BENZANTHRINS A AND B, A NEW CLASS OF QUINONE ANTIBIOTICS

II. ISOLATION, ELUCIDATION OF STRUCTURE AND POTENTIAL ANTITUMOR ACTIVITY

RONALD R. RASMUSSEN*, MERRILL E. NUSS, MICHAEL H. SCHERR, SANDRA L. MUELLER and JAMES B. MCALPINE

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

LESTER A. MITSCHER

Department of Medicinal Chemistry, The University of Kansas, Lawrence, Kansas 66045-2500, U.S.A.

(Received for publication June 19, 1986)

The benzanthrins, which were produced by *Nocardia lurida*, were extracted from the fermentation broth with CH_2Cl_2. Subsequent purification on Sephadex LH-20 and diol-bonded silica gel, followed by countercurrent chromatography, afforded analytically pure benzanthrins A and B. FAB-MS revealed that benzanthrins A and B were isomeric. It was demonstrated through degradative and spectroscopic studies that the benzanthrins were diglycosides of a trihydroxy benz[a]anthraquinone chromophore where one of the sugars was linked through carbon and the other through oxygen. Benzanthrins A and B differed in the stereochemistry of the *O*-glycosidic sugar. Both compounds inhibited the growth of Gram-positive bacteria and 9KB, 9PS and 9ASK tumor cells in tissue culture.

Fermentation studies with *Nocardia lurida*, a known producer of the ristocetins, resulted in the additional production of two new antibiotics, benzanthrins A and B. The discovery, fermentation and antibacterial activity of the benzanthrins is the subject of the preceding paper[1]. This paper will deal with the isolation, structure determination and antitumor activity of these novel antibiotics.

Experimental

General Procedures

NMR spectra were obtained with a General Electric GN 500 MHz spectrometer. The samples were run in CD_2Cl_2 and referenced on the lock solvent (5.32 ppm). The 2D proton-carbon chemical shift correlation (CSCM) experiment was performed on the GN 500 using a 5 mm C-13 probe and a model 1280 computer. The CSCM pulse sequence employed for the experiment was essentially that of FREEMAN and MORRIS[2] modified to deliver a composite 180 degree pulse. Ninety degree pulse widths on the 5 mm C-13 probe were 15 μseconds for carbon and 30 μseconds for proton. Fixed delays, delta 1 and delta 2, were set to 3.3 and 1.7 mseconds, respectively, with phase-cycling providing quadrature in both dimensions. The 2D homonuclear *J*-correlation experiment (COSY)[3] was run on the GN 500 spectrometer using the 5 mm H-1 probe. The ninety degree proton pulse for this probe was 12 μseconds. Mass spectra were determined on a Kratos MS-50 spectrometer. High resolution measurements were made at 10,000 resolving power. UV spectra were determined with a Perkin-Elmer Lambda 3B UV/VIS spectrophotometer and IR spectra were measured with a Perkin-Elmer Model 521 grating spectrometer.

Fig. 1. Isolation and purification of benzanthrins A and B.

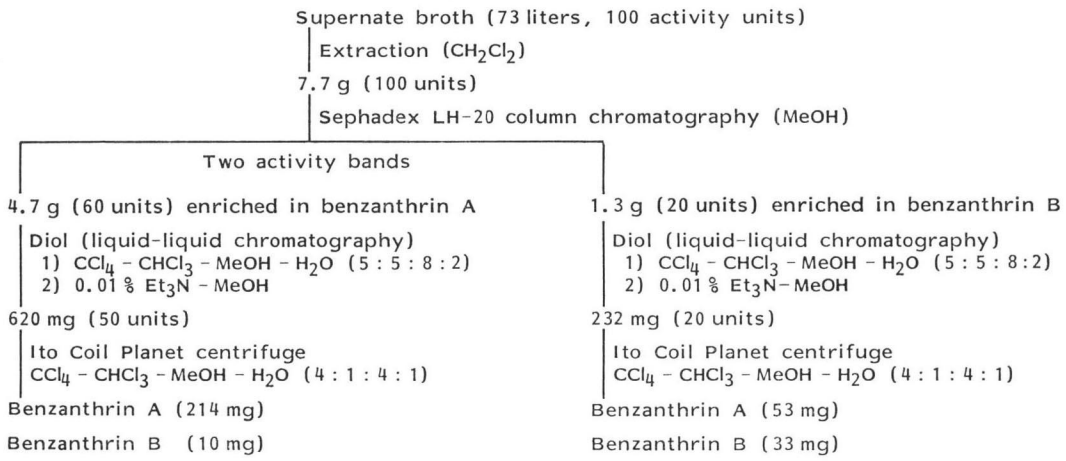


Table 1. Physico-chemical properties of benzanthrins A and B.

	Benzanthrin A	Benzanthrin B
Color: Solid state	Dark red	Dark red
Soln[a] (pH 5)	Red (orange)	Red (orange)
Soln[a] (pH 10)	Purple (blue)	Purple (blue)
Solubility[b]: pH 5	MeOH, DMSO, H_2O	MeOH, DMSO, H_2O
pH 9	MeOH, acetone, $CHCl_3$, Et_2O	MeOH, acetone, $CHCl_3$, Et_2O
FAB-MS (m/z) $(M+H)^+$	635.2957[c]	635.3001[c]
Molecular formula	$C_{35}H_{42}N_2O_9$	$C_{35}H_{42}N_2O_9$
UV: λ_{max}^{MeOH} nm (ε)	230 (47,000), 260 (24,000), 304 (25,500), 428 (8,200)	230 (49,400), 260 (25,200), 304 (26,700), 426 (8,400)
$\lambda_{max}^{MeOH+0.1N\ HCl}$ nm	230, 260, 304, 428	230, 260, 304, 426
$\lambda_{max}^{MeOH+0.1N\ NaOH}$ nm	230, 245, 290, 510	230, 243, 289, 510
TLC (Rf)[d]	0.30	0.46
HPLC (t_R, minutes)[e]	1.34	1.58

[a] aq MeOH.
[b] pH adjustment in aq MeOH followed by conc to dryness before checking solubility.
[c] Mass matched (theoretical, 635.2969, $C_{35}H_{43}N_2O_9$).
[d] $CHCl_3$ - MeOH - H_2O (70: 30: 1), pre-spotted 20 μl of NaCl satd MeOH before applying samples.
[e] See previous paper[1], t_R; retention time.

Isolation of Benzanthrins A and B

The isolation scheme for benzanthrins A and B is shown in Fig. 1. Activity units were determined by an agar diffusion assay using a *Staphylococcus aureus* 6538P inoculum. Supernate broth (73 liters) was adjusted to pH 8.5 and extracted with CH_2Cl_2 (2×16 liters). The extracts were combined and the CH_2Cl_2 evaporated under reduced pressure leaving a dark red residue. The residue was applied to a column of Sephadex LH-20 (4 liters bed vol) and two activity bands were eluted in MeOH, one enriched in benzanthrin A and the other in benzanthrin B. Each was purified further by liquid-liquid chromatography on diol-bonded silica gel (support weight/sample weight = 80/1). This separation method represented a new preparative technique for purifying microbial metabolites[4]. Elution was carried out first with the lower (less polar) phase of the two phase system (1) shown in Fig. 1 and then with the upper (more polar) phase. In the case of the benzanthrins a mixed-mode separation occurred (*i.e.* partitioning and adsorption), requiring final elution with 0.01% Et_3N - MeOH to get maximum recovery of activity. The final purification step was carried out in an Ito Coil Planet

Fig. 2. UV spectra of benzanthrin A (MeOH).

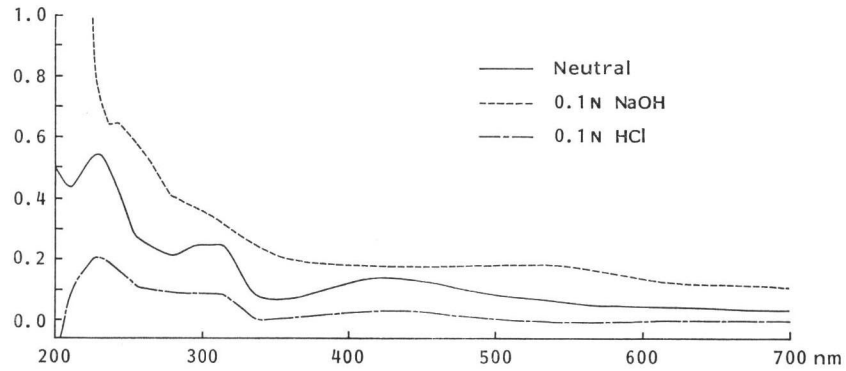
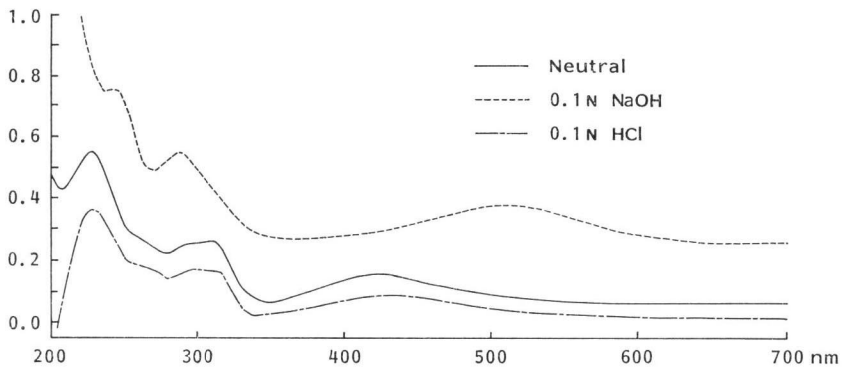


Fig. 3. UV spectra of benzanthrin B (MeOH).



centrifuge, a semi-preparative countercurrent chromatography (CCC) technique[5,6]. The lower phase of the two phase solvent system shown in Fig. 1 was the stationary phase and the upper was the mobile phase. Total recovery weights of pure benzanthrins A and B were 267 and 43 mg, respectively.

Hydrolysis of Benzanthrin A

Benzanthrin A (6 mg) was taken up in 1.5 ml of 0.1 N HCl and the resulting solution was heated on a steam bath for 3 hours. The reaction mixture was cooled to room temperature and the pH was adjusted to 9.5. The mixture then was extracted with CH_2Cl_2 (2×1 ml), the extracts were combined, and the CH_2Cl_2 was evaporated under reduced pressure leaving a dark blue-green residue. The residue was chromatographed in an Ito Coil Planet centrifuge; biphasic solvent system, CCl_4 - $CHCl_3$ - MeOH - H_2O (25: 25: 40: 10); stationary phase, lower layer; mobile phase, upper layer; flow rate, 5 ml/minute. Fractions were assayed by TLC (BuOH - HOAc - H_2O, 3: 1: 1) and those having a single component at Rf 0.41 were combined. The solvent was evaporated under reduced pressure to give 2 mg of a dark blue-green solid (benzanthrin pseudoaglycone): Electron impact (EI)-MS *m/z* 477.1764 (M^+), $C_{27}H_{27}NO_7$ (theory = 477.1788); UV λ_{max}^{MeOH} nm (ε) 235 (43,400), 264 (12,600), 326 (15,600), 450 (9,400); UV $\lambda_{max}^{MeOH+0.1N\ HCl}$ nm, no change; UV $\lambda_{max}^{MeOH+0.1N\ NaOH}$ nm 235, 258 (sh), 297 (sh), 317, 554; IR $\nu_{max}^{CHCl_3}$ cm^{-1} 3450 (OH, hydrogen bonded, single bridge), 3260 (OH), 3150 (OH), 1674 (C=O, non-hydrogen bonded), 1629 (C=O, hydrogen bonded).

Hydrolysis of Benzanthrin B

Benzanthrin B (4 mg) was taken up in 1.0 ml of 0.1 N HCl and treated as above. After CH_2Cl_2 extraction and purification on the Coil Planet centrifuge, 1 mg of material was obtained which was identical in all respects to benzanthrin pseudoaglycone above.

Fig. 4. IR spectrum of benzanthrin A ($CHCl_3$).

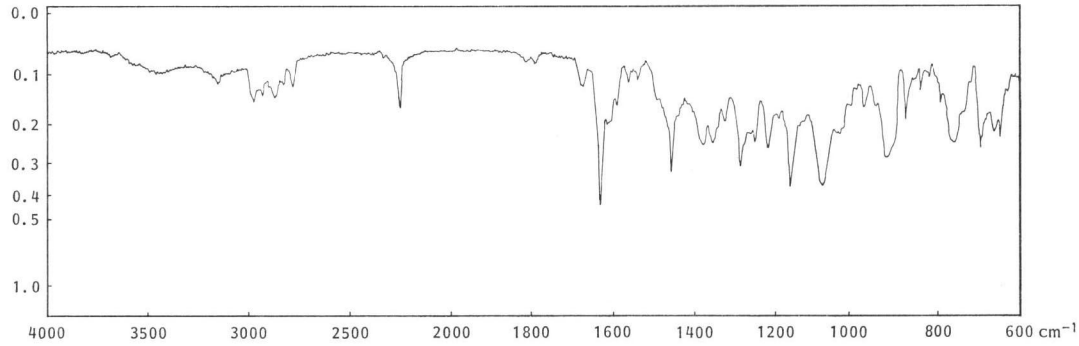
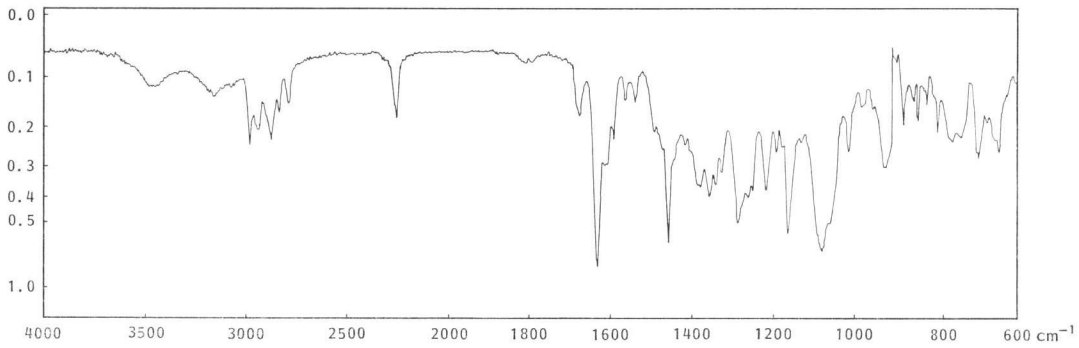


Fig. 5. IR spectrum of benzanthrin B ($CHCl_3$).



Zn Dust Distillation[7] of Benzanthrin A

Benzanthrin A (1 mg) was well mixed with 3 mg of freshly activated[8] Zn dust and was placed in a knee tube. The solid mixture was held in place with a small piece of glass wool. The bulb of the tube was heated over a microburner to a red glow and held there for 1 minute. After the tube cooled to room temperature, it was broken above the bend and the top piece containing the distillate was rinsed with CH_2Cl_2. The CH_2Cl_2 was evaporated and the residue was purified by preparative TLC (hexane development). The major component was collected and eluted from the silica gel with CH_2Cl_2. The residue remaining after evaporation of the CH_2Cl_2 was analyzed by UV and MS. UV λ_{max}^{EtOH} nm 267, 278, 288, 303, 316, 326, 344, 359, 386; EI-MS *m/z* 256 ($M^{\dagger}$). These data were consistent with a dimethylbenz[a]anthracene structure.

Physico-chemical Properties

The physico-chemical properties of benzanthrins A and B are summarized in Table 1, UV and IR spectra are shown in Figs. 2~5. These data allowed us to characterize benzanthrins A and B as novel isomeric hydroxyquinonoid antibiotics consisting of one non-hydrogen bonded carbonyl group and one hydrogen bonded carbonyl group positioned between peri hydroxyl groups[9~11].

Biological Properties

Benzanthrins A and B were shown to have potent *in vitro* Gram-positive antibacterial activity[1]. They were also assayed in tissue culture against three types of tumor cells†. The results are shown in Table 2. Benzanthrin B appeared to be 10-fold more cytotoxic than benzanthrin

† See footnote in p. 1519.

Table 2. *In vitro* antitumor activity of benzanthrins A and B*.

Test	ED_{50} (μg/ml)	
	Benzanthrin A	Benzanthrin B
9KB	0.3	0.3
9PS	0.01	0.01
9ASK	Cytotoxic at 100 >71% reversal at 10	Cytotoxic at 10 No reversal

* See footnote †.

A toward 9ASK cells (AC glioma tumor cell line), but it was interesting that benzanthrin A caused reversal of adenosine cyclic 3′,5′-monophosphate-induced morphological changes in 9ASK cells at 10 μg/ml while no reversal was observed with benzanthrin B. This suggested that benzanthrin A could have selective toxicity for certain types of tumors. Benzanthrin A was evaluated for *in vivo* activity and was found inactive in animal models for sarcoma, leukemia and lung tumors††.

Structure Determination

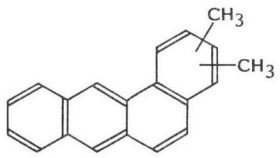
The nearly identical UV and IR spectra of benzanthrins A and B (Table 1 and Figs. 2~5) suggested that they had the same hydroxyquinonoid type chromophore. This was verified by separately hydrolyzing benzanthrins A and B in dilute mineral acid to obtain the same CH_2Cl_2 extractable product ($C_{27}H_{27}NO_7$) which was characterized as a pseudoaglycone (included all of the structural features of the chromophore by UV and IR analysis plus an additional sugar moiety by NMR analysis). The structure of the carbon skeleton of the chromophore was determined by carrying out a Zn dust distillation on benzanthrin A. The UV spectrum of the major product **1** was in close agreement with the Zn dust distillation product of tetrangulol, which had been characterized as 3-methylbenz[a]anthracene (**2**)[7] (UV results in Table 3). This established the ring system as a benz[a]anthracene. The mass spectrum of **1** (M^+, 256) supported a dimethyl structure which indicated that the ring system of the chromophore consisted of two branched carbon-carbon bonds.

The remaining structural features of the pseudoaglycone (hence the chromophore) were elucidated by 500 MHz NMR (CD_2Cl_2). A 2D *J* correlated map (COSY), and the accompanying ^1H spectra on the x and y axes is shown in Fig. 7. The results of the COSY, combined with the knowledge of the oxygenation pattern on rings B and D, (UV and IR) provided good evidence for assigning the three coupled aromatic protons as H-9, H-10, and H-11 on ring D, (**3**). This was supported by a comparison of ^{13}C chemical shifts of rings C and D with those of 1-hydroxy-8-methoxyanthraquinone[12], Table 4. The positions of the two aromatic singlet protons and the aromatic methyl in structure **3**

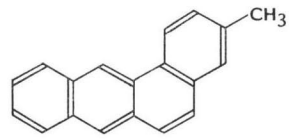
Table 3. UV λ_{max}^{EtOH} nm data of compound **1** and 3-methylbenz[a]anthracene (**2**).

1	267	278	288	303	316	326	344	359	386
2	268	273	287	300	314	328	342	359	385

Fig. 6. Zn dust distillation products of benzanthrin A and tetrangulol.



1



2

† JACOBSEN, L. & J. CASSADY: Purdue University Cancer Center, personal communication.

†† These *in vivo* activities are the results of screening performed under the auspices of the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland.

Fig. 7. 2D *J* correlated (COSY) NMR spectrum of benzanthrin pseudoaglycone in CD_2Cl_2 at 500 MHz.

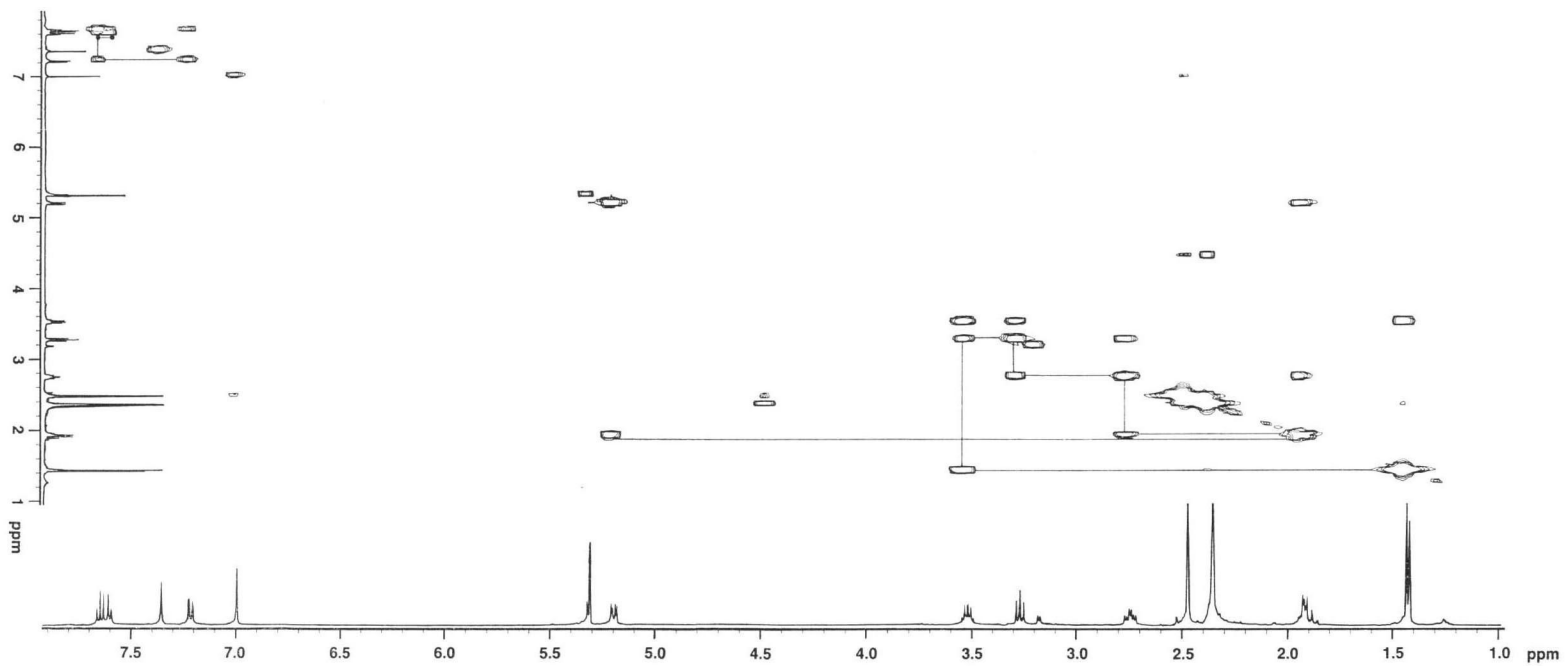
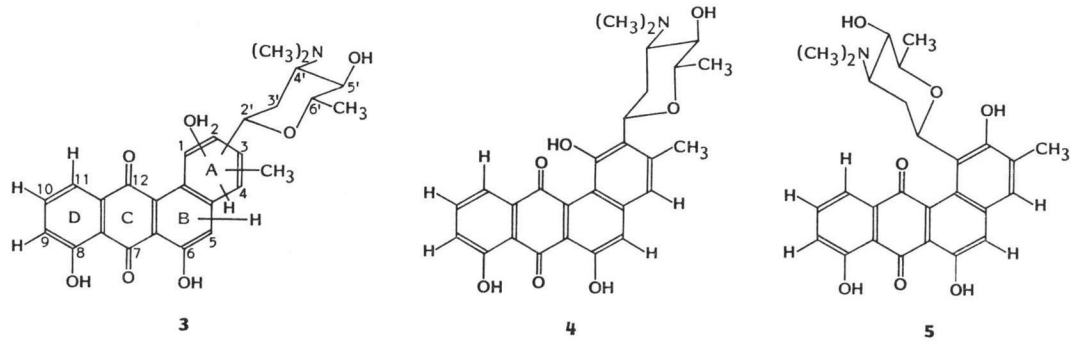


Fig. 8. Structures of benzanthrin pseudoaglycone.



3 4 5

Table 4. ^{13}C Chemical shifts of rings C and D of benzanthrin pseudoaglycone (CD_2Cl_2) and the corresponding values of 1-hydroxy-8-methoxyanthraquinone ($CDCl_3$).

	Benzanthrin pseudoaglycone	1-Hydroxy-8-methoxy-anthraquinone
7	193.2	188.62
7a	115.2	116.00
8	162.0	162.65
9	124.2	124.70
10	138.0	135.76
11	120.2	118.86
11a	Broad	133.06
12	187.3	182.58

Table 5. ^{13}C Chemical shifts of *C*-glycosidic angolosamine in benzanthrin pseudoaglycone (CD_2Cl_2) and in hedamycin ($CDCl_3$).

	Benzanthrin pseudoaglycone	Hedamycin
2′	76.4	75.2
3′	26.2	28.3
4′	67.8	67.4
4′-$N(CH_3)_2$	40.6	40.4
5′	71.8	71.9
6′	78.9	77.3
6′-CH_3	18.9	18.9

could not be assigned at this point in the analysis. After obtaining a chemical shift correlation map (CSCM), shown in Fig. 9, the non-aromatic protons could be unambiguously assigned to a 2-deoxy-3-dimethylaminohexose which was shown to be angolosamine on the basis of relative stereochemistry. The fact that there were only two unassigned aromatic protons indicated, by default, that the angolosamine was equatorially ($J_{2'-3'ax}$=10.5 Hz) linked to the benz[a]anthraquinone skeleton through carbon rather than oxygen. This also was in agreement with the generic structure of the Zn dust distillation product (**1**). The chemical shift of C-2′ (δ 76.4) in the CSCM which correlated with H-2′ (δ 5.2) is the expected value for carbon 2 of a *C*-glycoside[13]. Furthermore, the rest of the carbons for angolosamine matched well with those of the same *C*-glycosidic sugar in hedamycin[13] (Table 5). Most of the ambiguity of substitution on the A and B rings in **3** was resolved by nuclear Overhauser effect (NOE) differences measured for H-5, H-4 (13.1%) and H-4, 3-CH_3 (10.5%). These results were consistent with either structure **4** or **5**. The former was supported by the UV spectrum of benzanthrin pseudoaglycone which was almost identical to that of a rabelomycin dehydration product[14] having a benz[a]anthraquinone structure with the same oxygenation pattern as **4**. Thus the pseudoaglycone was formulated as structure **4**.

The ^{13}C chemical shift data for benzanthrins A and B and the pseudoaglycone (Table 6) provided strong evidence that C-1 was the site of *O*-glycosidation for benzanthrins A and B. Neither C-6 nor C-8 were viable alternatives as the chemical shifts of both carbons were nearly invariant

Fig. 9. Chemical shift correlation map (CSCM) NMR spectrum of benzanthrin pseudoaglycone in CD_2Cl_2 at 500 MHz.

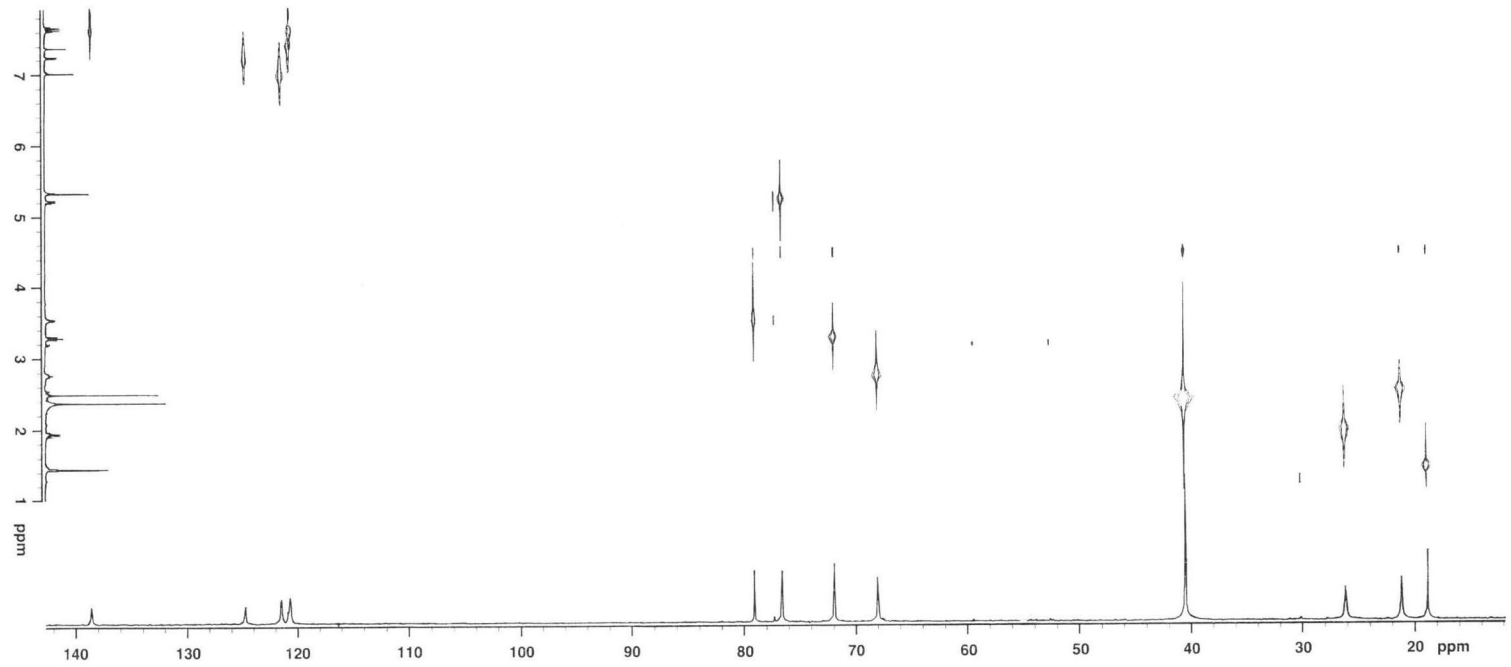
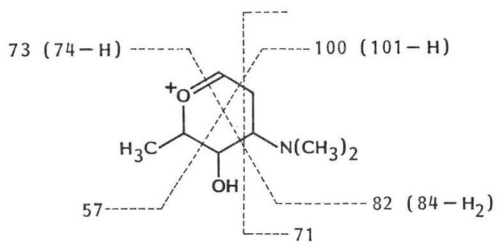


Table 6. ^{13}C NMR results (ppm) for benzanthrins A and B.

	Benzanthrin pseudoaglycone	Benzanthrin A	Benzanthrin B	
1, 1a, 2, 3, 4, 4a	Broad	Broad	Broad	
5	120.5	125.6	125.6	
6	156.8	156.8	156.8	
6a	118.0	115.5	115.7	
7	193.2	193.9	193.9	Chromophore
7a	115.2	115.7	115.7	
8	162.0	161.8	161.9	
9	124.2	122.7	122.8	
10	138.0	138.0	137.9	
11	120.2	119.8	118.9	
11a	136.1	131.8	131.9	
12	187.3	185.3	185.0	
12a		139.9	140.0	
3-CH_3	21.2	21.8	21.9	
2′	71.8	74.4	74.5	
3′	26.2	25.5	25.5	
4′	67.8	68.3	68.3	
5′	71.8	72.0	72.1	*C*-Glycosidic sugar
6′	78.9	78.2	78.2	
6′-CH_3	18.9	19.0	19.0	
4′-$N(CH_3)_2$	40.6	40.5	40.5	
1″		104.9	105.0	
2″		31.0	27.8	
3″		64.4	65.5	
4″		65.5	71.0	*O*-Glycosidic sugar
5″		72.1	74.1	
6″		16.0	17.4	
3″-$N(CH_3)_2$		42.5	40.5	

Fig. 10. Fragmentation of *O*-glycosidic sugar.



m/z 158$^+$, $C_8H_{16}NO_2$

in the three structures. Moreover, the data indicated that benzanthrins A and B differed in the structure of the *O*-glycosidic sugar. EI-MS demonstrated that both benzanthrins A and B gave the same fragment ion at m/z 158 ($C_8H_{16}NO_2$) due to a sugar. In each case, the sugar fragmented further to give ions derived from a 2-deoxy-3-dimethylaminohexose (Fig. 10). This suggested that benzanthrins A and B were stereoisomers. The proton assignments shown in Fig. 11 were based on the results of homo and hetero nuclear 2D NMR experiments. Coupling constants for the protons of the *O*-glycosidic (double primed) sugars of benzanthrins A and B revealed that the two antibiotics differed in their stereochemistry at the 4″ position. For benzanthrin A, $J_{4''-5''}<1.0$ Hz indicating 4″-H equatorial and

Fig. 11. ^1H NMR spectra in CD_2Cl_2 at 500 MHz.

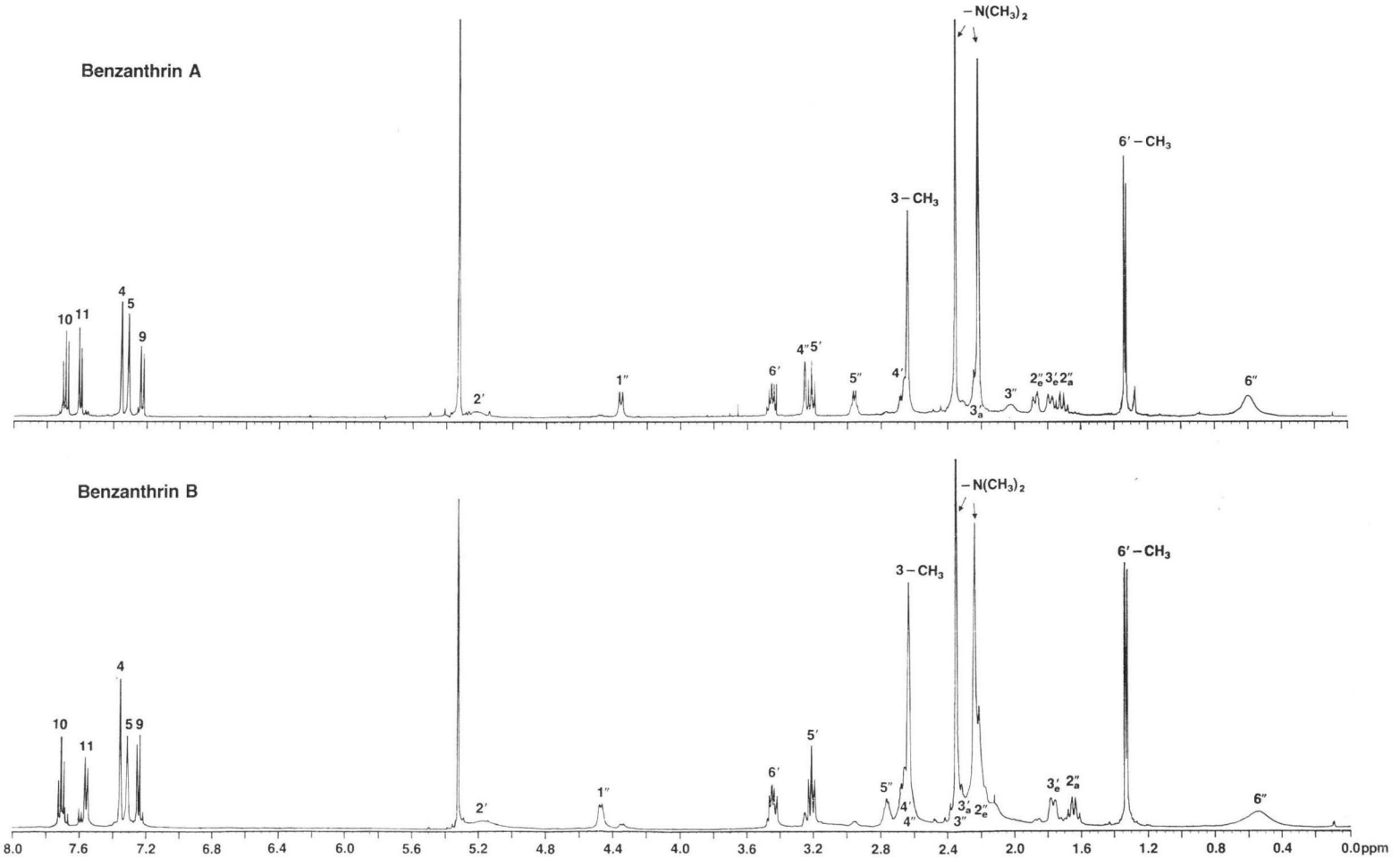
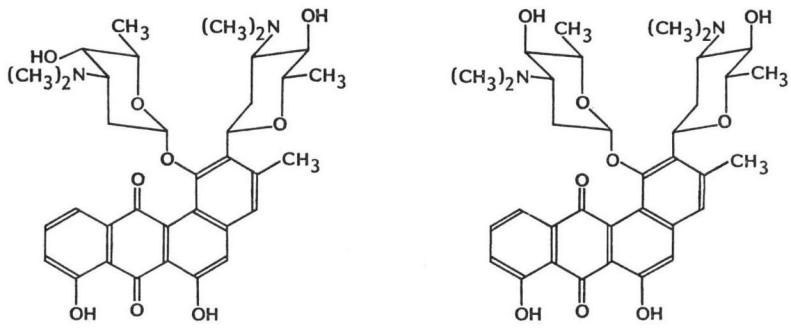


Fig. 12. Benzanthirns A and B.



Benzanthrin A (**6**) Benzanthrin B (**7**)

for benzanthrin B, $J_{4''-5''}=7.0$ Hz indicating 4″-H axial. The relative stereochemistry of the *O*-glycosidic sugar in the case of benzanthrin A (**6**) was that of rhodosamine and for benzanthrin B (**7**) was angolosamine. Accordingly the structures and relative stereochemistry of benzanthrins A and B were formulated as **6** and **7**, respectively.

The benzanthrins are structurally related to tetrangomycin[7], rabelomycin[14], the fujianmycins[15] and PD 116740[16]. Unlike the benzanthrins, none of these antibiotics are fully aromatized nor do they possess sugars. Nogalamycin[17] and the benzanthrins appear to be the only examples of antibiotics having both *O*-glycosidic and *C*-glycosidic linkages to an aglycone.

Acknowledgments

The authors thank Dr. J. HOCHLOWSKI for helpful discussions; Mr. S. CEPA and P. HILL for MS analyses; R. ANDERSON for IR spectra.

References

1) THERIAULT, R.; R. R. RASMUSSEN, W. L. KOHL, J. F. PROKOP, T. B. HUTCH & G. J. BARLOW: Benzanthrins A and B, a new class of quinone antibiotics. I. Discovery, fermentation and antibacterial activity. J. Antibiotics 39: 1509~1514, 1986
2) FREEMAN, R. & G. A. MORRIS: Experimental chemical shift correlation maps in nuclear magnetic resonance spectroscopy. J. Chem. Soc. Chem. Commun. 1978: 684~686, 1978
3) BAX, A., R. FREEMAN & G. MORRIS: Correlation of proton chemical shifts by two-dimensional fourier transform NMR. J. Magn. Reson. 42: 164~168, 1981
4) RASMUSSEN, R. & M. SCHERR: Diol-bonded silica gel as an inert support for low pressure liquid-liquid chromatography. A preparative technique which is complementary to the Ito Coil Planet centrifuge for the separation of complex mixtures. Abstracts of Papers of Tenth Int. Symp. on Col. liq. Chromatogr. No. 4103, San Francisco, 1986 and J. Chromatogr., in press
5) ITO, Y.: Toroidal coil planet centrifuge for counter-current chromatography. J. Chromatogr. 192: 75~87, 1980
6) ITO, Y.: The toroidal coil planet centrifuge without rotating seals applied to countercurrent chromatography. Anal. Biochem. 102: 150~152, 1980
7) KUNTSMANN, M. P. & L. A. MITSCHER: The structural characterization of tetrangomycin and tetrangulol. J. Org. Chem. 31: 2920~2925, 1966
8) FIESER, L. F. & M. FIESER: Reagents for Organic Synthesis. Vol. 1., p. 1276, John Wiley & Sons, Inc., New York, 1967
9) MORTON, R. A. & W. T. EARLAM: Absorption spectra in relation to quinones. 1: 4-Naphthaquinone, anthraquinone and their derivatives. J. Chem. Soc. 1941: 159~169, 1941
10) SPRUIT, C. J. P.: Absorption spectra of quinones. II. Anthraquinone and heterocyclic analogues. Recl. Trav. Chim. Pays Bas 68: 325~335, 1949

11) BLOOM, H., L. H. BRIGGS & B. CLEVERLEY: Physical properties of anthraquinone and its derivatives. Part 1. infrared spectra. J. Chem. Soc. 1959: 178~185, 1959
12) BERGER, Y.; A. CASTONGUAY & P. BRASSARD: Carbon-13 nuclear magnetic resonance studies of anthraquinones. Organic Magnetic Resonance 14: 103~108, 1980
13) SÉQUIN, U. & M. FURUKAWA: The structure of the antibiotic hedamycin—III. ^{13}C NMR spectra of hedamycin and kidamycin. Tetrahedron 34: 3623~3629, 1978
14) LIU, W.-C.; W. L. PARKER, D. S. SLUSARCHYK, G. L. GREENWOOD, S. F. GRAHAM & E. MEYERS: Isolation, characterization, and structure of rabelomycin, a new antibiotic. J. Antibiotics 23: 437~441, 1970
15) RICKARDS, R. W. & J.-P. WU: Fujianmycins A and B, new benz[a]anthraquinone antibiotics from a *Streptomyces* species. J. Antibiotics 38: 513~515, 1985
16) WILTON, J. H.; D. C. CHENEY, G. C. HOKANSON & J. C. FRENCH: A new dihydrobenz[a]anthraquinone antitumor antibiotic (PD 116740). J. Org. Chem. 50: 3936~3938, 1985
17) WILEY, P. F.; R. B. KELLY, E. L. CARON, V. H. WILEY, J. H. JOHNSON, F. A. MACKELLAR & S. A. MIZSAK: Structure of nogalamycin. J. Am. Chem. Soc. 99: 542~549, 1977