ. To isolate A4696-Brα, 1 g of II was dissolved in 10 ml of H_2O and processed as just described. Active fractions were combined, concentrated, desalted and lyophilized to give 172 mg of partially purified A4696-Brα. Rechromatography of that material using identical conditions but a smaller column gave 84 mg of pure A4696-Brα.

Table 1. Analytical HPLC of A4696-Brα and A4696-Brβ.

Factor	Retention time (minutes)
A4696-Brβ	4.27
A4696-Brα	9.22

Column: Stainless steel, 4.5×50 mm, IBM, 5 μm, ODS. Solvents: (A) CH_3CN - aqueous 0.1 M $NH_4H_2PO_4$ (5 : 95), pH 4.7. (B) CH_3CN - aqueous 0.1 M $NH_4H_2PO_4$ (15 : 85), pH 4.7. Gradient: Isocratic 25% B to 50% B in 10 minutes. Flow rate: 1.0 ml/minute. Detection: 254 nm.

Structure Elucidation

Actaplanins have a common peptide core, the amino sugar ristosamine and various neutral sugars (Fig. 1). The two new antibiotics, A4696-Brα and A4696-Brβ, were examined by fast atom bombardment mass spectrometry (FAB-MS) and the results were compared with molecular weights expected for actaplanins. The integer molecular weight for A4696-Brβ was found to be 1,996 or 44 mass units greater than that for actaplanin B_1. The increase of 44 mass units suggests the loss of Cl and gain of a Br in the actaplanin B_1 molecular formula. The integer molecular weight observed for A4696-Brα was 1,850, suggesting that this substance may be a bromo-analog of either actaplanin B_2 or B_3.

The A4696-Brα material (15 mg) was hydrolyzed for 3.5 hours in 5 ml of refluxing 0.5 N aqueous HCl under N_2, followed by adjustment of the pH of the cooled solution to 6.4 with anion exchange resin. After filtration the gum was analyzed for carbohydrates. Mannose and glucose were found to be present by both paper chromatography and HPLC (Waters Carbohydrate Analysis Column, refractive index monitor). The HPLC analysis indicated that mannose and glucose were present in a 2 : 1-ratio, which is the same as that found in actaplanins B_2 and B_3 (Fig. 1).

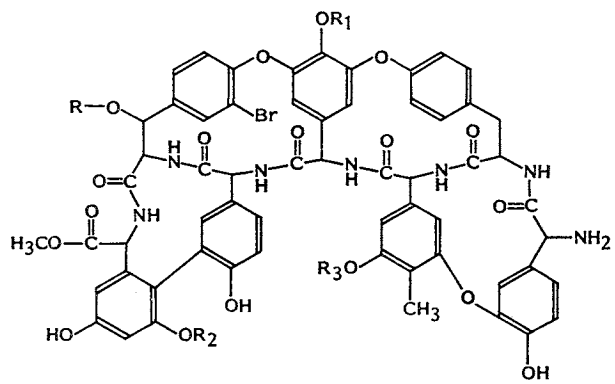
An additional 25 mg of A4696-Brα were subjected to acidic methanolysis (3% HCl in methanol, refluxed) and treated according to methods previously described[9] to prepare 12.5 mg of the ψ-aglycon dihydrochloride of A4696-Brα. The bromo-ψ-aglycon was examined by FAB-MS and 470 MHz ^1H NMR spectroscopy. The FAB-MS of the bromo-ψ-aglycon indicated an integer molecular weight of 1,364, or 44 mass units higher than the actaplanin ψ-aglycon (Fig. 1). The bromine is therefore associated with the nucleus of the antibiotic. A comparison of

Table 2. ^{1}H NMR resonance assignments (DMSO-d_6, δ).

Assignment[a]	Actaplanin ϕ-aglycon		Bromo-ϕ-aglycon from A4696-Brα		Assignment[a]	Actaplanin ϕ-aglycon		Bromo-ϕ-aglycon from A4696-Brα	
G-4	9.98	Phenol	9.99	Phenol	D-4	6.42	s	6.43	s
F-5	9.57	Phenol	9.60	Phenol	F-2	6.40	s	6.40	s
D-3	9.51	Phenol	9.52	Phenol	F-6	6.38	s	6.37	s
B-4	9.47	Phenol	9.47	Phenol	D-2	6.06	s	6.06	s
E-4	9.31	Phenol	9.32	Phenol	Rist-OH	6.00		6.03	s
D-NH	9.05		9.08		B-2	5.63	s	5.63	s
D-5	8.97	Phenol	8.97	Phenol	B-1′	5.61	d	5.60	d
G-NH_3^+	8.57	Intense	8.57	Intense	G-1′	5.51	br s	5.53	br s
E-NH	8.57	peak	8.57	peak	F-1′	5.27	d	5.26	d
C-NH	7.98	d	7.97	d	A-1′	5.07	s	5.06	s
C-2	7.85	d	7.88	d	B-6	5.03	s	4.98	s
A-2	7.70	s	7.85	s	C-2′	4.92	br s	4.91	br s
F-NH	7.62	d	7.65	d	Rist No. 1	4.79	br s	4.78	s
B-NH	7.60	d	7.59	d	E-1′	4.49	d	4.47	d
A-NH	7.49	d	7.53	d	D-1′	4.41	d	4.40	d
Rist-NH_3^+	7.40	br s	7.40	br s	A-2′	4.26	br d	4.24	br d
A-6	7.33	AB	~7.43	Overlapped	D-OCH_3	3.73	s	3.69	s
A-5	7.32	quartet	7.36		Rist No. 3	3.54	br s	3.53	br
C-5	7.19		7.26	d	Rist No. 4	3.33			DOH
G-6	7.19		7.20	Shoulder	C-1′	3.33			overlap
E-2	7.19	s	7.19	s	Rist No. 5	3.26	m	3.24	d?
G-5	7.09	d	7.09	d	C-1″	2.85	d	2.85	d
C-6	7.05	d	7.05	d	Rist No. 2	2.18	d	2.17	d
C-3	6.90	d	6.99	d	Rist No. 2	~2.00		~1.96	br
E-6	6.74	d	6.74	d	F-CH_3	1.96	s	1.96	s
G-2	6.72	s	6.71	s	Rist-CH_3	1.22	d	1.21	d
E-5	6.69	d	6.68	d					

[a] Actaplanin ϕ-aglycon chemical shifts and assignments from ref 2.

Fig. 2. Structures of the bromo-actaplanins.



R=Ristosamine

Bromo-actaplanin	R_1	R_2	R_3
A4696-Brα (bromo-B_2)	Glucose	Mannose	Mannose
A4696-Brβ (bromo-B_1)	Rhamnosyl-glucose	Mannose	Mannose
Bromo-ϕ-aglycon	H	H	H

the ^{1}H NMR assignments for the bromo-ψ-aglycon (470 MHz, DMSO-d_6) with those for the actaplanin ψ-aglycon[2] indicate a virtual identity of the two materials (Table 2). The two must differ only in the substitution of bromine for chlorine. The three ring A proton resonances show slight changes due to the halogen substitution. The neutral sugar distribution of the intact A4696-Brα was deduced to match that in actaplanin B_2, rather than actaplanin B_3, on the basis of chemical shift patterns for the proton resonances arising from rings D and F[3]. When the D-5 phenol is free, as it is for both pseudoaglycons in Table 2, the two ring D proton resonances occur at ~6.42 ppm and ~6.06 ppm. When no sugar is attached through the F-5 phenol the ring F proton peaks are both near 6.40 ppm. When either phenol has mannose attached (Fig. 1), the corresponding ring proton resonances shift downfield. Compounds A4696-Brα and A4696-Brβ were both examined by ^{1}H-^{1}H two-dimensional (2D) correlation NMR (DMSO-d_6, 270 MHz for Brα and 250 MHz for Brβ) and the aromatic proton regions of the two spectra were virtually superimposable. Both antibiotics had D-ring resonances at 6.23 and 6.78 and F-ring peaks at 6.58 and 6.70, indicating that both compounds have sugars attached at the D-5 and F-5 phenols. This distribution for A4696-Brα matches that for actaplanin B_2 rather than that for actaplanin B_3 (Fig. 1). Although the carbohydrate content of A4696-Brβ was not determined by chemical analysis, comparison of the ^{1}H NMR spectra of A4696-Brβ and of actaplanin B_1 suggests that with the exception of the halogen atom the two materials are identical. The structures of the brominated glycopeptides are summarized in Fig. 2.

Acknowledgments

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