BENZANTHRINS A AND B, A NEW CLASS OF QUINONE ANTIBIOTICS

I. DISCOVERY, FERMENTATION AND ANTIBACTERIAL ACTIVITY

ROBERT J. THERIAULT*, RONALD R. RASMUSSEN, WILLIAM L. KOHL, JOSEPH F. PROKOP, THOMAS B. HUTCH and GRANT J. BARLOW

Pharmaceutical Products Division, Department 47P, Abbott Laboratories, Abbott Park, Illinois 60064, U.S.A.

(Received for publication June 19, 1986)

Nocardia lurida has been shown to produce two novel quinone antibiotics, benzanthrins A and B. The antibiotics were discovered in concentrated butanol extracts of fermentation broths and were separated by TLC and HPLC. Benzanthrins A and B were produced in a fermentation medium consisting of glucose, yeast, selected peptones and $CaCO_3$. The antibiotics were present primarily at 66 hours in shake flask fermentations and from 66 to 162 hours in 14-liter fermentors. Benzanthrins A and B inhibited a number of Gram-positive pathogenic bacteria but were inactive against Gram-negative bacteria.

Nocardia lurida, which is known to produce the glycopeptide antibiotics ristocetins A and B[1~4], was examined for the production of other antibiotic substances. Two novel quinone antibiotics benzanthrins A and B were discovered in concentrated butanol extracts of fermentation broths by TLC and bioautography against *Staphylococcus aureus* 6538P. This paper reports the discovery, analysis, fermentation and antibacterial activity of benzanthrins A and B. The isolation, determination of structure and antitumor activity of benzanthrins A and B are described in an accompanying paper[5].

Materials and Methods

Microorganisms

A phage-resistant variant of *N. lurida* NRRL 2430 designated B7 was used in fermentation studies for the production of benzanthrins A and B. The bacterial strains used to determine the antibacterial spectrum of benzanthrins A and B were from the stock culture collection in our laboratory and from the American Type Culture Collection (ATCC).

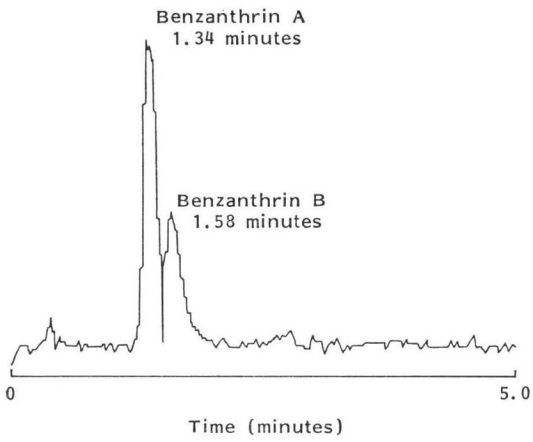
Fermentation Studies

N. lurida B7 was grown on a slant medium consisting of glycerol 1%, L-(+)-arginine 0.25%, NaCl 0.1%, K_2HPO_4 0.1%, $FeSO_4 \cdot 7H_2O$ 0.01%, $MgSO_4 \cdot 7H_2O$ 0.01%, $CaCO_3$ 0.02%, starch 0.25% and agar 2%. Slants were incubated for 7 days at 28°C and then stored at 4°C until needed.

Agar slant cultures of *N. lurida* B7 were used to inoculate 500-ml Erlenmeyer flasks containing 100 ml of seed medium consisting of glucose monohydrate 1.5%, soybean meal 1.5%, NaCl 0.5%, and $CaCO_3$ 0.1% in distilled water. No pH adjustment was made prior to sterilization. Seed flasks were incubated at 24°C for 48 hours on a rotary shaker at 250 rpm (3.2 cm stroke). Five percent vegetative inoculum was then used to inoculate fermentation flasks or New Brunswick 14-liter fermentors. Seed and fermentation flasks were closed with rayon plugs and sterilized for 30 minutes at 121°C and 1.06 kg/cm^2. Fermentation flasks were incubated on a rotary shaker at 250 rpm (3.2 cm stroke).

Fermentations in New Brunswick 14-liter fermentors were charged with a medium which consisted of glucose monohydrate (added post sterilization) 3%, Lexein F-152 liquid peptone (Inolex) 2%, whole yeast (Red Star) 0.5%, NaCl 0.5%, $CaCO_3$ 0.1% and P-2000 antifoam (Dow) 0.01% in distilled

Fig. 1. HPLC separation of benzanthrins A and B.



water. No pH adjustment was made prior to sterilization for 90 minutes at 121°C and 1.06 kg/cm^2. Fermentor volume, agitation and aeration were varied. Fermentors were incubated at 24°C.

Packed cell volumes were determined by centrifuging at $600 \times g$ for 30 minutes in 15 ml conical tubes. Glucose concentrations were determined by the method of HOFFMAN[6].

TLC/Bioautography

Fermentation broths were adjusted to pH 7, saturated with NaCl and extracted three times with 1/2 vol of BuOH. The combined BuOH extracts were evaporated to dryness under reduced pressure and the residue was reconstituted in MeOH at a concentration 25 times greater than the original fermentation broth. The extracts were then applied at a level of 25 μl on 20× 20 cm Fisher Redi-Plate silica gel GF 250 μm TLC plates. The plates were developed for 1 hour in a solvent system consisting of $CHCl_3$ - MeOH - H_2O (70: 30: 1). The TLC plates were air dried and bioautographed against *S. aureus* 6538P. A contact time of 1 hour was allowed between TLC plates and seeded agar, and the agar plates were incubated at 32°C overnight.

HPLC

HPLC analyses were performed on a Perkin-Elmer system consisting of a Series 4 liquid chromatograph, LC-85 spectrophotometric detector with LC autocontrol (set at 430 nm), an ISS 100 autosampler and a 3600 data station. Benzanthrins A and B were chromatographed on a high speed Regis 3 μm C-18 column (4.6×30 mm) using a quaternary solvent system consisting of 0.1 M NaOH in H_2O - CH_3CN (50: 50) in reservoir A, 0.1 M Et_3N in H_2O - CH_3CN (50: 50) in reservoir B, 0.1 M H_3PO_4 in H_2O - CH_3CN (50: 50) in reservoir C and H_2O in reservoir D. By systematically varying the volumes isocratically delivered from the four reservoirs, it was possible to study the effects of Na^+, H^+, Et_3N, CH_3CN, H_2O and total ionic strength on the separation[7]. An optimum system (pH 3.5) was found where %A=7.5, %B=19, %C=34.5 and %D=39 at a flow rate of 1.5 ml/minute (Fig. 1).

Fermentation samples selected for HPLC analysis were adjusted to pH 8.5 and extracted with 1/2 vol of CH_2Cl_2 three times. The CH_2Cl_2 extracts were combined, solvent was removed under reduced pressure and the residue was reconstituted in MeOH to a concentration 25 times that of the original fermentation broth. Each MeOH sample was then filtered through a Millipore SJHV syringe filter. Quantitative analyses were conducted using an external standard consisting of 46% benzanthrin A and 54% benzanthrin B. The data were processed with Perkin Elmer Chromatographics 2 software. Repeated 5 μl injections of the external standard demonstrated a 10% margin of error in the analysis.

In Vitro Potency Determinations

The MICs of benzanthrins A and B were determined using the standard agar dilution procedure[8] on brain heart infusion agar.

Results and Discussion

Discovery and Analysis of Benzanthrins A and B

A number of *N. lurida* isolates were grown in flasks in various fermentation media and examined for the presence of antibiotics other than ristocetins A and B. Concentrated butanol extracts, which were chromatographed on silica gel TLC plates in a solvent system consisting of $CHCl_3$ - MeOH - H_2O (70 : 30 : 1), revealed two orange colored products, benzanthrins A and B, at Rf's 0.15 and 0.23,

Table 1. Effect of different nitrogen sources on yields of benzanthrins A and B in shake flasks.

Nitrogen source	Level (%)	Age (hours)	Peak yield (mg/liter)	
			Benzanthrin A	Benzanthrin B
Lexein F-152 liquid peptone (Inolex)	2.0	66	170	60
Lexein F-1000 liquid peptone (Inolex)	2.0	66	185	43
Lexein F-159 liquid peptone (Inolex)	2.0	66	<1	<1
Lactalbumin	2.0	66	<1	<1
Fish flour	2.0	66	<1	<1
Spray dried lard water	2.0	66	<1	<1
SB (Beef) peptone (Marcor)	1.0	66	52	19
Peanut digest (Marcor)	1.0	66	9	5
Beef extract (Marcor)	1.0	42	52	26
Soybean meal	2.0	66	<1	<1

Basal medium: Glucose monohydrate (added post sterilization) 3%, yeast (Red Star) 0.5%, NaCl 0.5%, $CaCO_3$ 0.1%, distilled water, no pH adjustment.
Volume/flask: 50 ml/500 ml-Erlenmeyer flask, rayon plugs.
Incubation temperature: 24°C.

Table 2. Effect of temperature and volume of medium on the production of benzanthrins A and B in shake flasks.

Temperature (°C)	Volume/500-ml flask (ml)	Age (hours)	Benzanthrin A (mg/liter)	Benzanthrin B (mg/liter)
24	50	42	<1	<1
24	50	66	170	60
24	50	90	2	<1
24	50	114	<1	<1
28	50	42	17	1
28	50	66	<1	<1
28	50	90	<1	<1
28	50	114	<1	<1
30	50	42	<1	<1
30	50	66	4.3	<1
30	50	90	<1	<1
30	50	114	<1	<1
24	100	42	<1	<1
24	100	66	<1	<1
24	100	90	<1	<1
24	100	114	<1	<1

Medium: Glucose monohydrate (added post sterilization) 3%, yeast (Red Star) 0.5%, Lexein F-152 liquid peptone (Inolex) 2%, NaCl 0.5%, $CaCO_3$ 0.1%, distilled water, no pH adjustment.

respectively. Both inhibited *S. aureus* 6538P by bioautography and each antibiotic could be quantitatively extracted with CH_2Cl_2. This provided a useful method of fermentation broth treatment to obtain relatively clean samples for quantitative HPLC analysis. The high speed HPLC assay which was developed (see Materials and Methods) allowed for rapid analysis of large numbers of fermentation samples resulting from yield improvement studies.

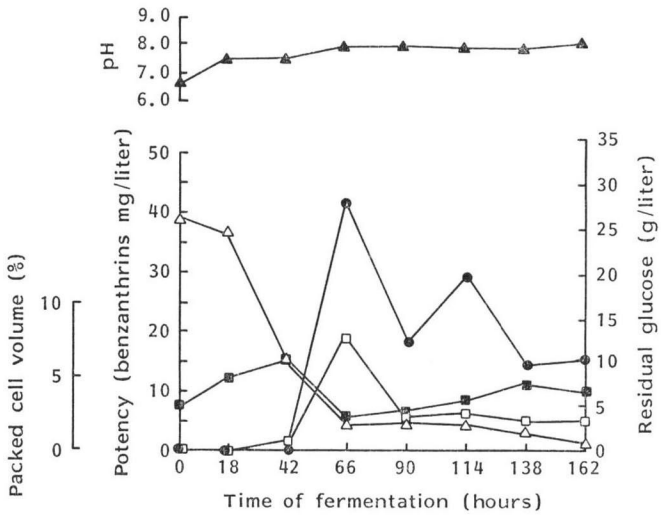
Fermentation

N. lurida B7, which produced the highest levels of benzanthrins A and B among strains examined

Table 3. Effect of agitation, aeration and volume of medium on yields of benzanthrins A and B in New Brunswick 14-liter fermentors.

Fermentor volume (liters)	Agitation (rpm)	Aeration (vol/vol/minute)	Age (hours)	Peak yield (mg/liter)	
				Benzanthrin A	Benzanthrin B
10	300	1	162	20	11
7	300	1	162	4	2
10	300	0.5	66	42	19
7	300	0.5	66	70	<1
10	150	1	114	17	17
7	150	1	114	23	10
10	150	0.5	114	<1	<1
7	150	0.5	114	<1	<1

Fig. 2. Time course of fermentation in a 14-liter fermentor.
●; Benzanthrin A, □; benzanthrin B, ■; packed cell volume, △; residual glucose, ▲; pH.



in shake flasks, was used for all fermentation studies reported here. The effect of ten different nitrogen sources on yields in shake flasks is shown in Table 1. Fermentation broth samples were analyzed at 42, 66, 90 and 114 hours and peak levels of benzanthrins A and B always occurred at 66 hours (Table 2). Lexein F-1000 and F-152 liquid peptones gave the highest peak benzanthrin A yields of 185 and 170 mg/liter, respectively. Benzanthrin B levels were significantly lower. Five of the nitrogen sources failed to produce any detectable benzanthrin A or B.

The effect of different temperatures and volumes of fermentation medium in flasks is shown in Table 2. The highest levels of benzanthrins A and B were produced by 66 hours using 50 ml of medium per flask and incubating at 24°C. Antibiotic levels were reduced drastically by increasing the temperature or volume of the medium.

Results with varied agitation, aeration and volumes of medium in New Brunswick 14-liter fermentors are shown in Table 3. The highest levels of benzanthrin A were obtained at 66 hours with 300 rpm and an air rate of 0.5 vol/vol/minute. Under these conditions, benzanthrin B was not observed with the lower volume of medium. Benzanthrins A and B were not detected when lower

Table 4. *In vitro* potency of benzanthrins A and B against a variety of aerobic bacteria.

Organism	MIC (μg/ml)	
	Benzanthrin A	Benzanthrin B
Staphylococcus aureus ATCC 6538P	3.1	3.1
S. aureus CMX 686B	3.1	1.56
S. aureus A5177	3.1	1.56
S. aureus 45	3.1	1.56
S. epidermidis 3519	3.1	3.1
Lactobacillus casei ATCC 7469	0.78	0.78
Streptococcus faecium ATCC 8043	1.56	1.56
S. bovis A5169	0.39	0.2
S. agalactiae CMX 508	0.78	0.39
S. pyogenes EES 61	1.56	0.39
S. pyogenes 930	0.2	0.2
Micrococcus luteus 9341	1.56	1.56
Escherichia coli JUHL	>100	>100
E. coli SS	3.1	6.2
E. coli DC-2	>100	>100
E. coli H560	>100	>100
E. coli KNK 437	>100	>100
Enterobacter aerogenes ATCC 13048	>100	>100
Klebsiella pneumoniae ATCC 8045	>100	>100
Pseudomonas aeruginosa BMH #10	>100	>100
P. aeruginosa 5007	>100	>100
P. aeruginosa K799/WT	>100	>100
P. aeruginosa K799/61	>100	>100
P. cepacia 296	>100	>100
Acinetobacter sp. CMX 669	>100	>100

agitation and aeration rates were used. Benzanthrins A and B were not observed until the glucose levels fell below 10 g/liter. These results are shown graphically in Fig. 2.

Antimicrobial Activity

The MICs of benzanthrins A and B against a variety of aerobic Gram-positive and Gram-negative bacteria are shown in Table 4. Benzanthrin A was more potent than benzanthrin B. Both antibiotics were quite active against all of the Gram-positive bacteria. With the exception of the supersensitive strain of *Escherichia coli*, both were inactive against Gram-negative bacteria.

References

1) GRUNDY, W. E.; A. C. SINCLAIR, R. J. THERIAULT, A. W. GOLDSTEIN, C. J. RICKHER, H. B. WARREN, Jr., T. J. OLIVER & J. C. SYLVESTER: Ristocetin, microbiological properties. *In* Antibiotics Annual 1956~1957. *Eds.*, H. WELCH & F. MARTI-IBAÑEZ, pp. 687~692, Med. Encyclopedia, Inc., New York, 1957
2) GRUNDY, W. E.; E. F. ALFORD, E. J. RDZOK & J. C. SYLVESTER: Ristocetin, the development of resistance and bactericidal activity. *In* Antibiotics Annual 1956~1957. *Eds.*, H. WELCH & F. MARTI-IBAÑEZ, pp. 693~698, Med. Encyclopedia, Inc., New York, 1957
3) PHILIP, J. E.; J. R. SCHENCK & M. P. HARGIE: Ristocetins A and B, two new antibiotics, isolation and properties. *In* Antibiotics Annual 1956~1957. *Eds.*, H. WELCH & F. MARTI-IBAÑEZ, pp. 699~705, Med. Encyclopedia, Inc., New York, 1957
4) ROMANSKY, M. J.; B. M. LIMSON & J. E. HAWKINS: Ristocetin, a new antibiotic, laboratory and clinical studies. *In* Antibiotics Annual 1956~1957. *Eds.*, H. WELCH & F. MARTI-IBAÑEZ, pp. 706~715, Med. Encyclopedia, Inc., New York, 1957
5) RASMUSSEN, R. R.; M. E. NUSS, M. H. SCHERR, S. L. MUELLER, J. B. MCALPINE & L. A. MITSCHER:

Benzanthrins A and B, a new class of quinone antibiotics. II. Isolation, elucidation of structure and potential antitumor activity. J. Antibiotics 39: 1515~1526, 1986

6) HOFFMAN, W. S.: A rapid photoelectric method for determination of glucose in blood and urine. J. Biol. Chem. 120: 51~55, 1937

7) KIEL, J. S.; S. F. MORGAN & R. K. ABRAMSON: Effect of amine modifiers on retention and peak shape in reversed phase high performance liquid chromatography. J. Chromatogr. 320: 313~323, 1985

8) WASHINGTON, J. A. & V. C. SUTTER: Dilution susceptibility test: Agar and macro-broth dilution procedure. *In* Manual of Clinical Microbiology. *Ed.*, E. H. LENNETTE *et al.*, pp. 453~458, Am. Soc. for Microbiol., Washington D.C., 1980