IDENTITY OF ALBOMYCIN δ_2 AND ANTIBIOTIC Ro 5-2667

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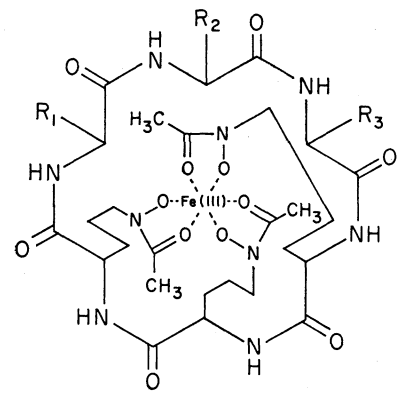
The major constituent of the albomycin complex is albomycin δ_2. Albomycins δ_1 and ε are degradation products of component δ_2. Antibiotic Ro 5-2667 has now been found to be identical with albomycin δ_2; the minor antibiotic components, Ro 7-7730 and Ro 7-7731, correspond to albomycins δ_1 and ε, respectively. The present studies demonstrate that previously proposed structures of albomycins are inconsistent with our findings.

The chemistry of the albomycins was initially investigated in the laboratories of ŠORM[1,2] and PODDUBNAYA[3]. Structures of albomycins proposed by both research groups resembled compounds of the ferrichrome type (1-4), namely Fe(III) trihydroxamate complexes with three linear hydroxamate functions connected head to head[4]. The first proposal for a partial structure of albomycin[3] was apparently deduced by analogy to an early proposal for a ferrichrome structure[5], whereas the latest one (4)[1] resembled ferrichrysin (3)[6].

The iron-binding center of ferrichrome-type compounds consists of a tripeptide portion containing three N^5-acetyl-N^5-hydroxyornithine moieties, which, together with three simple amino acids, contributes to a cyclic hexapeptide structure. Ferrichrome (1) contains three glycine units and no serine[7], but ferricrocin (2) and ferrichrysin (3) possess both glycine and serine moieties[6].



Ferrichrome (1) $R_1=R_2=R_3=H$
Ferricrocin (2) $R_1=HOCH_2$, $R_2=R_3=H$
Ferrichrysin (3) $R_1=R_2=HOCH_2$, $R_3=H$
(4) $R_1=R_2=R_3=HOCH_2$

Albomycin hydrolyzates have been reported to contain serine and ornithine in equimolar ratios[8,3,2,9]. Furthermore, selective degradation of albomycin by ŠORM and coworkers[1] was reported to yield small peptides, including a tripeptide consisting of three serine units, resulting in assignment of a ferrichrome-type structure (4) to the peptide portion of albomycin.

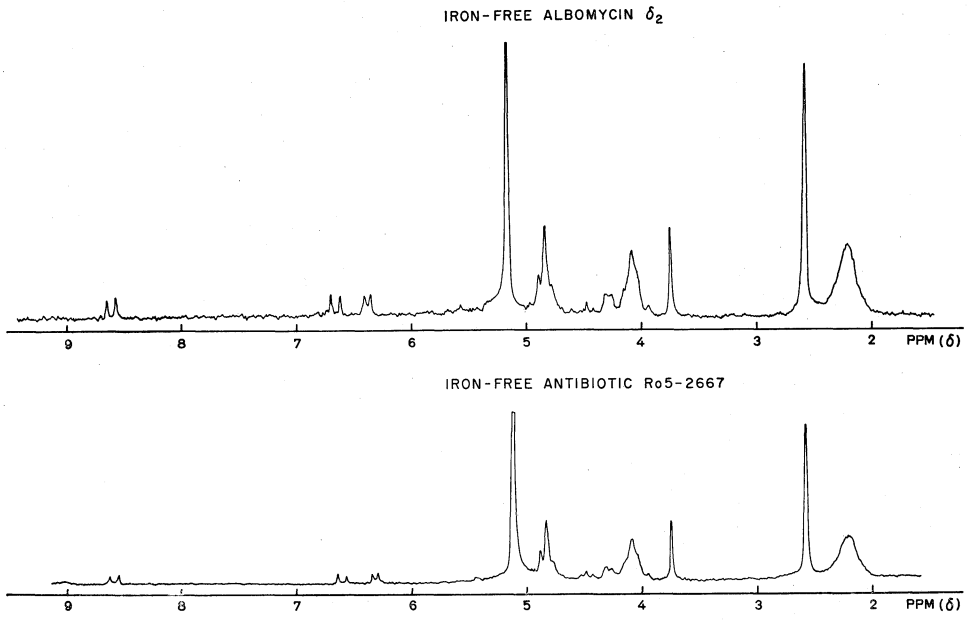
We have previously reviewed naturally occurring hydroxamic acids and hydroxamates with emphasis on the present status of the chemistry of albomycins and antibiotics Ro 5-2667, Ro 7-7730 and Ro 7-7731[4]. This paper presents evidence demonstrating identity of albomycin δ_2 and antibiotic Ro 5-2667; albomycin δ_1 and ε are shown to be identical with antibiotics Ro 7-7730 and Ro 7-7731 respectively.

More specifically, contrary to published results[8,3,2,9], authentic albomycins and our antibiotic samples contain only one serine moiety and three N^5-acetyl-N^5-hydroxy-ornithine units each by amino acid anylyses of acid hydrolysates and nmr spectroscopy. Thus, the representation of the amino acid portion of albomycins (4)[1] must be revised.

Experimental

Reported retention volumes of column chromatograms are identical with U* values as defined by RIEMAN[10]. Thin-layer chromatography was performed on pre-coated MN-Polygram CEL 300 plastic sheets (Brinkmann Instruments), followed by bioautographic detection with the gram-negative bacterium, *Serratia* sp. No. 101*. NMR spectra were recorded on a Varian HA-100 spectrometer with deuterium oxide as solvent. Difficulties encountered in producing good nmr spectra at low sample concentrations arose from the necessity of using either an external TMS reference glass-capillary or the residual HDO peak for field/frequency stabilization. Both methods offer disadvantages. Commercially available capillaries have relatively large diameters and, therefore, produce large spinning sidebands on all peaks in the spectrum by creating inhomogeneities in the magnetic field which cannot be compensated electronically. Locking onto the residual HDO peak eliminates spinning sidebands but produces beat frequencies extending over considerable distances on either side of the HDO peak and obscures peaks in this area of the spectrum which, similar to spinning sidebands, complicates interpretation and integration. Satisfactory spectra were obtained employing sealed TMS glass-capillary tubes of 200 mm length and outer and inner diameters of 0.76 and 0.51 mm, respectively, held concentrically with specially machined Nylon spacers. With these capillaries 1 volt of lock signal could be attained and spinning sidebands were no larger than those normally present with internal TMS as reference. To reduce the extent of the residual HDO peak further and hence its spinning sideband intensity, all samples were freeze-dried five times from D_2O solutions.

Fig. 1.



* In a previous publication[11], this culture was referred to as *Proteus vulgaris*.

A 4-mg sample of iron-free albomycin δ_2, after deuterium exchange, was dissolved in 0.25 ml D_2O. The spectrum of iron-free albomycin δ_2 (Fig. 1) is the result of 36 time-averaged scans with manual homogeneity optimization on every second scan. A spectrum of iron-free antibiotic Ro 5-2667 (Fig. 1), representing 12 time-averaged scans, was obtained under similar conditions. Amino acid analyses were performed on a Technicon Auto Analyzer employing a 75 cm column and a 5.5-hour gradient system.

Purification and Separation of Albomycin Complex

A sample of albomycin complex[4] was purified by chromatography on a CM Sephadex G-25 column as described previously for Ro 5-2667 and related antibiotics[11]. Elution of a 20×485 mm column, previously charged with 110 mg of crude albomycin complex, yielded four distinct, colored zones with retention volumes of 67, 104, 148 and 277 ml, respectively. The first zone exhibited the characteristic color of Fe(III) hydroxamate complexes but lacked biological activity. The second zone contained albomycins δ_2 and δ_1, the third was biologically inactive and was of greenish color, whereas the fourth zone represented crude albomycin ε. After desalting, the albomycin δ and albomycin ε fractions weighed 15 mg and 2 mg, respectively, with virtually quantitative recovery of biological activity.

Albomycins δ_2 and δ_1 were separated by liquid-liquid chromatography[11] on a 25×46 mm column. Sephadex G-25 superfine was employed as support, the lower phase of 1-butanol, 2-propanol, 0.2 M aqueous ammonium sulfate, 2 : 1 : 1, v/v, served as stationary phase; the column was developed with the upper phase. Fourteen mg of the albomycin δ mixture as previously obtained were dissolved in 0.1 ml of lower phase and enough dry Sephadex G-25 was added to form a thick paste which was applied to the column top. Two biologically active zones with retention volumes of 258 and 519 ml were obtained upon elution, representing albomycins δ_1 and δ_2, respectively. These fractions yielded, after desalting and freeze-drying, 2 mg of albomycin δ_1 and 5 mg of albomycin δ_2.

Table 1.

Solvent system	Rf · 100	
	Albomycin δ_1	Albomycin δ_2
1-Butanol - acetic acid - water, 4 : 1 : 5, upper phase	34	26
t-Butanol - 0.004 N hydrochloric acid - sat. aq. sodium chloride solution, 2 : 1 : 1, upper phase*	11	3
Ethanol - water, 2 : 1 containing 2 % sodium chloride	73	65
1-Propanol - pyridine - water, 15 : 1 : 10	63	52
Pyridine - 1-pentanol - water, 7 : 7 : 6	50	40
2-Propanol - water, 7 : 2**	19	7

* Plate pre-treatment with acetone - water - sat. aq. sodium chloride solution, 16 : 3 : 1

** Plate pre-treatment with 0.2 M ammonium sulfate. The solvent ratio of this system was erroneously printed as 7 : 3 (Ref. 11).

Thin-layer Chromatography

Six thin-layer chromatographic systems, previously proven to be of value in the analysis of sideromycins[12,13,11], differentiated albomycins δ_1 and δ_2. Both antibiotics were detected bioautographically but bioautographic detection of albomycin ε was difficult due either to very hazy zones or to antagonism in the presence of albomycin δ_1 and δ_2[4]. Solvent systems and mobilities are summarized in Table 1.

Iron-free Albomycins

To a solution of 5 mg of albomycin in 100 μl of water was added a solution of 5 mg of 8-hydroxyquinoline[14] (E. Merck, Darmstadt, test substance for elemental analysis, distributed by Brinkmann Instruments) in 75 μl of methanol. The mixture was stirred at 4°C for 16 hours; the black precipitate removed by filtration and the filtrate collected directly in a micro extraction tube, together with six protions of 20 μl wash water. The filtrate was extracted continuously with ether for 4 hours, the aqueous phase evaporated to a small volume under reduced pressure and freeze-dried to yield 4.6 mg of white, amorphous, deferrialbomycin.

Acid Hydrolysis of Albomycins

A sealed tube containing 0.1~0.2 mg of the sideromycin and 0.75 μl of 47 % hydriodic

acid was heated at 100°C for 10 hours. The hydriodic acid was evaporated under reduced pressure, the residue dissolved in water, diluted with an equal volume of 25 % sucrose solution containing internal standards and subjected to amino acid analysis by the Auto Analyzer.

Hydrolysis of performic acid oxidized deferrialbomycin

A micro tube with 0.1~0.2 mg of iron-free sideromycin in 200 μl of a 4 : 1 mixture of 80 % formic acid and 30 % hydrogen peroxide[15] was kept at room temperature overnight. The contents of the tube were evaporated to dryness, redissolved in 100 μl of 6 N hydrochloric acid, sealed and kept at 100°C for 18 hours, then evaporated to dryness and analyzed as described above.

Results and Discussion

Upon chromatography on the CM Sephadex G-25 column, previously used for the purification of crude albomycin complex, a mixture of antibiotics Ro 5-2667, Ro 7-7730 and Ro 7-7731 exhibited the same retention volumes as did the albomycin mixture. Similarly, identical retention volumes were observed for albomycin δ_1 and antibiotic Ro 7-7730 as well as albomycin δ_2 and antibiotic Ro 5-2667 upon liquid-liquid column chromatographic comparison. Direct thin-layer chromatographic comparison of albomycins δ_1 and δ_2 with antibiotics Ro 7-7730 and Ro 5-2667, respectively, revealed identical mobilities of each pair in all six solvent systems tested.

Albomycins δ_1, δ_2 and ε exhibited the same UV spectra as did antibiotics Ro 7-7730, Ro 5-2667 and Ro 7-7731[11] and a comparison of the nmr spectra of deferrialbomycin δ_2 with iron-free antibiotic Ro 5-2667 (Fig. 1) confirmed that the two antibiotics are indeed identical. Nine acetyl protons giving rise to a sharp singlet at δ 2.57, a broad 12 proton envelope due to six methylene groups attached to carbon, centered at δ 2.18, and six protons at δ 4.08, representing three methylene groups attached to nitrogen, confirm three N^5-acetyl-N^5-hydroxyornithine moieties. At slightly lower field one observes the absorption due to the methylene group of serine. Analogous signals with comparable relative chemical shifts were reported for the hydroxymethyl groups of serine in deferriferrichrome, deferriferrichrysin[16], and deferriferricrocin[6] (*cf.* structures 1~3). A comparison of absolute chemical shift values is not feasible due to different bulk susceptibility factors[17].

It is evident from nmr spectra that only one serine residue is contained in albomycin. This observation was fully corroborated by amino acid analysis of hydrolysates. Upon hydriodic acid hydrolysis of albomycins δ_2 and δ_1, ornithine and serine were obtained in molar ratios of 3 : 1. Iron-free albomycins δ_2 and δ_1 yielded, after performic acid oxidation and hydrochloric acid hydrolysis, ornithine and glutamic acid in molar ratios of 3 : 1. The same amino acid compositions were exhibited by antibiotics Ro 5-2667, Ro 7-7730 and Ro 7-7731[11].

These findings clearly indicate the identity of albomycins with the iron-containing antibiotics isolated in our laboratory[11]. In view of extensive efforts by Russian workers concerned with chemical syntheses in the albomycin field[18], a reexamination of the chemistry of albomycins would be of particular merit.

Acknowledgement

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