A STUDY OF SOME PHYSICOCHEMICAL PROPERTIES OF THE POLYPEPTIDE ANTIBIOTICS A-128-OP AND A-128-P

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UDC 615.779.931.011

It has been shown previously that the polypeptide antibiotic A-128 (neotelomycin) [1-4] consists of at least two components. By gel filtration on Sephadex G-25 it has been possible to isolate two biologically active components – the antibiotics A-128-OP and A-128-P [5, 6], differing only in their content of proline amino acids. The component A-128-OP contains, in addition to ten other amino acids common to both components, trans-3-hydroxyproline, while A-128-P contains proline.

In the present paper we give experimental results on a study of some physicochemical properties of the components of the antibiotic A-128 that we have isolated (Table 1). It can be seen from the table that the antibiotics have extremely similar properties. They are light cream-colored crystals readily soluble in aqueous alcohols and butanol and in dilute acids and alkalis and insoluble in acetone, benzene, and toluene; the solubility of the antibiotic A-128-P is somewhat higher than that of A-128-OP. They possess coincident electrophoretic and chromatographic properties, showing the predominance of basic over acidic properties. The hydrophilic nature of the antibiotics is due to the fact that they each contain one amine and one carboxy group, while A-128-OP contains five hydroxy groups and A-128-P contains four. The number of free functional groups was determined by the method of partial substitution in association with paper electrophoresis. The results of elementary analysis agreed well with the calculated figures obtained for 11 amino-acid residues and one lactone bond.

In actual fact, mild alkaline treatment of the antibiotics revealed a new carboxy group belonging to cis-3-hydroxyproline, which obviously forms a lactone bond with the hydroxy group of one of the hydroxy amino acids. The existence of a lactone bond is confirmed by the presence in the IR spectra of both antibiotics of the absorption band at 1747-1745 cm^{-1} characteristic for an ester grouping, this band disappearing when the antibiotics are treated with dilute alkali. In addition, the antibiotic A-128-OP has six hydroxy amino acids and A-128-P five, while the method of partial substitution indicates one OH group less in each case (see Table 1).

Consequently, one hydroxy group in each antibiotic is blocked; it may be assumed that it forms an ester bond with the C-terminal amino acid of the peptide chain of the antibiotic. It is also interesting to observe that the N-terminal aspartic acid forms a peptide bond with its β-carboxy group in each case. This was shown by an analysis of the products of the hydrazinolysis of the antibiotics, among which the β-hydrazide of aspartic acid was found. The results obtained permit the conclusion that the antibiotics A-128-P and A-128-OP are peptidocyclolactones, since they each contain aspartic acid at the N end and an amino acid (cis-3-hydroxyproline) revealed only after alkaline treatment at the C end.

The antibiotics studied differ from the antibiotics telomycin [7] and LL-A-0341 B [8] studied previously by the fact that they each contain three D amino acids: D-serine, D-aspartic acid, and D-allothreonine; the other amino acids are present in the L configuration. In all known antibiotics containing allothreonine, this is present in the D form [9], with the sole exception of telomycin, which contains L-allothreonine [7].

M. V. Lomonosov Moscow State University. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 815-819, November-December, 1972. Original article submitted February 15, 1971.

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TABLE 1. Some Physicochemical Properties of the Antibiotics A-128-OP and A-128-P

Properties	Antibiotic	
	A-128-OP	A-128-P
Empirical formula	$C_{59}H_{77}O_{19}N_{13}$	$C_{59}H_{77}O_{19}N_{13}$
mp, °C	240—246	240 —246
$[\alpha]_D^{20}$ (c 0.5; 4 N CH_3COOH), deg	—38	—42,6
Mol. wt.	1250	1230
UV spectrum, λ_{max}, nm	225; 277; 290; 337	225; 277; 290; 337
Number of NH_2 groups	1 (aspartic acid)	1 (aspartic acid)
Number of OH groups	5	4
Number of COOH groups	1 (α-COOH group of the aspartic acid)	1 (α-COOH group of the aspartic acid)
Proline amino acids	cis-3-Hydroxyproline trans-3-Hydroxyproline	cis-3-Hydroxyproline trans-3-Hydroxyproline
D isomers of amino acids	Asp; Ser: aThr	Asp; Ser· aThr
Biological activity on St. aureus 209, γ/ml	10	10
Electrophoretic mobility, $cm^2 \cdot sec^{-1} \cdot V^{-1} \cdot 10^{-5}$		
pH 2.4	0,91	0,91
pH 6.5	0,62	0,62
pH 8.4	0	0
Rf value in the n-butanol—CH_3COOH—H_2O (4 :1 : 1)system	0,79	0,79

Finer differences between A-128-OP and A-128-P and also between these antibiotics and telomycin and antibiotic LL-A-0341 B can be traced only after an analysis of the amino-acid sequence of these antibiotics.

EXPERIMENTAL

Neotelomycin was separated into the components A-128-P and A-128-OP by gel filtration on Sephadex G-25 [5]. The initial substance was given to us by Z. I. Belova of the Institute of Epidemiology and Microbiology of the Academy of Sciences of the USSR.

D-Aminooxidase (0.2 unit) was obtained from the firm Reanal, and its activity was checked with respect to the oxidation of D-α-valine. The IR spectra were taken on a UR-20 instrument and the UV spectra on an SF-4 spectrophotometer. The specific rotations were determined on a Roussel Jouan polarimeter. Paper electrophoresis was performed in apparatuses of the Durrum type [10] in the following electrolytes: 1) 1 N CH_3COOH (pH 2.4), 2) pyridine—CH_3COOH—H_2O (10:0.4:90) (pH 6.5), 3) 1% $(NH_4)_2CO_3$ (pH 8.4), and 4) HCOOH—CH_3COOH—H_2O (28:20:52) and chromatography in a thin layer of silica gel or cellulose [11] on 13×18-cm plates and Whatman No. 4 paper. The amino-acid composition of the components was determined after acid (6 N HCl, 24 h, 107°C) and alkaline [sat. $Ba(OH)_2$, 18 h, 107°C] hydrolyses in evacuated sealed tubes on a Hitachi type KLA-3B automatic analyzer in the laboratory directed by V. P. Korzhenko.

Determination of the Configurations of the Amino Acids in the Antibiotics A-128-P and A-128-OP. A solution of 60 mg of D-aminooxidase in 2 ml of 0.1 N phosphate buffer with pH 8.0 was added to the acid (alkaline) hydrolyzate that had been obtained from 4 mg of the antibiotic A-128-P (or A-128-OP) and had been thrice evaporated to dryness with water, and the mixture was thermostated at 37°C for 6-8 h with the constant passage of a current of oxygen through the solution. Then the solution was neutralized, the precipitate was separated by centrifuging and was washed with water, and the supernatant liquid was desalted on the cation-exchange resin Dowex 50×4 in the H^+ form (column 1×3 cm) as described in the literature [12]. The eluates were evaporated to dryness and the amino-acid composition of the desalted hydrolyzates was determined on the amino-acid analyzer. Threonine and allothreonine were separated by chromatography on Whatman No. 4 paper in the methyl ethyl ketone—butan-1-ol—water—NH_4OH (conc.) (3:5:1:1) system. In this system, threonine has R_f 0.12 and allothreonine 0.25.

A control experiment with D-α-alanine was performed similarly. β-Methyltryptophan was determined by the "amino-acid-map" method on plates with a thin layer of cellulose [11].

Preparation of the DNP* Derivative of the Antibiotic A-128-OP. To a solution of 65 mg of the antibiotic A-128-OP in 2 ml of ethanol and 4 ml of 3% $NaHCO_3$ were added 1.5 g of recrystallized urea and 4

*Here and below, DNP denotes 2,4-dinitrophenyl.

ml of an ethanolic solution of 1-fluoro-2,4-dinitrobenzene (20 mg/ml). The mixture was kept at 20°C for 30 min and at 37°C for 1 h with constant shaking at a pH of 9.0-9.5. Then the ethanol was evaporated off in vacuum, the excess of reagent was extracted with purified ether, the solution was acidified with 2 N hydrochloric acid to pH 1.0, and the DNP derivative of the antibiotic was extracted with a mixture of ethyl acetate and butan-1-ol (1:1) (3×5 ml). Then the organic layer was extracted with 2 N hydrochloric acid, washed with water, dried over Na_2SO_4, and evaporated to dryness. The DNP-A-128-OP was reprecipitated from propanol. Yield 60 mg, mp 362-365°C (decomp.); on chromatography in the butan-1-ol $-CH_3COOH-H_2O$ (4:1:1) system, the DNP-A-128-OP had R_f 0.37; in UV light λ_{max} 277, 290, and 355 nm; in an acid hydrolyzate of the DNP-A-128-OP only aspartic acid was absent, and this was detected in the form of the DNP derivative when an ethereal extract from the acid hydrolyzate of the DNP-A-128-OP was chromatographed on silica gel.

The preparation of DNP-A-128-P was performed similarly; its UV spectrum, amino-acid composition, and chromatographic and electrophoretic properties coincided completely with those of the DNP-A-128-OP described above.

The numbers of free amino groups in the antibiotics A-128-OP and A-128-P were determined by partial dinitrosulfophenylation with the subsequent electrophoretic investigation of the products of partial substitution [13]. Both for A-128-OP and for A-128-P two spots were obtained on paper electrophoresis in electrolyte 4 (240 V, 2 h): the unsubstituted antibiotic, moving towards the cathode (1.5 cm) and its mononitrophenylsulfo derivative with charge −1, moving to the anode and colored yellow (1.3 cm). Each antibiotic has one free NH_2 group.

The number of free carboxy groups was determined by partial methylation, using diazomethane [14] and a methanolic solution of HCl [15]. The reaction mixture was studied by electrophoresis in pyridine$-CH_3COOH-H_2O$ (1:4:995), 2 h at pH 5.6. Each antibiotic, after treatment with a 0.4% solution of ninhydrin in ethanol, showed two spots: one of the unsubstituted antibiotic (migration towards the cathode by 1.5 cm) and the other at a distance of 4.5 cm from the starting line and also moving towards the cathode due to the monomethyl ester of the antibiotic. Each antibiotic has one free COOH group.

The numbers of free hydroxy groups in A-128-OP and A-128-P were determined by the previously described method of partial sulfation [16] with subsequent electrophoretic analysis in electrolyte 4 of the mixture of $O-SO_3H$ esters formed at the hydroxy groups of the hydroxy amino acids present in the antibiotics.

In the case of the antibiotic A-128-OP, five spots of $O-SO_3H$ derivatives moving towards the anode were obtained and in the case of the antibiotic A-128-P four such spots. Consequently, in the antibiotic A-128-OP there are five free OH groups and in A-128-P four.

The molecular weights of A-128-OP and A-128-P were determined from the optical densities of solutions of the DNP derivatives of the antibiotics of accurately known concentrations measured at 355 nm, taking $\varepsilon = 16{,}000$, as described by Battersby and Craig [17] (see Table 1).

The C-terminal amino acids in A-128-OP and A-128-P were determined by Akabori's method [18]. The hydrazides of the amino acids were eliminated with benzaldehyde. The residue after treatment with benzaldehyde was studied in the amino-acid analyzer. No free amino acids were detected.

The type of peptide bond of the aspartic acid in A-128-OP and A-128-P was determined by hydrazinolysis. A solution of 5 mg of the antibiotic in 0.5 ml of anhydrous hydrazine in a sealed tube was kept in the thermostat at 105°C for 5 h, and was then evaporated to dryness and the residue was analyzed by thin-layer chromatography on silica gel in the pyridine–water (65:35) system and by paper electrophoresis in 1 N CH_3COOH (600 V, 2 h) with markers, as the α- and β-monohydrazides of aspartic acid, R_f 0.43 and 0.34, respectively, and the dihydrazide of aspartic acid (R_f 0.54) (revealing agent: 0.4% ninhydrin). After treatment with ninhydrin the β-hydrazide of aspartic acid appears in the form of a bright carnation-red spot [19]. The hydrazides of aspartic acid isolated after the hydrazinolysis of A-128-OP and A-128-P had the size and color characteristic for the β-hydrazides.

Preparation of the Acid of A-128-OP. A solution of 21 mg of A-128-OP in 0.5 ml of 50% ethanol was treated with 3 ml of 0.1 N NaOH solution, and the mixture was kept at 37°C for 45 min. Then it was acidified with 0.1 N hydrochloric acid to pH 4.0, and the acid of A-128-OP was extracted with a mixture of ethyl acetate and butan-1-ol (2:1) (3×5 ml), and the organic layer was washed with water, dried over $MgSO_4$, and evaporated in vacuum. Yield 14.7 mg, mp 238-240°C (decomp.). The A-128-OP acid obtained was electro-

phoretically homogeneous in electrolytes 1 and 2 and gave a single spot with R_f 0.52 on thin-layer chromatography in silica gel containing gypsum in the pyridine – butan-1-ol – CH_3COOH – H_2O (30:20:6:28) system, $[\alpha]_D^{20}$ 21.6° (c 0.5; glacial CH_3COOH), λ_{max} 277, 288, and 337 nm.

The acid of antibiotic A-128-P was obtained similarly.

The C-terminal amino acids in the linear peptides (the A-128-OP acid and the A-128-P acid) were determined by Akabori's method [18]. The C-terminal amino acids were identified in the amino-acid analyzer. In each case the C-terminal amino acid was cis-3-hydroxyproline.

SUMMARY

Some physicochemical properties of the natural polypeptide antibiotics A-128-OP and A-128-P have been studied.

It has been shown that these antibiotics have similar properties but differ in the type of proline amino acids, and they are cyclopeptidolactones, their N-terminal amino acid being D-aspartic acid.

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