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OLIVOMYCIN AND RELATED ANTIBIOTICS

X. Isolation and Acid Degradation of Olivomycins A, B, C, and D*

Yu. A. Berlin, S. E. Esipov, O. A. Kiseleva, and M. N. Kolosov

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In 1962, Gauze and Brazhnikova and their colleages reported the isolation from Actinomyces olivoreticuli of a new antibiotic which they called olivomycin [3, 4]. From its medical, biological, and physicochemical properties, it belongs to the little-studied group including aureolic acid [5], aburamycin [6], LA-7017 [7], M5-18903 [8], NSCA-6409 [9], chromomycin [10], mithramycin [11], and antibiotics 232, 3014, 7193, 11296 [12] and 2410 [13]. Olivomycin is capable of retarding the growth of certain malignant tumors, which has made it of practical use in oncology, and it possesses a high antibiotic activity against Gram-positive bacteria [3, 14]. The mechanism of its antitumor action apparently consists of the inhibition of the DNA-dependent synthesis of RNA [15]. In the first chemical study of olivomycin, it was established that it consists of a mixture of substances of similar properties and the main component

Table 1

Antibiotic and its empirical formula	R_f^*	Minimum concentration (mg/l) to suppress			
		S. aureus	B. mycoides	E. coli	C. albicans
Olivomycin A $C_{58}H_{84}O_{26}$	0.70	0.1	0.01	>100	>100
Olivomycin B $C_{56}H_{80}O_{26}$	0.59	0.5	0.03	>100	>100
Olivomycin C $C_{56}H_{82}O_{25}$	0.43	0.3	0.05	>100	>100
Olivomycin D $C_{47}H_{66}O_{22}$	0.34	1.5	1.5	>100	>100

*In thin-layer chromatography on silica gel (activity grade IV) in the benzene-acetone (1:1) system.

of this mixture, which was called olivomycin 1 was obtained in the pure state [4, 16]. By thin-layer chromatography on silica gel we succeeded in separating native olivomycin into components and in isolating four individual antibiotics, olivomycins A (identical with olivomycin 1), B, C, and D, in a ratio of approximately 30:4:1:1. Information on the chromatographic mobilities and antibacterial activities of these antibiotics is given in Table 1. All the olivomycins possess very similar IR spectra and can hardly be distinguished by their UV spectra, the latter depending little on the acidity of the solution. A determination of the molecular weight of olivomycin A by the thermoelectric method in ethyl acetate gave a value of 1150-1200, and potentiometric titration in 10% ethanol gave an equivalent weight of 1200-1250 and a pK_a value of 7.3. By microanalysis and by methanolysis with subsequent gas-liquid chromatography, one O-isobutyl group and one O-acetyl group have been found in olivomycin A and two acetoxy groups in olivomycin B.

Since they are feebly acidic compounds, the olivomycins dissolve in aqueous solutions of bases, but they are unstable at high pH values because of their tendency to oxidize. In addition to this, they do not undergo substantial fragmentation in an alkaline medium, suffering only the loss of O-acyl groups under these conditions. Conversely, the olivomycins are readily decomposed under acid conditions with the formation of a mixture of substances one of which, from its spectroscopic characteristics, is similar to the initial antibiotic and consists of the aglycone olivin, while the others are carbohydrates or derivatives of them.** Depending on the olivomycin taken (A, B, C, or D) and the method

*For previous communications, see [1]; for Communication IX, see [2].

**The acid hydrolysis of olivomycin 1 (i.e., olivomycin A) was first carried out by M. G. Brazhnikova, et al., [17], who isolated a "chromophore" spectroscopically similar to the antibiotic; they did not suggest that the hydrolyzate from olivomycin contained any modified sugars.

of its cleavage (hydrolysis or alcoholysis), these carbohydrates, which we have called olivomycose, olivomose, olivose, and oliose, are isolated in the form of the free sugars or their glycosides. Two of them, olivomycose and olicose, in the form of O-acyl derivatives (isobutyrate and acetate) or the products of their hydrolysis.

Table 2

Volume of fraction, ml	Wt. of residue after evaporation, mg	Composition of the fraction
1050	168	Unidentified oily substances
150	33	Olivomycin A contaminated with chromatographically more mobile substances
450	328	Olivomycin A
450	780	Olivomycins A and B
150	45	Olivomycin B contaminated with a small amount of olivomycin C
150	52	Olivomycin C
150	25	Olivomycins C and D
150	40	Olivomycin D
150	22	Olivomycin D and substances with a lower chromatographic mobility

We have studied the acid degradation of the olivomycin in detail in the case of olivomycin A, the main component of the olivomycin complex. The most convenient method of its degradation proved to be alcoholysis with 0.05 N methanolic HCl at 70° C, which gives olivin and a mixture of methyl glycosides of the carbohydrate components of the antibiotic, the O-acetyl residue being split off completely but the O-isobutyryl group being unaffected. From this mixture of glycosides, by repeated chromatography on alumina in various systems, we have isolated methyl isobutyryl olivomycosides and methyl olivomosides in the pure state. To separate the methyl olivosides and methyl oliosides, which have very similar chromatographic mobilities, we used the difference in their chemical properties; it was found that the oliosides give isopropylidene derivatives under the action of acetone in the presence of $CuSO_4$, while the olivosides remain unchanged under these conditions and can be separated chromatographically.

Table 3

Benzene-acetone system	Volume of the fraction, ml	Wt. of residue after evaporation, mg	Composition of the fractions
3:1	200	170	Substances chromatographically more mobile than olivomycin A
3:1	500	1000	Olivomycin A
2:1	300	160	Olivomycins B and C contaminated with a small amount of olivomycin A
1:1	500	60	Olivomycins C and D contaminated with chromatographically less mobile substances

The carbohydrate components of the olivomycins are liberated in the free state when the antibiotics are hydrolyzed with aqueous acetic acid. The cleavage of the olivomycins by this method, which was first used by Japanese chemists in a study of the antibiotic chromomycin A_3 [18], takes place with the retention of the ester groups and leads to olivin and a mixture of monosaccharides which are separated relatively readily by adsorption or partition chromatography. Consequently, we used this method of degradation not only for the qualitative determination of the monosaccharides in the compounds investigated but also for their quantitative determination. A complete component analysis of olivomycin A showed that this antibiotic contains one residue each of olivin $C_{20}H_{22}O_9$, isobutyrylolivomycose $C_{11}H_{20}O_5$, olivomose $C_7H_{14}O_4$, and acetyloliose $C_8H_{14}O_5$, and two residues of olivose $C_6H_{12}O_4$. Since, as can be seen from the hydrolysis conditions, all these fragments are joined to one another by glucosidic bonds, olivomycin A must possess the empirical formula $C_{58}H_{84}O_{26}$. A similar quantitative determination of the compositions of the other olivomycins permitted the conclusion that olivomycin B is acetylolivomycosyl-olivomosyl-olivosyl-olivosyl-acetyloliosylolivin $C_{56}H_{80}O_{26}$, olivomycin C is isobutyrylolivomycosyl-olivomosyl-olivosyl-olivosyl-oliosyl-olivin $C_{56}H_{82}O_{25}$, and olivomycin D is

olivomosyl-olivosyl-olivosyl-acetyloliosyl-olivin $C_{47}H_{66}O_{22}$.

Experimental

Chromatography was carried out with neutral alumina (activity grade V) and silica gel of the "aqueous silicic acid" type (activity grade IV) smaller than 150 mesh. Thin-layer chromatography was carried out in a nonfixed layer of adsorbent 0.5 mm thick for analytical purposes and 0.5–2 mm thick for the preparative separation of mixtures. The R_f values given for the substances isolated by thin-layer chromatography relate to that system in which the separation was carried out. Partition chromatography was carried out by the descending method on Whatman No. 2 paper in the 1-butanol–ethanol–water (4:1:5, upper layer) system. The reagents used for revealing the spots were aniline hydrogen phthalate, antimony trichloride, and triphenyltetrazolium chloride. The melting points were determined on a Kofler block.

Production of the crude olivomycin. The fermentation of A. olivoreticuli (the strain of A. olivoreticuli was provided by the Institute for the Search for New Antibiotics of the Ministry of Health of the USSR) was carried out under the conditions described previously [3]. The filtrate of the culture was acidified with HCl to pH 3.5 and extracted with butyl acetate and the combined extracts were passed through a column of Al_2O_3, after which they were washed with 10% $Na_2S_2O_4$ solution, dried with Na_2SO_4, and evaporated, and the residue was freed from fats by trituration with petroleum ether. One hundred liters of culture filtrate gave 40 g of crude olivomycin in the form of a yellow-brown powder containing about 60% of a mixture of antibiotics.

Table 4

Antibiotic, amount, μmole		Found, μmole					
		isobutyrylolivomycose	acetylolivomycose	olivomose	olivose	acetyloliose	oliose
Olivomycin A	0.13	0.15	—	0.13	0.26	0.14	—
	0.21	0.18	—	0.20	0.41	0.21	—
Olivomycin B	0.13	—	0.13	0.11	0.28	0.12	—
	0.21	—	0.22	0.18	0.49	0.20	—
Olivomycin C	0.13	0.15	—	0.13	0.25	—	0.12
	0.22	0.22	—	0.22	0.44	—	0.21
Olivomycin D	0.15	—	—	0.14	0.28	0.15	—
	0.25	—	—	0.24	0.45	0.28	—

Separation of olivomycins A, B, C, and D. A. By preparative thin-layer chromatography on silica gel in the benzene–acetone (1:1) system, 2 g of crude olivomycin yielded 800 mg of olivomycin A, 100 mg of olivomycin B, 30 mg of olivomycin C, and 20 mg of olivomycin D and also 250 mg of various mixtures of olivomycins which were used for reseparation. The residual part of the initial sample consisted of foreign materials which were not investigated further.

B. Two grams of crude olivomycin was chromatographed on 400 g of silica gel (column 650 × 55 mm) in the benzene–acetone (2:1) system, 150-ml fractions being collected (Table 2).

C. By using gradient desorption, satisfactory results were obtained on a smaller amount of adsorbent. Table 3 gives the results of the chromatography of 2 g of crude olivomycin on a 50 × 50 mm column containing 30 g of silica gel.

D. Separation of the crude olivomycin by countercurrent distribution was carried out by means of 400 transfers in the ethanol–methyl acetate–n-hexane–water (10:14:10:13) system, the distribution coefficients of olivomycins A, B, C, and D being 0.76, 0.24, 0.22, and 0.16, respectively.

Olivomycins. Olivomycin A is a yellow crystalline substance with mp 160°–165° C (from a mixture of ethyl acetate and hexane), $[\alpha]_D^{23}$ −35.5°, $[\alpha]_{578}$ −36°, $[\alpha]_{546}$ −37° (c 0.5; in ethanol); ν_{max} 1060, 1510, 1582, 1640, 1738, 3380 cm^{-1}; λ_{max}^{EtOH} 228, 277, 308 shoulder, 318, 330 shoulder, 406 mμ (log ε 4.35, 4.67, 3.72, 3.81, 3.59, 4.05. Equivalent weight 1200–1250 pK_a 7.2 (by potentiometric titration with 0.1 N NaOH in 10% aqueous ethanol); mol. wt. 1150–1200 (by the thermoelectric method for a 0.03 M solution in ethyl acetate).

Found, %: C 57.7, H 7.0; CH_3O 5.1; $CH_3(C)$ 9.5; $CH_3CO(O)$ 7.2 (from the results of gas-liquid chromatography–1 CH_3COO and 1 $(CH_3)_2CHCOO$). Calculated for $C_{58}H_{84}O_{26}$, %: C 58.2; H 7.1; 2 CH_3O 5.2; 8 $CH_3(C)$ 10.0; 2 $CH_3CO(O)$ 7.2; mol. wt. 1197.

Determination of the acidity of the borate complex of olivomycin A: A 0.18 N solution of H_3BO_3 in 75% aqueous alcohol had pH 5.55; when olivomycin A was dissolved in it to a concentration of 0.009 M, the pH fell to 3.05.

Olivomycin B is a yellow amorphous substance $[\alpha]_D^{20}$ −28°, $[\alpha]_{578}$ −26.5°, $[\alpha]_{546}$ −22.5° (c 1; in ethanol); ν_{max} 1060, 1510, 1582, 1639, 1740, 3370 cm^{-1}; λ_{max}^{EtOH} 228, 276, 308, (shoulder), 318, 330 (shoulder), 406 mμ (log ε 4.34, 4.64, 3.71, 3.78, 3.61, 4.02).

Found, %: C 57.5; H 7.2; $CH_3CO(O)$ 7.0. Calculated for $C_{56}H_{80}O_{26}$, %: C 57.5; H 7.0; 2 $CH_3CO(O)$ 7.3.

Olivomycin C is a yellow amorphous substance, $[\alpha]_D^{20}$ −17°, $[\alpha]_{578}$ −16.5°, $[\alpha]_{546}$ −15° (c 0.3; in ethanol); ν_{max} 1060, 1512, 1584, 1637, 1720, 3370 cm^{-1}; λ_{max}^{EtOH} 228, 277, 308 (shoulder), 319, 330 (shoulder), 406 mμ (log ε 4.36, 4.67, 3.73, 3.80, 3.69, 4.04).

Found, %: C 58.3; H 7.4; $(CH_3)_2CHCO(O)$ 6.3. Calculated for $C_{56}H_{82}O_{25}$, %: C 58.2; H 7.2; 1$(CH_3)_2CHCO(O)$ 6.2.

Olivomycin D is a yellow amorphous substance, $[\alpha]_D^{20}$ −25°, $[\alpha]_{578}$ −24°, $[\alpha]_{546}$ −20° (c 0.8; in ethanol) ν_{max} 1060, 1510, 1582, 1638, 1727, 3360 cm^{-1}; λ_{max}^{EtOH} 227, 275, 308 (shoulder), 319, 330 (shoulder), 406 mμ (log ε 4.34, 4.68, 3.77, 3.80, 3.38, 4.03).

Found, %: C 56.3; H 6.8. Calculated for $C_{47}H_{66}O_{22}$, %: C 56.4; H 6.8.

Methanolysis of olivomycin A. A solution of 1.2 g (1 mmole) of olivomycin A in 100 ml of 0.05 N methanolic HCl was boiled for 2.5 hr and, after cooling, it was neutralized with Ag_2CO_3 and the silver salt was filtered off. The filtrate was evaporated and the residue was dissolved in 20 ml of water and extracted with ethyl acetate (a total of 40 ml). The residue after the evaporation of the extract was triturated with 50 ml of ether and the olivin that precipitated was filtered off, an additional amount of the material being obtained from the filtrate by dilution with an equal volume of hexane. The total yield of olivin was 349 mg (86%), mp 187°–189° C (from acetonitrile), $[\alpha]_D^{25}$ + 60.5° (c 0.5, in ethanol).

Found, %: C 58.8; H 5.6. Calculated for $C_{20}H_{22}O_2$, %: C 59.1; H 5.5.

After evaporation of the solvent, the mother liquor yielded 368 mg of a mixture of substances (so-called ether fraction). The aqueous solution was evaporated, the residue was dissolved in ethyl acetate, and the solution was filtered and again evaporated; the weight of residue was 480 mg (so-called aqueous fraction). As was shown by analytical chromatography, the ether fraction contained, in addition to a small amount of olivin, a mixture of glycosides in which methyl isobutyrylolivomycosides predominated. The latter were almost absent from the aqueous fraction, the main components of which were methyl olivomosides, olivosides, and oliosides. The initial separation of the two fractions was effected by thin-layer chromatography on Al_2O_3 in the benzene–acetone (1:1) system, 180 mg (74%) of methyl isobutyrylolivomycosides (R_f 0.89), 98 mg (56%) of methyl olivomosides (R_f 0.61), and 360 mg (74%) of methyl olivosides and methyl oliosides (R_f 0.40–0.25) being isolated.

Methyl isobutyrylolivomycosides. 180 mg of a mixture of methyl isobutyrylolivomycosides was chromatographed on 180 g of Al_2O_3 (column 300 × 40 mm) elution being carried out with methylene chloride. After additional purification by thin-layer chromatography on Al_2O_3 (activity grade II) in ethyl acetate, 115 mg of the α-glycoside and 40 mg of the β-glycoside were isolated.

α-Methyl isobutyrylolivomycoside: $[\alpha]_D^{26}$ −115° (c 0.7, in ethanol).

Found, %: C 58.7; H 9.1. Calculated for $C_{12}H_{22}O_5$, %: C 58.5; H 9.0.

β-Methyl isobutyrylolivomycoside: $[\alpha]_D^{23}$ + 27° (c 1.7, in ethanol).

Found, %: C 58.7; H 8.9.

Methyl olivomosides. By thin-layer chromatography on Al_2O_3 in the benzene–acetone (1:1) system, 350 mg of a mixture of methyl olivomosides yielded 260 mg of α-methyl olivomoside (R_f 0.61) and 52 mg of the β-anomer (R_f 0.54).

α-Methyl olivomoside: mp 97°–98° C (from hexane), $[\alpha]_D^{22}$ + 160° (c 0.5, in ethanol).

Found, %: C 54.8; H 9.3. Calculated for $C_8H_{16}O_4$, %: C 54.5; H 9.2.

β-Methyl olivomoside: mp 152°–153° C (from hexane), $[\alpha]_D^{26}$ −35° (c 0.4, in ethanol).

Found, %: C 54.7; H 9.3.

Methyl olivosides. A solution of 264 mg of a mixture of methyl olivosides and methyl oliosides in 30 ml of acetone was boiled with 0.5 g of anhydrous copper sulfate with stirring for 12 hr. After filtration and evaporation, the

residue was chromatographed on Al_2O_3 in the benzene–acetone (1:1) system. The fraction with the mobility of the initial mixture (R_f 0.25–0.40) was subjected to rechromatography under the same conditions, which led to the isolation of 101 mg of α-methyl olivoside (R_f 0.38) and 25 mg of β-methyl olivoside (R_f 0.27) (the α-glycoside could also be isolated from the initial mixture without preliminary treatment with acetone).

α-Methyl olivoside: $[\alpha]_D^{25}$ + 131° (c 0.7, in ethanol).

Found, %: C 51.8; H 8.7. Calculated from $C_7H_{14}O_4$, %: C 51.8; H 8.7.

β-Methyl Olivoside: mp 84° C (from ethyl acetate–hexane), $[\alpha]_D^{22}$ −85° (c 1, in ethanol).

Found, %: C 51.8; H 8.6.

Methyl oliosides. After the separation of the methyl olivosides, 75 mg of a mixture of isopropylidene methyl oliosides was isolated from the fraction with R_f 0.85–0.90. A solution of this mixture in 5 ml of 0.05 N methanolic HCl was heated at 75° C for 4 hr and after being cooled it was neutralized with Ag_2CO_3, filtered and evaporated, and the residue was chromatographed on Al_2O_3 in the benzene–acetone (1:1) system. The zone with R_f 0.25–0.35 yielded 50 mg (87%) of anomeric methyl oliosides.

Found, %: C 52.4; H 8.2. Calculated for $C_7H_{14}O_4$, %: C 51.9; H 8.6.

Hydrolysis of olivomycin A. A solution of 1.2 g (1 mmole) of olivomycin A in 100 ml of 50% acetic acid was heated at 75° C for 3 hr and evaporated, and the residue was dissolved in 35 ml of ethyl acetate and extracted three times with water (a total of 30 ml). The dried ethyl acetate solution was concentrated to half its volume and diluted with two volumes of hexane, and the precipitate that deposited was filtered off. This gave 360 mg of the solvate of olivin with acetic acid (contaminated with the product of the complete hydrolysis of the antibiotic), while the mother liquor after the elimination of the solvent gave 230 mg of crude isobutyrylolivomycoside. The combined aqueous extract was evaporated and the residue (730 mg) was chromatographed on silica gel in the benzene–acetone (1:1) system. This yielded 70 mg of isobutyrylolivomycose with R_f 0.77 (contaminated with olivin), 80 mg of acetyloliose (R_f 0.38), 33 mg of its isomerization product (R_f 0.54), 120 mg of olivomose (R_f 0.28), and 213 mg of olivose (R_f 0.14). Both portions of isobutyrylolivomocose (230 mg and 70 mg), in order to free them from olivin, were rechromatographed in the same system on Al_2O_3, as a result of which 190 mg of analytically pure material with R_f 0.72 was obtained.

Isobutyrylolivomycose: $[\alpha]_D^{23}$ − 43° → −33.5° (c 0.5, in water); R_f 0.84 (on paper).

Found, %: C 56.4; H 8.6. Calculated for $C_{11}H_{20}O_5$, %: C 56.8; H 8.7.

Olivomose: mp 158°–162° C (from acetone), $[\alpha]_D^{23}$ + 113° → + 90° (c 1, in water), R_f 0.65 (on paper).

Found, %: C 51.4; H 8.7. Calculated for $C_7H_{14}O_4$, %: C 51.8; H 8.7.

Olivose: $[\alpha]_D^{23}$ +31° (c 1, in water), R_f 0.54 (on paper).

Found, %: C 48.1; H 8.3. Calculated for $C_6H_{12}O_4$, %: C 48.6; H 8.2.

Acetyloliose: $[\alpha]_D^{23}$ + 89° → + 81° (c 1, in water); R_f 0.70 (on paper).

Found, %: C 50.6; H 7.6. Calculated for $C_8H_{14}O_5$, %: C 50.5; H 7.4.

Acetylolivomycose, which is formed together with isobutyrylolivomycose in the hydrolysis of olivomycin B, has $[\alpha]_D^{21}$ − 30° (c 1, in water), R_f 0.80 (on paper). This sugar is identical with the substance obtained from α-methyl olivomycoside by acetylation and hydrolysis.

Oliose, which is formed together with acetyloliose in the hydrolysis of olivomycin C, has $[\alpha]_D^{20}$ + 51° (c 0.7, in water), R_f 0.44 (on paper).

Found, %: C 48.6; H 8.2. Calculated for $C_6H_{12}O_4$, %: C 48.6; H 8.2.

Quantitative determination of the monosaccharide composition of the olivomycins. The individual monosaccharides in amounts of 25–100 μg were chromatographed on paper under the standard conditions described above. After drying, the chromatogram was passed through a mixture of equal volumes of 4% triphenyltetraolium chloride solution and 1 N methanolic NaOH and was heated at 65° C for 1 hr. The triphenylformazans were extracted from the spots corresponding to the various monosaccharides by means of 5 ml of methanol acetic acid (10:1) and the optical densities of the extracts obtained were determined at 480 mμ against the eluate from the background of the chromatogram as comparison solvent. Calibration curves (in the form of plots of the optical density of the solutions against the molar amounts of sugars) practically coincided for all the carbohydrate components of the olivomycins with the exception of olivomose.

To determine its monosaccharide composition, the antibiotic was hydrolyzed with 50% acetic acid (5 hr at 75° C), the hydrolyzate was evaporated to dryness, and the residue was dissolved in ethanol and chromatographed on paper, after which the sugars were determined quantitatively as described above. The results of the analyses are given in Table 4.

Summary

1. From the culture fluid of the ray fungus Actinomyces olivoreticuli four individual antibiotics, olivomycins A, B, C, and D have been isolated.

2. The acid degradation of olivomycins A, B, C, and D has been carried out with the formation of the aglycone olivin and the carbohydrates olivomycose, olivomose, olivose, oliose, and (or) their derivatives.

3. The qualitative and quantitative monomeric composition of olivomycins A, B, C, and D has been elucidated and their empirical formulas have been established.

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Institute of the Chemistry of Natural Compounds, AS USSR