GLYCOSIDES OF VACCARIA SEGETALIS

III. Enzymatic and Alkaline Cleavage of Vacsegoside

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The isolation from the seeds of Vaccaria segetalis (Neck.) Carke of two triterpene glycosides, vacsegoside and segetoside, has been reported previously [1, 2]. It has been shown that on hydrolysis wi th dilute sulfuric acid vascegoside gives the β-D-glucopyranoside of gypsogenin, vaccaroside [1], and the monosaccharides D-glucose, L-arabinose, D-xylose, D-fucose, and L rhamnose. All the sugars were obtained in the crystalline state [2].

The molecular weight of vacsegoside was calculated by a method based on the adjusted integral intensities of the stretching vibration bands of the carbonyl groups in the IR spectra of vaccaroside and vacsegoside, and lies within the range 1600-1650. The results of measuring the integral intensity of the ester band of the glycoside acetate in the 1700-1800 cm^{-1} region [3] shows that vacsegoside contains 18-20 acetylatable hydroxyl groups. Photometric determination of the porportions of the sugars by the aniline phthalate method [4] and also the amount of vaccaroside obtained by the hydrolysis of the vacsegoside with dilute sulfuric acid show that the sugar part of the glycoside contains 2 molecules each of D-glucose and D-xylose and 1 molecule each of L-arabinose, D-fucose, L-rhamnose, and D-glucuronic acid. Consequently, vacsegoside is an octaoside of gypsogenin and must have the empirical formula $C_{75}H_{98}O_{40}$ and, correspondingly, mol. wt. 1659.77. As with the majority of triterpene glycosides, the substance forms stable crystal solvates which decompose only on prolonged heating under vacuum. The formula $C_{75}H_{98}O_{40}(CH_3CO)_{20}$ corresponds to vacsegoside acetate, which has been described previously [2]. Further information on the structure of vacsegoside has been obtained by enzymatic and alkaline hydrolysis of the glycoside.

The action of the pancreatic juice of the snail Helix plectotropis on vacsegoside forms a crystalline substance (IV). Paper chromatography of the aqueous hydrolyzate showed the presence of D-glucose, D-xylose, D-glucuronic acid, and a disaccharide of unknown structure. The product (IV) was subjected to hydrolytic decomposition with 2% sulfuric acid under conditions corresponding to the conditions of the hydrolysis of vacsegoside to vaccaroside [2]. This yielded gypsogenin (II) and the sugars D-fucose, L-rhamnose, L-arabinose, and D-xylose. Here, the formation of gypsogenin (II) in place of vaccaroside (III) shows that substance (IV) is an O-acylglycoside in which the sugar chain is linked to the carboxyl group of gypsogenin. From the results of a photometric determination, the 28-O-acylglycoside contains one molecule each of D-glucose, L-rhamnose, L-arabinose, and D-xylose. Consequently, substance (IV) is a 28-O-acyltetraoside of gypsogenin.

The alkaline hydrolysis of the acyglycoside (IV) gave gypsogenin. The tetraoside associated with it could not be detected by the usual reagents for reducing sugars. Consequently, after the separation of the gypsogenin the alkaline hydrolyzate was a acidified with sulfuric acid and heated. Among the hydrolysis products only L-rhamnose, L-arabinose, and D-xylose were found. The absence of D-fucose indicates that this particular sugar, partially decomposed on alkaline hydrolysis is, very probably, directly attached to the carboxyl group of the gypsogenin.

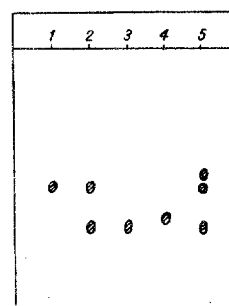
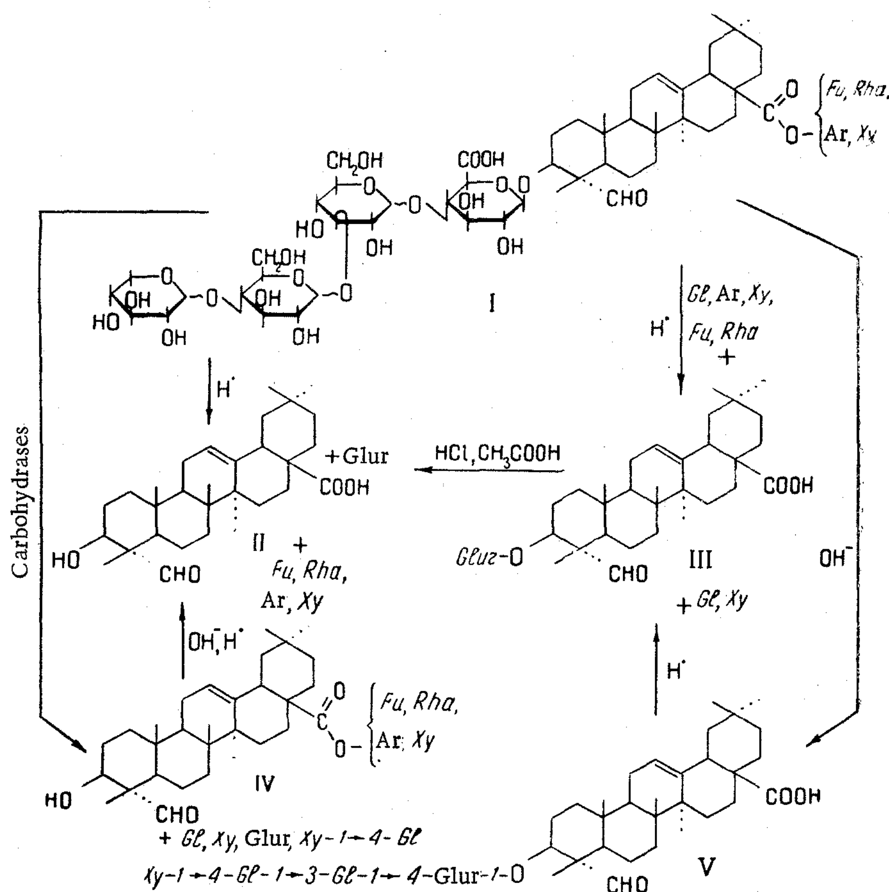


Fig. 1 Control chromatograms of methylated sugars on paper. System: n-butyl alcohol saturated with water. Time 29 hr. 1) 2, 3, 6-Tri-O-methyl-D-glucose; 2) hydrolyzate of the methylated sugar obtained by the enzymatic hydrolysis of vacsegoside; 3) 2, 3, 4-tri-O-methyl-D-xylose; 4) 2, 3, 4, 6-tetra-O-methyl-D-glucose; 5) hydrolyzate of methylated vaccarotetraoside.

The sugar fraction of the hydrolyzate obtained by the enzymatic cleavage of vacsegoside was separated on a cellulose column. After the removal of the monosaccharides, D-glucuronic acid, D-glucose, and D-xylose, a chromatographically homogeneous disaccharide was eluted from the column. Hydrolysis of the disaccharide with dilute sulfuric acid gave D-glucose and D-xylose. The disaccharide was subjected to exhaustive methylation. The methylation product was hydrolyzed with dilute sulfuric acid, and the methlylated sugars were compared by paper chromatography with various methyl derivatives of D-xylose and D-glucose. 2, 3, 4-Trimethyl-D-xylose and 2, 3, 6-trimethyl-D-glucose were identified (Fig. 1). Thus, the disaccharide obtained by the enzymatic hydrolysis of vacsegoside is 4-(D-xylopyranosido)-D-glucopyranoside. To determine the structure of the sugar moiety linked to the hydroxyl group at C_3 of gypsogenin, vacsegoside was hydrolyzed with caustic soda in aqueous alcholic solution. A single substance (V) was obtained. The acid hydrolysis of this compound gave vaccaroside (III) and a number of sugars including D-glucose and D-xylose. The amount of vaccaroside formed on hydrolysis and the photometric determination of the propoportions of the sugars show

that the glycoside (V) contains, in addition to D glucuronic acid, two molecules of D-glucose and one molecule of D-xylose. Consequently, we have provisionally called the glycoside vaccarotetraoside.



Scheme of the hydrolytic conversions of vacsegoside (I)

Gl – glucose; Glur – glucuronic acid; Fu – fucose; Rha – rhamnose; Ar – arabinose; Xy – xylose

Vaccarotetraoside (V) was oxidized with periodic acid, and the oxidation product was hydrolyzed with dilute sulfuric acid. Gypsogenin was deposited and D-glucose was found in the hydrolyzate. The fact that after periodic-acid oxidation D-glucose was found to be unaffected by the oxidation shows that there is branching in the sugar chain or the third hydroxyl group of one of the molecules of glucose is substituted. To elucidate this question, compound (V) was subjected to exhaustive methylation. When the methanolysis products of the methylated vaccarotetraoside were chromatographed on paper, three spots appeared. Two of them were identified as 2, 3, 4-trimethyl-D-xylose and 2, 3, 6-trimethyl-D-glucose (see Fig. 1). These methylated sugars evidently correspond to the two sugars of 4-(D-xylopyranosido)–D-glucopyranose obtained by the enzymatic cleavage of vacsegoside. But the third spot, with a R_f value less than that of 2, 3, 6-trimethyl-D-glucose must, judging from [5], be assigned to 2, 4, 6-trimethyl-D-glucose.

Since the exhaustive methylation of vaccarotetraoside (V) gave only one fully methylated sugar, 2, 3, 4-trimethyl-D-xylose the D-xylose molecule must be the terminal one in the sugar chain. The production of 4-(D-xylopyranosido): D-glucopyranose shows that the xylose is connected to the fourth hydroxyl group of one of the two glucose molecules. The other molecule of glucose occupies the linking position between the disaccharide and gypsogenin β-D-glucuronoside (III). The disaccharide is attached to its third hydroxyl group. In its turn, this molecule of glucose is most probably connected with the fourth or else with the second hydroxyl group of the glucuronic acid. The 1-3' bond is excluded, since in this case the periodic- acid oxidation of vaccarotetraoside would not affect the glucuronic acid. The nature of the glycosidic bonds is still obscure, except for the bond of the glucuronic acid with the gypsogenin (β-glycosidic bond [1]).

All that has been said enables us to put forward the partial structural formula (I) for vaccegoside.

Experimental

In all cases, including determinations of molecular weight and number of acetyl groups, the substance under investigation was pressed into tablets with potassium bromide (0.5-1.0 mg of substance to 150 mg of KBr). Silica gel of type

KSK was used for the thin-layer chromatography [6]. The following systems of solvents (by volume) were used for chromatography on paper and in a thin fixed layer of silica gel: 1) butan-1-ol—acetic acid—water (4 : 1 : 5) butan-1-ol—ethanol—water (1 : 1 : 3); 3) butan-1-ol—ethanol—water (7 : 2 : 5).

Vacsegoside (for preparation, see [2]), mp 217-219°C (decomp). $[\alpha]_D^{26}$ − 7.5 ± 1° (c 3.16, 30% acqueous methanol).

Found, %: C 54.32; 54.58; H 7.53; 7.54. Calculated for $C_{75}H_{118}O_{40}$, %: C 54.26; H 7.16.

793 mg of vacsegoside gave 322 mg of vaccaroside, $C_{75}H_{118}O_{40}$, (mol. wt. 1659). Calculated 326 mg.

Vaccegoside acetate (for preparation, see [2]), mp 182-186°C, $[\alpha]_D^{27}$ − 32.3 ± 1° (c 3.09; chloroform).

Found, %: C 56.06; 55.77; H 6.59; 6.83. Calculated for $C_{115}H_{158}O_{60}$, %: C 55.33; H 6.36.

Gypsogenin 28-O-tetraoside (IV). One ml of the pancreatic juice of the snail Helix plectotropis was added to 3 g of vacsegoside in 250 ml of water. The mixture was kept in a thermostat at 34-36°C for 2 days. Then 0.3 ml of snail juice was added and the reaction mixture was left for another 8 days at the same temperature. Subsequently, chromatography in system 3 gave the spot of a new substance in addition to the spot of the initial compound. The fermentation products were extracted with butanol (three 150-ml portions). The emulsion which formed was broken by the addition of a small amount of methanol. The combined butanolic extract was concentrated under vacuum until the appearance of turbidity. The precipitate which was deposited (the initial vacsegoside) was separated off, and the mother liquor was evaporated to dryness. The residue was transferred to a column 1.5 cm in diameter charged with 15 g of silica gel. The column was washed with a mixture of chloroform and methanol (3 : 1). The first fractions, which showed the spot of the new compound, were combined and concentrated. The residue was dissolved in 10 ml of methanol and was precipitated with a tenfold amount of petroleum ether. The precipitate was dissolved in 15 ml of methanol, 15 ml of butyl alcohol was added, and part of the methanol was evaporated off. A white precipitate was deposited. Repetition of this operation gave small white needles which, after drying under vacuum at 80°C, had mp 253-255°C, $[\alpha]_D^{26}$ − 8.2 ± 2° (c 1.72, 80% aqueous ethanol). A comparative chromatogram is shown in Fig. 2.
IR spectrum: 3400 (OH), 1735 (C=O) (water of hydration), 1250 cm^{-1} (C—O—C).

Found, %: C 58.70; 58.60; H 7.86; 8.16. Calculated for $C_{52}H_{82}O \cdot 2H_2O$, %: C 58.69; H 8.14.

After the extraction with butanol, the enzymatic hydrolyzate was shown by paper chromatography in systems 1 and 2 to contain D-glucuronic acid, D-glucose, D-xylose, and an unknown disaccharide.

Gyposgenin 28-O-tetraoside acetate (IV). A mixture of 50 mg of the tetraoside, 1 ml pyridine, and 1 ml of acetic anhydride was left at room temperature for 3 days. After the usual procedure, the product that was deposited in ice water was dissolved in 1 ml of benzene, and the solution was poured dropwise into 20 ml of low-boiling (bp 40-60°C) petroleum ether. The dried white precipitate had mp 153-155°C.

Acid and alkaline hydrolysis of gyposgenin 28-O-tetraoside. Methanol was added dropwise to a suspension of 50 mg of substance (IV) in 5 ml of 2% sulfuric acid until the solid material had completely dissolved. The reaction mixture was heated on a boiling water bath for 4.5 hr, and the methanol was evaporated off. Crystals were deposited which, by their chromatographic behaviour (see Fig. 2) and melting point, were gypsogenin. A mixture with authentic gypsogenin gave no depression of the melting point. Paper chromatography in systems 1 and 2 of the hydrolyzate neutralized with barium carbonate showed the presence of L-arabinose, D-xylose, D-fucose, and L-rhamnose.

40 mg of the tetraoside (IV) was heated on a boiling water bath with 2 ml of 10% aqueous alcoholic caustic potash solution for 3 hours. The cooled solution was acidified with 2% sulfuric acid to pH 2 and evaporated until a precipitate was produced. The precipitate was washed with water to neutrality.

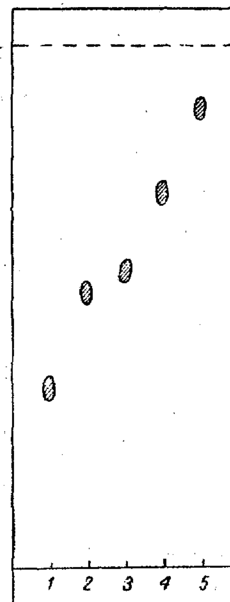


Fig. 2 Combined chromatogram of the product of vacsegoside cleavage on fixed silica gel. n-Butyl alcohol—ethanol—water (7 : 2 : 5) system; time 5 hr. 1) vacsegoside; 2) 28-O-tetraoside; 4) vaccaroside; 5) gypsogenin.

When chromatographed on silica gel in systems 1 and 3, a sample of the substance obtained migrated to the same level as gypsogenin. The hydrolyzate was additionally acidified to pH 1 and heated for 4 hours. After neutralization of the sulfuric acid with barium carbonate, L-arabinose, D-xylose, and L-rhamnose were detected by paper chromatography in system 1.

4-(D-xylopyranosido)-D-glucopyranose. The enzymatic hydrolyzate, which, after the extraction of the tetraoside (IV) with butanol, contained D-glucuronic acid, D-glucose, D-xylose, and a disaccharide, was filtered and evaporated to dryness with the aid of the azeotropic mixture with butyl alcohol. A solution of the residue in 5 ml of water was treated with 3 ml of methanol mixed with 10 g of cellulose powder, and transferred to an 85-cm column charged with 200 g of cellulose. The cellulose had previously been washed with the organic layer (phase) of solvent system 1. The charged column was washed with the same solvent mixture. Fractions with a volume of 20-25 ml were collected and were analyzed by paper chromatography in system 1. Fractions 1-5 contained no sugars or glycosides, fractions 6-22 were found to contain unchanged vacsegoside, 23-28 contained xylose, 29-32 a mixture of xylose and glucose, 33-38 glucose, 34-41 a mixture of glucose and the disaccharide, 42-46 the disaccharide, and, finally, fractions 47-49 contained a mixture of disaccharide with traces of glucuronic acid.

Fraction 42-46 containing the disaccharide were combined and evaporated to dryness under vacuum. The residue consisted of an amorphous powder.

A solution of 15 mg of the disaccharide in 2 ml of 2% sulfuric acid was heated at 100°C for 2 hours. Paper chromatography of the hydrolyzate neutralized with barium carbonate showed the presence of D-glucose and D-xylose.

Methylation of 4-(D-xylopyranosido)-D-glucopyranose. A solutuon of 100 g of the disaccharide in 7 ml of water was treated with 1 ml of dimethyl sulfate and dropwise with 1 ml of 40% caustic soda, the medium being kept alkaline and the temperature not above 30°C during this process. The reaction mixture was left overnight. Then a further 2 ml of dimethyl sulfate was added and the mixture was heated to 70°C, and 2 ml of 40% caustic soda was added dropwise over 2 hours. The mixture was heated for 45 min on a boiling water bath. The cooled solution was extracted with chloroform, and the chloroform extract, after being dried over Na_2SO_4, was evaporated to the syrupy state. The syrup of the partially methylated sugar was dissolved in absolute ether and several pieces of sodium metal were added [7] and then, dropwise, 1 ml of dimethyl sulfate diluted with absolute ether. As soon as the initially vigorous reaction had abated, the heating of the reaction mixture was begun (1 hour at 50°C). The excess of sodium was removed, and the ether was distilled off. The residue was treated with 4 ml of 10% caustic soda, and the mixture was heated for 1 hr at 60°C and for a further 20 min at 90°C. The cooled reaction mixture was extracted with chloroform. The chloroform extract was dried with Na_2SO_4 and evaporated. The completely methylated disaccharide obtained, chromatography of which on silica gel in system 2 showed only one spot (revealed by sulfuric acid), was dissolved in 3 ml of 2% sulfuric acid and heated for two hours on a boiling water bath. After neutralization of the sulfuric acid with the anion-exchanger "Dowex-1" (CO_3—form), the hydrolyzate was concentrated and the product obtained was compared by paper chromatography with methylated derivatives of xylose and glucose. 2, 3, 6-Trimethyl-D-glucose and 2, 3, 4-trimethyl-D-xylose were identified (cf. Fig. 1).

Vaccarotetraoside (V). A solution of 8 g of vacsegoside in 16 ml of water was treated with 122 ml of a 10% aqueous-alcoholic solution of caustic potash (13 g of KOH in 12 ml of water and 110 ml of 96% alcohol); the mixture was heated for 3 hours on a boiling water bath, cooled, and the solution was then neutralized with 20% sulfuric acid. The precipitate which was deposited was filtered off and the solution was extracted with butyl alcohol. As the ethyl alcohol was removed under vacuum, the aqueous butanol separated into layers. After the water had been separated off, the butanol was evaporated until the appearance of turbidity. The precipitate which was deposited proved to be the initial saponin. The mother solution was evaporated to dryness, and the residue was dissolved in methanol and decolorized with activated carbon. The methanol was driven off and the residue was ground to a pale brownish powder that gave a single spot on fixed silica gel in systems 1 and 3 (see, Fig. 2).

0.5 g of vaccarotetraoside (V) was acetylated in 3 ml of pyridine and 3 ml of acetic anhydride with heating for 4 hours on a boiling water bath. After the usual working up, the non-crystalline acetate, precipitated from benzene solution with petroleum ether, had $[\alpha]_D^{28} - 15.6 \pm 2°$ (c 2.17; chloroform).

Hydrolysis of vaccarotetraoside. A solution of 100 mg of the tetraoside (V) in 5 ml of 2% sulfuric acid was heated at 100°C for 4 hours. When chromatographed on silica gel in systems 1 and 3, the precipitate which had been deposited migrated to the same level as vaccaroside (see Fig. 2) D-Glucose and D-xylose were detected in the solution neutralized with the anion-exchanger "Dowex-1" (CO_3^{-}form) by paper chromatography in system 1.

From 138.0 mg of vaccarotetraoside 81.9 mg of crude vaccaroside was obtained. Calculated for $C_{53}H_{82}O_{24}$: 85.4 mg. By the aniline phthalate method [4] it was found that the ratio of D-xylose to D-glucose in the neutralized hydrolyzate was 1 : 2.

Oxidation of vaccarotetraoside with periodic acid. A solution of 150 mg of periodic acid in 5 ml of water was partially neutralized with sodium bicarbonate to pH 3-4, 40 mg of caccarotetraoside was added, and the solution was left at room temperature for 10 days. The excess was neutralized with the anion-exchanger "Dowex-1", the solution was evaporated to dryness, and the residue was dissolved in 5 ml of 2% sulfuric acid and heated at 100°C for 3 hr. When chromatographed on supported silica gel in system 3, the precipitate that was deposited gave a spot at the same level as that of gypsogenin. After neutralization of the sulfuric acid with barium carbonate, D-xylose was found in the hydrolyzate.

Methylation of vaccarotetraoside. A solution of 1 g of vaccarotetraoside (V) in 20 ml of methanol was treated with 20 ml of methyl iodide and 15 g of silver oxide. The mixture was left for 2 days with constant stirring, being heated to a gentle boil from time to time. The silver oxide was filtered off and fresh portions of methyl iodide and silver oxide in the same proportions as before were added to the reaction mixture. The mixture was left for 4 days. The process was repeated three times, and then the methanol was distilled off. The fourth time, the methylated product was dissolved only in methyl iodide and, after the addition of silver oxide, the reaction mixture was kept for 3 days at a gentle boil. This operation was repeated twice more. The vaccarotetraoside methylated in this way showed a single diffuse spot on chromatography on a thin non-fixed layer of alumina in the toluene—ethanol (9 : 1) system. The yellow powder obtained was dissolved in benzene and the solution was filtered through a small layer of alumina. After the benzene had been distilled off, a light yellow powder was left which, in the above-mentioned solvent system, gave one diffuse spot, $[\alpha]_D^{28}$ −9.9 ± 2° (c 3.01; chloroform).

300 mg of the isolated compound was dissolved in 40 ml of a mixture of methanol and 42% perchloric acid (5: 1), and the mixture was heated at 100°C for 5 hours. The cooled solution was diluted with 60 ml of water. The precipitate which was deposited was separated off, and the hydrolyzate was neutralized with the anion-exchanger "Dowex-1" (CO_3—form) and evaporated under vacuum to a volume of 20 ml. Then 15 ml of 2% sulfuric acid was added and, with the mixture heated to 65°C, it was evaporated to 15 ml, after which heating was continued for a further 2 hours at 80°C. The solution, neutralized with the same anion-exchanger, was concentrated to a syrup and dissolved in 2 ml of water; this solution was filtered and concentrated again. Paper chromatography showed the presence in the concentrated solution of 2, 3, 4-trimethyl-D xylose and 2, 3, 6-trimethyl-D-glucose. The third spot (see Fig. 1), from its R_f value in the butan-1-ol—ethanol—water (5 : 1 : 4) system, must be ascribed to 2,4,6-trimethyl-D-glucose.

The IR spectra were taken on a UR-10 spectrophotometer.

Summary

The enzymatic hydrolysis of vaccegoside, a triterpene glycoside from Vaccaria segetalis, has given D-glucose, D-xylose, D-glucuronic acid, an unknown disaccharide, and a 28-O-acyltetraoside of gypsogenin, esterified with sugars at the carboxyl group, containing D-glucose, D-rhamnose, L-arabinose, and D-xylose. It has been shown that the disaccharide has the structure 4-(D-xylopyranosido)-D-glucopyranose. The alkaline hydrolysis of vacsegoside yielded a gypsogenin tetraoside – vaccarotertraoside. The structure of vaccarotetraoside has been established as gypsogenin-(3)-β-D-glucopyranuronosido-(4 or 2)-D-glucopyranosido-(3)-D-glucopyranosido-(4) d-xylopyranose.

REFERENCES

1. N. K. Abubakirov and K. Amanmuradov, ZhOKh, 34, 1661, 1964.
2. K. Amanmuradov and N. K. Abubakirov, Izv. AN TurkmSSR, ser. FTKh i GN, 6, 106, 1964.
3. Ya. V. Rashkes, ZhAKh, 20, 238, 1965.
4. G. N. Zaitseva and T. P. Afanas'eva, Biokhim., 22, 1035, 1957.
5. K. Macek, in: Paper Chromatography [Russian translation], Moscow, 285, 1962.
6. A. Ya. Khorlin, L. V. Bakinovskii, V. S. Vas'kovskii, A. G. Ven'yaminova, and Yu. S. Ovodov, Izv. AN SSSR, 11, 2008, 1963.
7. H. Bredereck, G. Hagelloch, and E. Hambsch, Ber., 87, 35 1954.

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