THE STRUCTURE OF THE POLYPEPTIDE ANTIBIOTICS A-128-OP AND A-128-P

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In a preceding paper [1] we reported some physicochemical properties of the natural polypeptide antibiotics A-128-OP and A-128-P. These antibiotics are eleven-membered peptidolactones and contain at the N end D-aspartic acid acylating the amino group of the following amino acid by its β-carboxy group. In the present paper we give experimental details of the analysis of the primary amino-acid sequence of the antibiotics A-128-OP and A-128-P.*

The antibiotics investigated proved to be stable to the action of proteolytic enzymes. The presence of a β-peptide link of aspartic acid at the N end of the antibiotic prevented the use of the stepwise splitting off of amino acids by Edmann's method. At the C end of the linear eleven-membered peptide (the "acid" of the antibiotic), obtained by mild alkaline treatment of the antibiotic, there is cis-3-hydroxyproline, which it was possible to determine by hydrazinolysis and by the action of carboxypeptidase C [3] from the proteolytic complex of Aspergillus oryzae.

The action of the enzyme complex on the "acid" of A-128-OP and A-128-P for 3-4 h split off cis-3-hydroxyproline and, in low yield, dehydrotryptophan, which was determined as tryptophan by thin-layer chromatography and also by qualitative reactions and by a comparison with reference samples. The action of the enzyme complex for 8 h yielded β-methyltryptophan. Consequently, at the C end of both antibiotics there is the amino-acid sequence β-MeTrp-ΔTrp-cis-3Hyp-OH.

The only possible method for obtaining the C-terminal peptides proved to be mild alkaline hydrolysis of the antibiotics with 2 N NaOH at 30°C for 35 h. This partial hydrolysis, after the neutralization of the hydrolyzate, formed a mixture of water-soluble and water-insoluble peptides. The amino-acid composition of the water-soluble fraction of the peptides was the same for both antibiotics; it contained aspartic acid, serine, threonine, alanine, and allothreonine; the water-insoluble peptides proved to be characteristic for the antibiotics, having a UV spectrum with λ_{max} 277, 290, 337 nm and consisting of glycine, dehydrotryptophan, β-methyltryptophan, cis-3-hydroxyproline, and β-hydroxyleucine and, in addition to these amino acids, A-128-OP included trans-3-hydroxyproline and A-128-P included proline. Since this fraction contained the cis-3-hydroxyproline and the dehydrotryptophan, it became clear that the water-insoluble fraction consists of the C-terminal fragment of the antibiotics. The fraction obtained proved to be nonhomogeneous: it contained several peptides fluorescing with different intensities in UV light.

By chromatography in a thin layer of silica gel, the hexapeptide present in greatest amount was isolated in the homogeneous state. It was composed of cis- and trans-3-hydroxyprolines, dehydrotryptophan, β-methyltryptophan, β-hydroxyleucine, and glycine in the case of A-128-OP and the same six amino acids with the exception of the replacement of the trans-3-hydroxyproline by proline in the case of A-128-P. Both peptides had glycine at the N end (DNP method) and cis-3-hydroxyproline at the C end (Akabori's method). The N-terminal sequence of both peptides was established by Edmann's subtractive method [4] using phenyl isothiocyanate. After each cleavage step, the amino-acid composition and UV spectrum of the free peptide

* For a paper on the structure of the antibiotics A-128-OP and A-128-P, see [2].

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was determined in an aliquot. In view of the results on the C-terminal sequence of the hexapeptide of the antibiotic A-128-OP, its structure may be represented in the following way:

H–Gly→t–3HyP→e3HyLe→βMeTrp→ΔTrp→c–3HyP–OH.

The hexapeptide from the antibiotic A-128-OP was studied similarly, and this has the structure

H–Gly→Pro→e3HyLe→βMeTrp→ΔTrp→c–3HyP–OH.

To determine the N-terminal sequence we used the partial acid hydrolyzate [mixture of concentrated HCl and CH_3COOH (1 : 1), 50°C, 4 h] of the N-DNS* derivatives of the antibiotics. The use of the DNS label, which is highly sensitive to UV light, enables the N-terminal peptides to be detected and isolated by electrophoresis in a thin layer of silica gel in amounts sufficient for amino-acid analysis. In this way, the analysis of the antibiotic A-128-OP yielded the peptides

DNS–Asp
β|→Ser

DNS–Asp
β|→Ser→aThr

DNS–Asp
β|→Ser→aThr→Thr

DNS–Asp
β|→Ser→aThr→Thr→Ala.

The sequence of amino acids of the N-terminal pentapeptide of the antibiotic A-128-OP is

H
\
Asp
/
OH β|→Ser→aThr→Thr→Ala–OH.

A N-terminal pentapeptide of this structure is also present in the antibiotic A-128-P. The results obtained enable the structure of the linear "acid" of antibiotic A-128-OP to be deduced:

H
\
Asp
/
OH β|→Ser→aThr→Thr→Ala→Gly→t–3HyP→e3HyLe→
→βMeThr→ΔTrp→c–3HyP OH.

The antibiotic A-128-P has a similar sequence of amino acids, except that the trans-3-hydroxyproline in position 7 is replaced by L-proline.

In order to establish the complete structure of one of these antibiotics it is necessary to know the size of the lactone ring; in other words, it is necessary to know the amino hydroxy acid with the hydroxy group of which the cis-3-hydroxyproline forms a lactone bond. For this purpose, we made use of Sheehan's original method [5], consisting in the chromium-trioxide oxidation of the antibiotic and its linear "acid" followed by the amino-acid analysis of the oxidation products. On oxidation, all amino hydroxy acids are decomposed with the exception of those forming the ester bond. In both cases, this amino acid proved to be L-threonine. In this way, the complete structural formulas of the antibiotics A-128-OP and A-128-P were established (see following page).

A-128-OP and A-128-P differ in structure from telomycin [6] and also from LL-AO341B [7] and are new natural antibiotics.

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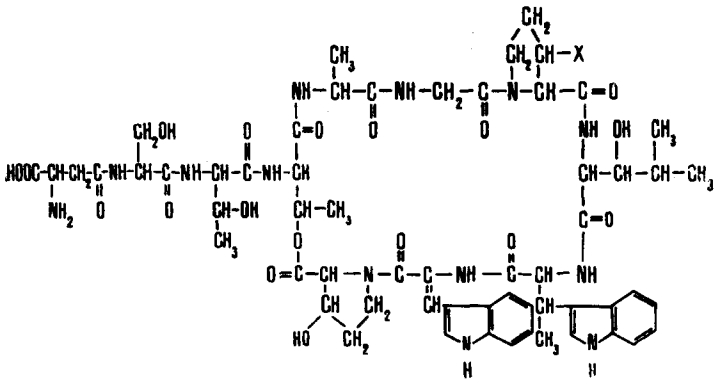
*Here and below, DNS means the 1-dimethylaminonaphthalene-5-sulfonyl derivative.



Structural formula of the antibiotics A-128-OP, X = OH (trans-3-hydroxyproline) and A-128-P, X = H (proline).

EXPERIMENTAL

Antibiotic A-128 (neotelomycin) was obtained from the N. F. Gamalei Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR [8]. It was separated into the components A-128-OP and A-128-P by gel filtration on Sephadex G-25 [9]. The reagents for the Edmann reaction were previously purified carefully by a published method [10].

Carboxypeptidase C, which forms part of the proteolytic complex of Aspergillus oryzae was kindly supplied by Dr. V. M. Stepanov (Protein Structure Laboratory, Institute of the Chemistry of Natural Compounds).

The preparation of the acids of the antibiotics A-128-OP and A-128-P has been described previously [1].

Determination of the C-Terminal Sequence in the Acid of Antibiotics A-128-OP and A-128-P by Hydrolysis with Carboxypeptidase C. A solution of 3 mg of the antibiotic and 1 mg of carboxypeptidase C from Aspergillus oryzae in 2 ml of acetate buffer with pH 5.3 was kept at 37°C for 10 h. Every 2 h, samples were taken from the solution and were investigated by thin-layer chromatography on cellulose in the butanol–acetic acid–water (4 : 1 : 1) system. As markers we took tryptophan (R_f 0.74), β-methyltryptophan (R_f 0.82), cis-3-hydroxyproline (R_f 0.34) and the complete hydrolyzate of the antibiotic.

Partial Alkaline Hydrolysis of the Antibiotics A-128-OP and A-128-P. A solution of 60 mg of the antibiotic under investigation in 20 ml of 2 N aqueous alcoholic (2 : 1) NaOH was thermostated at 30°C for 35 h and was then neutralized with hydrochloric acid at −19°C. The peptides that precipitated were repeatedly washed with water to free them completely from salts and from water-soluble peptides. Then they were separated off by centrifuging and were dried in vacuum. This gave 20 mg (67%) of a mixture of water-insoluble peptides. Their UV spectrum (λ_{max} 277, 290, and 337 nm) was identical with the UV spectra of the antibiotics in each case.

The water-soluble fraction of peptides was evaporated in vacuum in a rotary evaporator and the residue was stored in a desiccator over phosphorus pentoxide at + 4°C. Portions of 2 mg of the mixture of peptides from each fraction were hydrolyzed in evacuated sealed tubes in 6 N HCl at 105°C for 16 h and the total amino-acid composition of each fraction was determined by means of an amino-acid analyzer. The fraction of water-soluble peptides from each of the antibiotics A-128-OP and A-128-P contained aspartic acid, serine, two threonine, and alanine. The water-insoluble peptides contained glycine, dehydrotryptophan, β-methyltryptophan, cis-3-hydroxyproline, and erythro-β-hydroxyleucine and, in addition, trans-3-hydroxyproline in the case of A-128-OP and proline in the case of A-128-P.

Isolation of the C-Terminal Peptides from the Water-Insoluble Fraction. A solution of 37 mg of the mixture of peptides of the water-insoluble fraction in 90% methanol was deposited in narrow bands on four plates coated with a thin layer of silica gel. Chromatography was performed in the methyl ethyl ketone–butan-1-ol–water–ammonia (3 : 5 : 1 : 1) system. The tryptophan-containing peptides were detected by their

absorption in UV light at distances of 3.7, 3.1, and 2.8 cm from the point of deposition. The peptides were eluted from the silica gel with 90% methanol and the eluates were evaporated in a rotary evaporator. The homogeneity of the peptides isolated was checked by thin-layer chromatography in various systems. Of the three peptides isolated, the one present in largest amount was homogeneous. From 37 mg of the mixture of peptides of antibiotic A-128-OP, 20 mg of this peptide was obtained, and from 15 mg of the mixture of peptides from antibiotic A-128-P, 8 mg. Then the peptides were subjected to acid hydrolysis with 6 N HCl at 105°C for 16 h. After evaporation, the hydrolyzates were analyzed in an amino-acid analyzer by thin-layer electrophoresis in combination with chromatography. The peptides each contained the six amino acids of the C-terminal section of the acid of the antibiotics. The hexapeptide from A-128-OP differed from the hexapeptide from A-128-P by the fact that it contained trans-3-hydroxyproline in place of proline. In addition to these amino acids they each contained glycine, β-hydroxyleucine, dehydrotryptophan, β-methyltryptophan, and cis-3-hydroxyproline. The presence of dehydrotryptophan in the hexapeptides was shown by UV spectroscopy (λ_{max} 280 and 337 nm).

The determination of the N-terminal amino acid in each of the hexapeptides was performed by the dinitrophenylation method [12]. The N-terminal amino acid was identified by thin-layer chromatography. The results of the analysis showed that each of the hexapeptides had glycine at the N end.

Determination of the N-Terminal Sequence in the Hexapeptides by Edmann's Method. A solution of 12 mg of one of the hexapeptides in the dry state in 10 ml of a reagent containing 15 ml of pyridine, 15 ml of triethylamine, and 0.5 ml of phenyl isocyanate (all the reagents were carefully purified beforehand as described in the literature [10]) was heated at 40°C for 5 h, after which the solvent was evaporated off in a rotary evaporator, finally under vacuum (10^{-2} mm) at 40°C for 30 min. The residue was dissolved in 3 ml of anhydrous trifluoroacetic acid (in a current of nitrogen), and after 1 h (at 20°C) the trifluoroacetic acid was eliminated completely in vacuum with methanol. Then the residue dissolved in methanol was deposited on a column (1 × 5 cm) of Dowex 50 × 2 (H^+) previously washed with methanol. The excess of phenyl isothiocyanate and the phenylthiazolinone derivative of the N-terminal acid were first eluted from the column with methanol. Elution was continued until a sample no longer absorbed in UV light, which required approximately 200 ml of methanol. The shortened peptide was eluted from the column with a 2 N solution of ammonia in methanol until the ninhydrin reaction was negative. After separation, part of the peptide (1 mg) was removed and was hydrolyzed in a mixture of 3 mg of redistilled CH_3COOH and 1.5 ml of a 6 N HCl in a sealed tube at 107°C for 16 h. The hydrolyzate was evaporated and the amino-acid composition of the residual peptide was determined on the amino-acid analyzer. The shortened peptide was subjected to the following step of the Edmann degradation. A total of four steps was performed. The results of the determination of the N-terminal sequences in the hexapeptides from the antibiotics A-128-OP and A-128-P are given below.

Amino-acid composition*	Gly	t-3HyP	e3HyLe	βMeTrp	ΔTrp	c-3HuP
	1.0	0.9	0.7	1	1	1
Edmann's method						
1st step	0.3	0.9	0.7	1	1	1
2nd step	0.1	0.2	0.7	1	1	1
3rd step	0	0.1	0.1	1	1	1

Amino-acid sequence: Gly → t-3HyP → e3HyLe → βMeTrp → ΔTrp → c-3HyP

Similar results were obtained for the antibiotic A-128-P, except that Pro was found instead of t-3HyP.

The determination of the C-terminal amino acids in the hexapeptides from A-128-OP and A-128-P was performed by Akabori's method [13]; each hexapeptide has cis-3-hydroxyproline at the C end.

Preparation of the DNS Derivatives of the Antibiotics A-128-OP and A-128-P. To a solution of 40 mg of one of the antibiotics in 2 ml of a mixture of methanol and water (2 : 1) were added 3 ml of a 3% solution of $NaHCO_3$ (pH 9) and 22 mg of DNS chloride in 3 ml of acetone. The mixture was shaken at room temperature for 3 h. The completeness of the reaction was checked by paper electrophoresis. After the end of the reaction, the solution was brought to neutrality with 1 N hydrochloric acid and evaporated to dryness, and the DNS derivative was extracted with 10 ml of absolute ethanol (to eliminate salts). The residue was filtered off, and the filtrate was evaporated. The new residue was dissolved in 10 ml of a mixture of methanol and water (2 : 1) and was deposited on a 2 × 5-cm column of Dowex 2 × 8 ion-exchange resin in the Cl form. The DNS-antibiotics were eluted with methanol–water (2 : 1). The course of the reaction was followed

* The proportion of cis-3-HyP in the hydrolyzate was taken as 1.

from the luminescence in UV light. The eluate was evaporated on a rotary evaporator. The homogeneity of the DNS-antibiotics was checked by thin-layer electrophoresis on cellulose in 1 N CH_3COOH at 600 V for 1 h with DNS-amide, DNS-acid, and the initial antibiotic as markers. The DNS-antibiotic contained no impurities whatever. After hydrolysis, all the amino acids present in the initial antibiotic were found, apart from the N-terminal aspartic acid. The distances of migration from the point of deposition were 6.6 cm for DNS-amide and 3 cm for the DNS-antibiotic; the DNS-acid remained at the point of deposition.

The DNS-acids from the antibiotics A-128-OP and A-128-P were obtained by the same method. The DNS acid of the antibiotic under investigation was extracted with dry pyridine, and the solution was evaporated in a rotary evaporator. Yield 75%. The completeness of the reaction and the purity of the DNS-acid of the antibiotic were checked electrophoretically in a buffer with pH 6.5, 400 V, 2 h. The DNS-antibiotic was deposited on the electrophoregram as marker. The DNS-acid of the antibiotic was electrophoretically homogeneous in each case, and in the buffer with pH 6.5 it migrated 1 cm towards the anode, while the DNS-A-128-OP and the DNS-A-128-P remained at the point of deposition.

Partial Acid Hydrolysis of the DNS-Acid of the Antibiotics. A solution of 20 mg of the DNS-acid of the antibiotics in 10 ml of a mixture of concentrated CH_3COOH and 6 N HCl (1 : 1) was thermostated at 50°C in a sealed evacuated tube for 4 h. The hydrolyzate was evaporated in a rotary evaporator at 35°C and was investigated electrophoretically in a buffer with pH 6.5 at 450 V for 2 h.

The pure DNS-peptides (five) in the partial hydrolyzate of the DNS-acid of the antibiotics A-128-OP and A-128-P were determined by two-dimensional electrophoresis in a thin layer of cellulose. We have described the method and conditions elsewhere [14].

Preparative Separation of the DNS-Peptides. A solution containing the mixture of DNS-peptides (4 mg) was deposited in the form of a thin band on a plate coated with a thin layer of silica gel, and electrophoresis was performed in a buffer with pH 6.5 at 600 V for 70 min. The plate was dried under an infrared lamp. The zones of the DNS-peptides, which were located at distances of 2, 4.2, 6.5, 8, and 9 cm from the line of deposition were detected by examination in UV light. In each case, the silica gel with the DNS-peptide was transferred from the plate to a filter, and the DNS-peptide was eluted with a mixture of methanol and water (2 : 1). The solution of the peptide was evaporated in a rotary evaporator, and the homogeneity of the peptide was checked by electrophoresis and by chromatography in several systems of solvents and electrolytes. The nonhomogeneous DNS-peptides were purified by thin-layer electrophoresis on silica gel in a buffer with pH 2 at 500 V for 75 min.

Complete Hydrolysis of the DNS-Peptides. Each of the isolated DNS-peptides (0.5 g) was dissolved in 2 ml of 6 N HCl and hydrolyzed in a sealed evacuated tube at 105°C for 16 h. Then the hydrolyzate was evaporated in a rotary evaporator and the amino-acid composition of the DNS-peptide was investigated by paper electrophoresis in 1 N CH_3COOH (pH 2.4) at 600 V for 3 h and on the amino-acid analyzer. For the analysis of the peptides containing threonine and allothreonine (R_f 0.120 and 0.25, respectively) we used chromatography on Whatman No. 4 paper in the methyl ethyl ketone–butan-1-ol–25% ammonia (3 : 5 : 1 : 1) system.* The amino-acid composition of the peptides was determined by a combination of thin-layer electrophoresis and thin-layer chromatography on cellulose [15].

Oxidation of the Antibiotics A-128-OP and A-128-P and Their Acids with Chromium Trioxide [5]. The reagent for oxidation was 83.3 mg for CrO_3, 0.0167 ml of H_2O, 0.1 ml of pyridine, and 3 ml of CH_3COOH. The antibiotic or the acid of the antibiotic (5 mg) was dissolved in 1.5 ml of the oxidation mixture and left at room temperature for 16 h. Then the solution was evaporated in a rotary evaporator, and the residue was dissolved in 15 ml of n-butanol and the solution was extracted repeatedly (10-15 times) with 1% acetic acid saturated with butanol to remove the excess of chromium trioxide. The butanol extract was evaporated in a rotary evaporator. The oxidized antibiotic or acid of the antibiotic was subjected to acid hydrolysis (105°C, 6 N HCl, 12 h). The hydrolyzate was analyzed on the amino-acid analyzer and by paper chromatography in the methyl ethyl ketone–butan-1-ol–25% ammonia (3 : 5 : 1 : 1) system* on Whatman No. 4 paper. A complete hydrolyzate of the antibiotic and threonine and allothreonine were used as markers, the R_f value of threonine being 0.25 and that of allothreonine 0.120.

SUMMARY

The primary structures of two natural polypeptide antibiotics A-128-OP and A-128-P, which are tripeptidylcyclooctapeptidolactones, have been determined.

*As in Russian original – Publisher.

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