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IDENTIFICATION OF A 2,3-DIAMINO-2,3-DIDEOXYHEXOSE IN THE LIPID A COMPONENT OF LIPOPOLYSACCHARIDES OF *Rhodopseudomonas viridis* AND *Rhodopseudomonas palustris**

JOACHIM ROPPEL, HUBERT MAYER,
Max-Planck-Institut für Immunbiologie, D-78 Freiburg i. Br. (W. Germany)
AND JÜRGEN WECKESSER
Lehrstuhl für Mikrobiologie der Universität, D-78 Freiburg i. Br. (W. Germany)
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ABSTRACT

A hitherto unknown amino sugar (Compound A), detected in acid hydrolyzates of lipopolysaccharides of *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris*, is present in the Lipid A component but not in the O-specific part of the lipopolysaccharides. 2-Amino-2-deoxy-D-glucose is lacking in the purified Lipid A of both strains. Compound A, characterized by a very high migration in paper electrophoresis was obtained in a pure state by ion-exchange chromatography and shown by m.s. of the alditol acetate to be a 2,3-diamino-2,3-dideoxyhexose. G.l.c. and periodate oxidation excluded all possible stereoisomers with the exception of 2,3-diamino-2,3-dideoxyglucose and 2,3-diamino-2,3-dideoxyidose. G.l.c. of the alditol acetates of Compound A and of the glucose derivative suggests that Compound A is 2,3-diamino-2,3-dideoxyglucose. The significance of the occurrence of this new aminodeoxy sugar in the Lipid A component of *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris* O-antigens for the biological properties of the respective lipopolysaccharides and for the taxonomy of the *Rhodospirillaceae* family is discussed.

INTRODUCTION

Recent studies on photosynthetic gram-negative bacteria of the *Rhodospirillaceae* family have shown that these bacteria, like other gram-negative bacteria, possess O-antigens (lipopolysaccharides) in their outer cell-wall layer[1–3]. The lipopolysaccharides determine the serologic specificity of the respective strains. Recently, the lipopolysaccharides of the two closely related species *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris* were investigated in detail[1,4]. These studies, carried out with a number of different strains of either species, showed that the general composition of the lipopolysaccharides is characteristic for the species, even when

*Dedicated to Professor Michael Heidelberger in honor of his 87th birthday.

it comprises more than one serotype, like *Rhodopseudomonas palustris*[1,5]. O-Antigens of *Rhodopseudomonas palustris* contain the typical R-core constituents of enteric bacteria: L-*glycero*-D-*manno*-heptose and 2-keto-3-deoxy-octonic acid (KDO); those of *Rhodopseudomonas viridis*, however, contain KDO but lack the heptose component[1,4]. All strains of *Rhodopseudomonas viridis* so far investigated belong to a single chemotype and very probably to a single serotype[4]. Their lipopolysaccharides are characterized by the following sugar constituents: 3-*O*-methyl-L-xylose, 3-*O*-methyl-D-mannose, D-mannose, D-glucose, D-galactose, 2-amino-2-deoxy-D-glucose, 2-amino-2,6-dideoxy-D-glucose (quinovosamine), 2-amino-2-deoxygalacturonic acid, and KDO[4,6,7].

Lipid A of *Rhodopseudomonas palustris* and *Rhodopseudomonas viridis* can easily be split off from the isolated O-antigens by mild acetic acid hydrolysis, indicating that KDO forms the acid-labile bridge between the polysaccharide and the lipid moieties, like in enterobacterial lipopolysaccharides[8,9]. An analysis of the Lipid A moiety of both species revealed the complete absence of 2-amino-2-deoxy-D-glucose in the purified preparations. This finding was very surprising, since disaccharides composed of 2-amino-2-deoxy-D-glucose phosphate residues are known to form the backbone of the Lipid A component of nearly all lipopolysaccharides, so far investigated, from gram-negative bacteria[8,9]. Hydrolysis of the Lipid A of the two just mentioned *Rhodopseudomonas* species gave, however, an unusual aminodeoxy sugar showing a very high electrophoretic mobility, and thus differing in this property from all hitherto described aminodeoxy sugars of bacterial O-antigens. The isolation of this sugar, referred to as Compound A, and its characterization as a 2,3-diamino-2,3-dideoxyhexose is described in this communication.

EXPERIMENTAL

Cultivation of bacteria and isolation of lipopolysaccharides. — Strains of *Rhodopseudomonas palustris*[1] and *Rhodopseudomonas viridis*[4] were obtained from the strain collection of the Institute for Biology II, Freiburg i. Br. (Germany). *Rhodopseudomonas viridis*, strain F, was used throughout the study as source of Compound A. Mass cultivations were performed as described previously[1]. Lipopolysaccharides were extracted from lyophilized bacteria by the hot phenol–water method of Westphal *et al.*[10]: The lipopolysaccharides of *Rhodopseudomonas viridis* strains were extracted into the aqueous phase, whereas those of *Rhodopseudomonas palustris* strains were generally found in the phenol layer[1]. After extensive dialysis, the material obtained from *Rhodopseudomonas viridis* F in the aqueous layer was separated from an insoluble material by centrifugation (2,500*g*, 4 h, 3 times). The purified lipopolysaccharide was obtained from the pellet.

Isolation of Lipid A. — The lipopolysaccharide from *Rhodopseudomonas viridis* F (30 mg) was suspended in 1% acetic acid (20 ml) and the suspension was heated in a sealed tube for 2 h at 100°. The hydrolyzate was freeze-dried and resuspended in distilled water (200 ml). Lipid A, being insoluble in water, was recovered

by centrifugation (4,000*g*, 1 h), washed with water and then dissolved in a mixture of chloroform–methanol (9:1, v/v), which neither dissolves the undegraded lipopolysaccharide nor the polysaccharide moiety of the lipopolysaccharide.

Conditions of hydrolysis for the liberation of Compound A. — A systematic investigation of the conditions giving a maximal liberation of Compound A revealed that the hydrolysis with 4M hydrochloric acid for 18 h at 100° is optimal. These conditions were therefore generally used for the liberation of Compound A. Hydrochloric acid was removed in the presence of potassium hydroxide–phosphorus pentaoxide in a vacuum desiccator. The dry residue obtained from 1 mg of hydrolyzed lipopolysaccharide was dissolved in water (100 μl), and aliquots were subjected to paper high-voltage electrophoresis and paper chromatography.

Paper high-voltage electrophoresis and paper chromatography. — Paper high-voltage electrophoresis was performed in a vertical chamber system, according to Kickhöfen and Warth[11] at a field strength of 47 $V \cdot cm^{-1}$. The buffer systems were: (a) pyridine–acetic acid–water (5:2:43, v/v), pH 5.3, and (b) pyridine–acetic acid–formic acid–water (2:3:20:180, v/v), pH 2.8 (Ref. 12). Descending paper chromatography on Whatman No. 1 paper was performed in the solvent system of Fischer and Nebel[13]. Aminodeoxy sugars were detected either with alkaline silver nitrate or with 0.25% ninhydrin in acetone.

Column chromatography on ion-exchange resin. — Compound A ($\sim$300 μg) was obtained from 5 mg of hydrolyzed lipopolysaccharide by ion-exchange chromatography on a column (1 × 1 cm) of Dowex 50 (H^+) ion-exchange resin, according to Wheat[14]. The hydrolyzate, dissolved in 0.1M hydrochloric acid (1–2 ml) was applied to the column, which had previously been equilibrated with 0.1M hydrochloric acid. The column was subsequently washed with water and then with 0.5M and 2M hydrochloric acid (30 ml of each). Compound A was exclusively found in the last eluate, together with some diamines that were removed by subsequent chromatography of the *N*-acetyl derivative in the Fischer–Nebel solvent system[13].

Preparation, g.l.c., and m.s. of alditol acetates. — Compound A and suitable standards were converted into the alditol acetates by *N*-acetylation according to Roseman and Ludowieg[15], followed by reduction with sodium borohydride or with sodium borodeuteride in deuterium oxide, and then *O*-acetylation[16,17]. G.l.c. was performed with a Varian Aerograph (model 1520B), equipped with a glass column (0.3 × 150 cm) containing either ECNSS-M (3% on Gas-chrom Q) or OV 17 (3% on Varoport), at column temperatures of 200° or 180°, respectively. M.s. was performed with a Perkin–Elmer mass spectrometer (model 270B) coupled to a gas–liquid chromatograph (OV-17 column). Mass spectra were recorded with a Honeywell visicorder (model 3508) at the maximum of the chromatographic peak and at an electronic energy of 70 eV and an ionization current of 80 μA.

Periodate oxidation studies. — The *N*-acetylated and reduced aminodeoxy sugars were oxidized in 25mM sodium periodate solution at 4° or 20°, for various lengths of time. In order to obtain diaminodideoxypentoses (in admixture with the respective diaminodideoxytetroses), the oxidation was performed for 18 h at 4°, and

then was stopped by adding an excess of 1,2-ethanediol. Complete periodate oxidation, in order to obtain only diaminodideoxytetroses, was performed for 18 h at 4° and for an additional 18-h period at 20°. Reduction of the periodate oxidation products and their *O*-acetylation was performed as just described.

Colorimetric reactions. — The Elson–Morgan reaction was performed with the isolated, nonacetylated Compound A and with suitable standards according to Rondle and Morgan[18], whereas the *N*-acetylated products were tested with the Morgan–Elson reaction according to Reissig *et al.*[19]. The absorption spectra were recorded in the range of 400–600 nm with a Cary 15 automatic spectrophotometer.

RESULTS AND DISCUSSION

Strain F of *Rhodopseudomonas viridis* was selected for the isolation of Compound A because it gave the highest yield of lipopolysaccharide of all *Rhodopseudomonas viridis* strains tested (4% of the bacterial dry mass) and its lipopolysaccharide is almost exclusively extracted into the aqueous layer of the phenol–water extracts[4]. Crude Lipid A could be obtained by mild hydrolysis of the purified lipopolysaccharide in a yield of 30% of lyophilized lipopolysaccharide. Since a substantial loss of material occurred during the isolation and purification of Lipid A, Compound A was directly isolated from lipopolysaccharide hydrolyzates. It was detected on paper electrophoretograms of acid hydrolyzates by staining with alkaline silver nitrate, and it reacted also with ninhydrin to give an orange-brown color when heated at 100°. Compound A showed a high electrophoretic mobility (M_{GlcN} 1.16 at pH 5.3 and 1.49 at pH 2.8) and could, therefore, easily be separated from the other aminodeoxy sugar constituents of the *Rhodopseudomonas viridis* lipopolysaccharide[4,6], *i.e.* D-glucosamine, quinovosamine, and 2-amino-2-deoxygalacturonic acid by electrophoresis: however, an extensive decomposition occurred when Compound A was eluted from the paper, with either water or 0.1M hydrochloric acid, after electrophoretic separation. This was ascertained by subjecting the isolated sugar to a second electrophoresis under the same conditions; a fairly large proportion of a nonmigrating compound reacting with alkaline silver nitrate was detected. However, Compound A could be isolated in a pure state after chromatography on Dowex 50 (H^+) ion-exchange resin[14]. In a representative experiment performed with 200 mg of lipopolysaccharide of *Rhodopseudomonas viridis* F, 15 mg of crude Compound A was obtained. In order to prevent its decomposition, the free aminosugar was immediately *N*-acetylated[15], and then subjected to paper chromatography. About 10 mg of *N*-acetylated Compound A was recovered from the chromatogram after development for 18 h with the Fischer–Nebel solvent system[13]. Compound A gave a strong color in the Rondle and Morgan modification[18] of the Elson–Morgan reaction, showing an absorption maximum at 520 nm, a slight shift of the maximum from the 525 nm absorption maximum observed for 2-amino-2-deoxy-D-glucose. This reactivity establishes that Compound A is a 2-amino-2-deoxy sugar[20,21]. No color, however, was obtained in the Morgan–Elson reaction performed according to Reissig *et al.*[19]:

500 μg of the chromatographically pure *N*-acetylated Compound A gave less than 10% of the color yield obtained with 50 μg of 2-acetamido-2-deoxy-D-glucose. Compound A was converted into the alditol acetate for g.l.c. and m.s. studies, since alditol acetates have a rather high volatility and relatively simple fragmentation patterns[22,23]. Attempts to separate the respective derivative of Compound A on an ECNSS-M column were not successful because of the high retention times of aminodeoxy sugars on this column[24]. It was, however, possible to obtain this derivative as a symmetrical peak on the OV-17 column at 180°. The mass spectrum of the alditol acetate of Compound A is shown in a simplified form in Fig. 1. Only

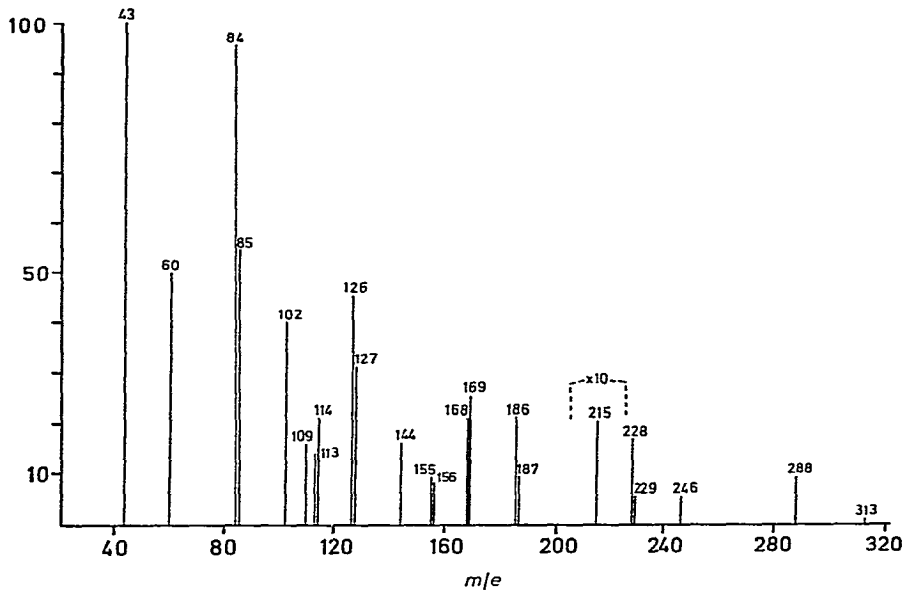
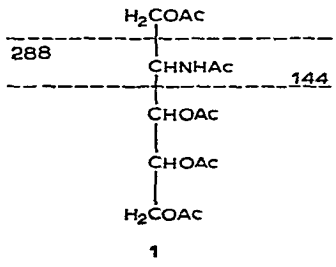
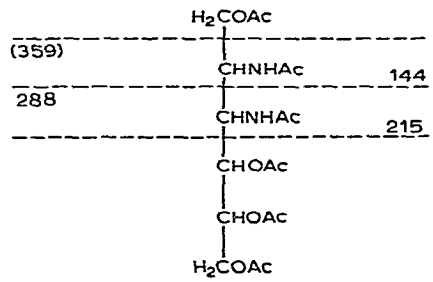


Fig. 1. Mass spectrum of Compound A as alditol acetate. The spectrum was taken at an electron energy of 70 eV and an ionization current of 80 μA. For simplification, only those peaks that had an intensity of >10% of the base peak (*m/e* 43) in the mass range of *m/e* 43–200 and >5% in the mass range >200 were recorded. Peak at *m/e* 215 is given in a tenfold magnification.

two classes of aminodeoxy sugars are expected to give a fragmentation pattern similar to that obtained with the alditol acetate of Compound A, namely the alditol acetates of 2-amino-2-deoxypentoses (**1**) and of 2,3-diamino-2,3-dideoxyhexoses (**2**)[24]. Scheme 1 shows the primary fragments expected from these two aminodeoxy sugars. The primary fragments at *m/e* 144 and 288 are common to both, the fragment at *m/e* 215, however, can only arise from **2**. This fragment is obtained as a minor fragment (reported in Fig. 1 in a ten-fold magnification). A number of secondary fragments, however, arising from it at *m/e* 156, 155, 114, and 113 by elimination of either acetamide (mol. wt. 59) or acetic acid (mol. wt. 60), or of either of them in addition to ketene (mol. wt. 42) (Ref. 22, 24) are rather prominent and confirm that



1



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Scheme 1

Compound A is a 2,3-diamino-2,3-dideoxyhexose. In the m.s. fragmentation of the deuterium-reduced, peracetylated Compound A, fragments at *m/e* 144, 102, and 84, and the just mentioned fragments arising from the primary fragment at *m/e* 215 show a shift of one mass unit due to the deuterium atom at C-1. The presence of 2-amino-2-deoxypentoses could be further excluded by the electrophoretic mobility of authentic standards. The separation in high-voltage electrophoresis at pH 2.8 gave values of M_{GlcN} 1.18 for 2-amino-2-deoxy-D-arabinose and 1.14 for 2-amino-2-deoxy-D-lyxose as compared to M_{GlcN} 1.49 for Compound A. This result clearly establishes that Compound A is much more basic than are the monoaminodeoxypentoses.

2,3-Diamino-2,3-dideoxyhexoses are hitherto unknown as natural constituents, but a number of them have been obtained by chemical synthesis[25–28]. Mass spectra of the alditol acetates of authentic 2,3-diamino-2,3-dideoxy-D-glucose and 2,3-diamino-2,3-dideoxy-D-allose showed the same fragments as observed with the alditol acetate of Compound A. The synthetic standards having the *gluco*, *galacto*, and *allo* configuration were separable on the OV-17 column as alditol acetates. The respective retention times are given in Table I. Compound A is clearly different from 2,3-diamino-2,3-dideoxy-D-allose and 2,3-diamino-2,3-dideoxy-D-galactose, but forms a single and symmetrical peak when added to the respective alditol acetate having the *gluco* configuration.

The *threo* or *erythro* configuration of the amino groups of Compound A was studied by periodate oxidation of the *N*-acetylated and subsequently reduced Compound A and of authentic 2,3-diacetamido-2,3-dideoxy-D-glucitol and 2,3-diacetamido-2,3-dideoxy-D-allitol. The 2,3-diacetamido-2,3-dideoxytetroses formed by periodate oxidation were converted into the tetritol acetates by reduction with sodium borohydride and subsequent *O*-acetylation. G.l.c. on an ECNSS-M column allowed the separation of the two 2,3-diacetamido-1,4-di-*O*-acetyl-2,3-dideoxytetritols. The periodate oxidation products of Compound A (as alditol acetate) gave a single peak when added to 2,3-diacetamido-1,4-di-*O*-acetyl-2,3-dideoxythreitol, thus showing that the adjacent amino groups of Compound A are in the *threo* configuration. The respective retention times are given in Table I. The number of possible

TABLE I

GAS–LIQUID CHROMATOGRAPHY DATA OF ALDITOL ACETATES OF 2,3-DIACETAMIDO-2,3-DIDEOXY SUGARS

Per-O-acetylalditol derivatives	*Retention times*[a]	
	ECNSS-M column[b]	*OV-17 column*[c]
2,3-Diamino-2,3-dideoxy-D-allose (3)		1.27
2,3-Diamino-2,3-dideoxy-D-glucose (4)		1.51
2,3-Diamino-2,3-dideoxy-D-galactose (5)		1.68
Compound A (6)		1.53
2,3-Diamino-2,3-dideoxy-D-erythrose (from 3)[d]	5.1	
2,3-Diamino-2,3-dideoxy-L-threose (from 4)[d]	4.6	
2,3-Diamino-2,3-dideoxytetrose (from 6)[d]	4.4	
2,3-Diamino-2,3-dideoxy-D-ribose (from 3)[e]	8.1	
2,3-Diamino-2,3-dideoxy-D-xylose (from 4)[e]	9.0	
2,3-Diamino-2,3-dideoxy-L-arabinose (from 5)[e]	8.6	
2,3-Diamino-2,3-dideoxypentose (from 6)[e]	8.9	

[a]Average values. [b]Relative to 1,2,3,4,5,6-hexa-*O*-acetyl-D-glucitol, 1.00. [c]Relative to 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-glucitol, 1.00. [d]Obtained by complete periodate oxidation of 3, 4, and Compound A (6). [e]Obtained by incomplete periodate oxidation of 3, 4, 5, and Compound A (6).

configurations of Compound A was therefore reduced to the *gluco*, *galacto*, *ido*, or *altro* configuration. 2,3-Diamino-2,3-dideoxy-D-galactose showed another retention time in g.l.c. and its structure, therefore, was also excluded.

Incomplete periodate oxidation of the 2,3-diacetamido-2,3-dideoxyhexitols gave small proportions of the corresponding 3,4-diacetamido-3,4-dideoxypentoses, besides diacetamidodideoxytetroses. Separation of the pentoses as alditol acetates was achieved on the ECNSS-M column (see Table I). The degradation product of Compound A had a retention time identical to that of 1,4,5-tri-*O*-acetyl-2,3-diacetamido-2,3-dideoxyxylitol but different from that of the corresponding alditol acetates of 2,3-diacetamido-2,3-dideoxy-ribose and -arabinose, which excluded the *2,3-diamino-2,3-dideoxygalacto* and *2,3-diamino-2,3-dideoxyaltro* configurations for Compound A. Complete and incomplete periodate oxidation of 2,3-diacetamido-2,3-dideoxyglucose and 2,3-diacetamido-2,3-dideoxyidose yield the same degradation products, *i.e.* 2,3-diacetamido-2,3-dideoxythreitol and 2,3-diacetamido-2,3-dideoxyxylitol, respectively, after sodium borohydride reduction. Since authentic 2,3-diamino-2,3-dideoxyidose is presently not available, it was not possible to decide unequivocally whether Compound A is 2,3-diamino-2,3-dideoxyglucose or 2,3-diamino-2,3-dideoxyidose. It is, however, much more likely that Compound A has the *gluco* configuration, since a mixture of the alditol acetates of Compound A and of 2,3-diamino-2,3-dideoxy-D-glucose gave a single peak on the OV-17 column. The alditol acetate of 2,3-diamino-2,3-dideoxyidose is expected to have a retention time higher

than that of the glucose derivative, because alditol acetates having the *ido* configuration are known to have the highest retention times[29]. Furthermore, paper chromatography of *N*-acetylated Compound A and of authentic 2,3-diacetamido-2,3-dideoxy-D-glucose showed identical rates of migration in the Fischer–Nebel solvent system[13] (R_{GlcNAc} 1.18). Like *N*-acetylated Compound A, 2,3-diacetamido-2,3-dideoxy-D-glucose was also found to give no color in the modification of Reissig *et al.*[19] of the Morgan–Elson reaction.

The identification of a 2,3-diamino-2,3-dideoxyhexose in the Lipid A moiety of the O-antigens of the two related species[30] *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris* is to the best of our knowledge the first report of this type of sugar in Nature. The capability of certain microorganisms to introduce amino groups at C-3 of sugar molecules, as manifested in antibiotics and bacterial O-antigens[31,32], prompted Baer and Neilson[28] to speculate about the possible existence of 2,3-diamino-2,3-dideoxyhexoses in microbial products. The present report documents that at least some of the photosynthetic bacteria have the enzymic equipment for synthesis of these sugars.

It is of interest that the 2,3-diamino-2,3-dideoxyhexose is part of the Lipid A component but not of the polysaccharide moiety of the O-antigens. 2-Amino-2-deoxy-D-glucose, which could not be found in purified samples of Lipid A, is part (esterified with a phosphate group) of the disaccharide which forms the Lipid A backbone of nearly all gram-negative bacteria investigated so far[8]. In enteric bacteria, the amino group of the 2-amino-2-deoxy-D-glucose residue is amidified with 3-hydroxymyristic acid[33], this being the single fatty acid found in lipopolysaccharides of *Rhodopseudomonas viridis* strains and occurring always in an amide-linked form[4]. It is also of importance, in this context, that Lipid A of *Rhodopseudomonas viridis* is practically free of phosphate groups[4]. Phosphate bridges form the cross-links between the 2-amino-2-deoxy-D-glucose disaccharides in enterobacterial Lipid A.

Most biological properties of lipopolysaccharides (endotoxins), such as lethal toxicity, pyrogenicity, adjuvantic activity, or mitogenicity are found to reside in the Lipid A moiety of lipopolysaccharides[34,35]. Recent observations have shown that the lipopolysaccharide of *Rhodopseudomonas viridis* is neither toxic for mice nor pyrogenic for rabbits[36].

Strains of other species of the *Rhodospirillaceae* family, including *Rhodopseudomonas spheroides*, *Rhodopseudomonas gelatinosa*, *Rhodopseudomonas capsulata*, *Rhodospirillum molischianum*, *Rhodospirillum tenue*, and *Rhodospirillum rubrum* were investigated for the presence of Compound A. They all lack this sugar and contain instead 2-amino-2-deoxyglucose in the Lipid A moiety. The occurrence of a 2,3-diamino-2,3-dideoxyhexose in all strains of *Rhodopseudomonas viridis* and *Rhodopseudomonas palustris* seems, therefore, to have taxonomical relevance within the family of *Rhodospirillaceae*[37].

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