ENZYMATIC SYNTHESIS OF *SALMONELLA* O-SPECIFIC POLYSACCHARIDE ANALOGS FROM MODIFIED POLYPRENYL PYROPHOSPHATE SUGAR ACCEPTORS

V. N. SHIBAEV, L. L. DANILOV, T. N. DRUZHININA, L. M. GOGILASHVILI*, S. D. MALTSEV and N. K. KOCHETKOV

N. D. Zelinsky Institute of Organic Chemistry, USSR Academy of Sciences, Moscow 117913 and **I. G. Kutakeladze Institute of Pharmacochemistry, Academy of Sciences of Georgian SSR, Tbilisi, USSR*

Received 11 January 1982

1. Introduction

Biosynthesis of O-specific polysaccharide chains of *Salmonella* lipopolysaccharides occurs through intermediate formation of polyprenyl pyrophosphate oligosaccharides followed by enzymatic polymerization of the oligosaccharides repeating units. Fig.1 shows some of the enzymic reactions which are characteristic for the bacteria of serogroups B and E and were demonstrated in vitro [1,2]. The main factor which determines the structures of the O-antigenic polysaccharides is probably specificity of the biosynthetic enzymes towards the structure of the glycosyl donors and acceptors. It was found (review [3]) that some modified monosaccharide residues may be incorporated into the trisaccharide repeating unit of the *Salmonella anatum* O-antigens with the use of

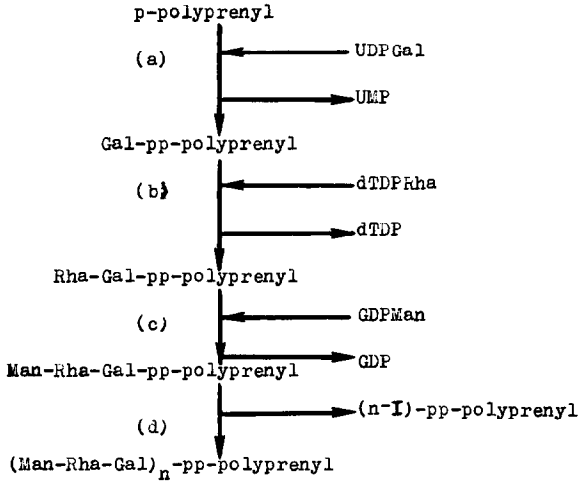


Fig.1. Some enzymatic reactions of O-specific polysaccharide biosynthesis in *Salmonella* serogroups B and E.

synthetic analogs of the glycosyl donors, GDPMan and dTDPRha. The chemical synthesis of polyprenyl pyrophosphate sugars [4] and ability of synthetic moraprenyl pyrophosphate sugars derived from mulberry leaf polyprenol (moraprenol) to serve as substrates for O-specific polysaccharide biosynthesis in *Salmonella* [5] allowed us to prepare modified O-antigens from the acceptor analogs. We report a chemical synthesis of several analogs of polyprenyl pyrophosphate galactose and conversion of these derivatives through reactions (b–d) (fig.1) into the analogs of O-specific polysaccharides, which may be of interest for immunochemical studies.

2. Materials and methods

GDP[^{14}C]Man (Amersham) was diluted by the non-radioactive sugar nucleotide (Calbiochem) to spec. radioact. 10 mCi/mmol. dTDPRha was prepared as in [6]. Chromatographic and analytical methods used for synthesis of polyprenyl pyrophosphate sugars are described in [4].

Cell envelope fractions and soluble glycosyltransferases were prepared according to [2] from *S. anatum*, *S. senftenberg* and *S. typhimurium*.

For preparation of the moraprenyl pyrophosphate trisaccharides through the reactions (b) and (c) (fig.1) an aliquot of methanolic solution of the moraprenyl pyrophosphate monosaccharide was evaporated and the residue was shaken with 0.5% aqueous solution of Tween 85 (15 μl) till homogeneity. Tris–acetate (pH 8.5) (5 μmol), $MgCl_2$ (1 μmol), dTDPRha (50 nmol) and GDP[^{14}C]Man (12 nmol) were added followed by the soluble glycosyltransferase preparation; incubation mixture was 0.1 ml final vol. After

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

incubation for 60 min at 25°C the reaction was stopped by addition of chloroform–methanol (2:1) mixture (2 ml). The lipid–oligosaccharide products were extracted as in procedure B of [2].

To convert the polyprenyl pyrophosphate trisaccharide into the polysaccharide (fig.1d) it was treated with 0.2% aqueous solution of Tween 85 (30 μl), Tris–maleate pH 6.0 (90 μmol), $MgCl_2$ (1.5 μmol) and the cell envelope preparation. The mixture (0.12 ml final vol.) was incubated for 1 h at 25°C and analysed with paper chromatography (ethanol–1 M ammonium acetate (pH 7.5), 7:3, system A) as in [2]. For gel filtration analysis the mixture was treated with 0.1 M acetic acid with 0.005 M EDTA (3 ml), the precipitate was collected by centrifugation (3000 rev./min, 30 min) and heated with 0.5 M acetic acid (0.5 ml) for 30 min at 100°C followed by centrifugation. The supernatant was diluted with water to 1 ml and applied to a column (34 × 1.5 cm) of Sephadex G-15, the column was eluted with water (cf. [7,8], treatment of the product with alkaline phosphatase was found to be unnecessary).

Mild acid hydrolysis was performed with 0.01 M HCl in 50% aqueous *n*-propyl alcohol (15 min, 100°C), 2 M HCl (2 h, 100°C) was used for complete acid hydrolysis. The conditions for degradation of polyprenyl pyrophosphate sugars with aqueous phenol and ammonia are described in [4]; for degradation with dilute alkali the product was dissolved in benzene (0.3 ml), 0.2 M aqueous NaOH (0.05 ml) and methanol (0.05 ml) were added and the mixture was heated at 100°C for 5 min.

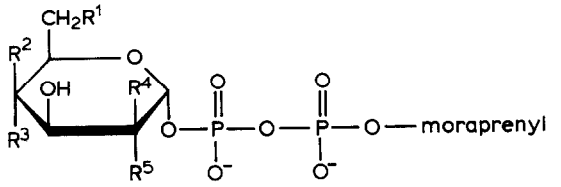
Reduction with sodium borotritiide (Isotope USSR, 3.9 Ci/mmol) was performed in 1 M borate (pH 9.5) for 3 h at room temperature, borate was removed after acidification with acetic acid by co-evaporation with methanol.

Mono- and oligosaccharides were analysed by paper chromatography in butanol–pyridine–water 6:4:3 (system B).

3. Results and discussion

The chemical synthesis of moraprenyl pyrophosphate derivatives of α-D-galactopyranose (Gal-pp-Mpr) and α-D-glucopyranose (Glc-pp-Mpr) through interaction of moraprenyl phosphoimidazolidate with the unprotected glycosyl phosphates was described in [4], Glc-pp-Mpr may be considered as an analog of Gal-pp-Mpr modified at C^4 of the monosaccharide residue. Using exactly the same procedure for the pyrophosphate synthesis and separation of the reaction products and 6-deoxy-α-D-galactopyranosyl phosphate [9], α-D-talopyranosyl phosphate [10] or α-D-mannopyranosyl phosphate [11] as starting glycosyl phosphates we achieved preparation of 3 new analogs of Gal-pp-Mpr modifications at C^6, C^2 or C^2 and C^4 of the monosaccharide residue (structural formula yields, analytical data and mobility on TLC plates, in table 1).

Table 1
Moraprenyl pyrophosphate monosaccharides studied as substrates for O-antigen biosynthesis in *Salmonella*



Abbreviations	R^1	R^2	R^3	R^4	R^5	Yield (%)	Moraprenyl / Phosphate	R_f[a]
Gal-pp-Mpr	OH	OH	H	H	OH	47[b]	1:1.8[b]	0.20[b]
Fuc-pp-Mpr	H	OH	H	H	OH	45	1:2.2	0.25
Glc-pp-Mpr	OH	H	OH	H	OH	65[b]	1:2.3[b]	0.20[b]
Tal-pp-Mpr	OH	OH	H	OH	H	49	1:1.85	0.20
Man-pp-Mpr	OH	H	OH	OH	H	58	1:1.75	0.20

[a] TLC on Kieselgel G-60 plates (Merck) with chloroform–methanol–water 60:25:4 (by vol.); [b] See [4]

Table 2
Incorporation of radioactivity from GDP[^{14}C]Man into moraprenyl pyrophosphate trisaccharides in the presence of dTDPRha and moraprenyl monophosphate monosaccharides

Moraprenyl pyrophosphate monosaccharide	Content in incubation mixture (nmol)	Radioactivity of lipid–oligosaccharide fractions (cpm × 10^{-3}) with the enzymes of:		
		S. anatum	*S. senftenberg*	*S. typhimurium*
Gal-pp-Mpr	1	7.80	5.80	8.60
Fuc-pp-Mpr	12	5.98	2.84	2.80
Glc-pp-Mpr	25	0.20	0.25	0.36
Tal-pp-Mpr	27	8.55	3.30	5.82
Man-pp-Mpr	25	0.22	0.20	0.40

Their structure was confirmed by the methods in [4]. In all cases treatment of the products with 40% aqueous phenol (10 min, 70°C) resulted in formation of the glycosyl pyrophosphates. Fuc-pp-Mpr was readily converted into moraprenyl phosphate and 1,2-cyclic phosphate of the monosaccharide after treatment with dilute ammonia in benzene–methanol mixture at room temperature [4]. As may be expected Tal-pp-Mpr and Man-pp-Mpr were stable under these conditions whereas their heating with dilute alkali gave rise to moraprenyl phosphate and P_i (cf. degradation of dolichyl pyrophosphate mannose under similar conditions [12]).

When analogs of Gal-pp-Mpr were incubated with dTDPRha and GDP[^{14}C]Man in the presence of the soluble glycosyltransferase preparations from *S. anatum* (serogroup E_1), *S. senftenberg* (serogroup E_4) or *S. typhimurium* (serogroup B) which are known to catalyze reactions (b) and (c) (fig.1) significant formation of radioactive, chloroform–methanol soluble products was observed for Fuc-pp-Mpr and Tal-pp-Mpr but not for Glc-pp-Mpr and Man-pp-Mpr (table 2). Mild acid hydrolysis of the products converted them into the water-soluble trisaccharides (R_{Gal} 0.45, system B). Treatment of the latter with NaB^3H_4 followed by complete acid hydrolysis resulted in formation of 6-deoxy[^{3}H]galactitol and [^{3}H]talitol identified by paper chromatographic comparison with the authentic samples (system B).

These results show that Fuc-pp-Mpr and Tal-pp-Mpr may serve as glycosyl acceptors in reaction (b) (fig.1), subsequent mannosyl transfer from GDP[^{14}C]-Man resulted in formation of the modified moraprenyl trisaccharides which contained Fuc or Tal residues instead of the Gal residue. It may be concluded that presence of OH-group at C^6 and correct configuration at C^2 of the galactose residue are not significant for effective interaction of Gal-pp-Mpr and Rha-Gal-pp-Mpr with the glycosyltransferases which catalyze reactions (b) and (c) whereas correct configuration at C^4 of the galactose residue seems to be essential for at least one of these enzymes.

The modified polyprenyl pyrophosphate trisaccharides with Fuc or Tal residues were found to serve as substrates for enzymatic polymerization (fig.1d) with enzymes from *S. anatum*, *S. senftenberg* and *S. typhimurium*. After incubation of the [^{14}C]Man-containing trisaccharide derivatives with the cell envelope prepa-

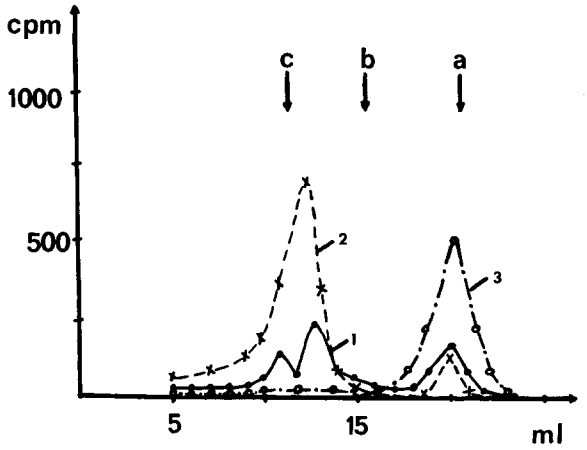


Fig.2. Radioactivity profile in gel filtration of the products obtained after incubation of [^{14}C]Man-Rha-Fuc-pp-Mpr with cell-envelope preparation of *S. anatum* (1) or [^{14}C]Man-Rha-Tal-pp-Mpr with cell-envelope preparation of *S. senftenberg* (2) followed by mild acid hydrolysis. (3) Biosynthetic [^{14}C]-Man-Rha-Fuc-pp-Mpr after mild acid hydrolysis: (a) raffinose; (b) the synthetic hexasaccharide Man–Rha–Gal–Man–Rha–Gal in [13]; (c) exclusion volume as determined with blue dextran or [^{14}C]$(GlcNAc)_2Man_9Glc_3$.

rations of the mentioned bacterial strains and analysis of the products with paper chromatography (system A) the main portion of radioactivity (50–70%) was present at the origin of a chromatogram as characteristic for a polymer formation [2]. Analysis of the reaction products after mild acid hydrolysis by gel filtration on Sephadex G-15 clearly demonstrated conversion of the trisaccharide derivatives into the higher oligosaccharides and polysaccharides (representative elution profiles are shown in fig.2), i.e., participation of the modified trisaccharide derivatives in the polymerization reaction.

These data show that the glycosyl transferases and the polymerase of O-antigen biosynthesis have no absolute specificity towards the structure of carbohydrate fragment of polyprenyl pyrophosphate sugars and demonstrate a possibility of enzymatic synthesis of modified O-specific polysaccharides with the use of modified polyprenyl pyrophosphate monosaccharides. This approach may be useful for preparation of models for immunochemical studies.

Acknowledgements

The authors are greatly indebted to Dr A. V. Nikolaev for a sample of the synthetic hexasaccharide, to Dr L. Lehle for a sample of $GlcNAc_2Man_9Glc_3$ and to Dr S. S. Roznova and Dr V. A. Kilesso for cooperation in growing bacteria.

References

[1] Robbins, P. W. and Wright, A. (1971) in: Microbial Toxins (Weinbaum, G. et al. eds) vol. 4, pp. 351–368, Academic Press, New York.
[2] Osborn, M. J., Cynkin, M. A., Gilbert, J. M., Müller, L. and Singh, M. (1972) Methods Enzymol. 28, 583–601.
[3] Shibaev, V. N. (1978) Pure Appl. Chem. 50, 1421–1436.
[4] Danilov, L. L., Maltsev, S. D., Shibaev, V. N. and Kochetkov, N. K. (1981) Carbohydr. Res. 88, 203–211.
[5] Shibaev, V. N., Danilov, L. L., Druzhinina, T. N., Torgov, V. I., Gogilashvili, L. M. and Utkina, N. S. (1982) Bioorg. Khim. in press.
[6] Shibaev, V. N., Utkina, N. S., Danilov, L. L. and Eliseyeva, G. I. (1980) Bioorg. Khim. 6, 1778–1781.
[7] Kent, J. L. and Osborn, M. J. (1968) Biochemistry 7, 4409–4419.
[8] Shibaev, V. N., Druzhinina, T. N., Popova, A. N., Roznova, S. S. and Kilesso, V. A. (1979) Eur. J. Biochem. 101, 309–316.
[9] Nordin, J. H., Salo, W. L., Bevill, R. D. and Kirkwood, S. (1965) Anal. Biochem. 13, 405–411.
[10] Shibaev, V. N., Eliseyeva, G. I., Krayevskaya, M. A. and Kochetkov, N. K. (1981) Bioorg. Khim. 7, 376–380.
[11] Hill, E. C. and Ballou, C. E. (1977) J. Biol. Chem. 241, 895–902.
[12] Warren, C. D. and Jeanloz, R. W. (1975) Biochemistry 14, 412–419.
[13] Kochetkov, N. K., Nikolaev, A. V. and Dmitriev, B. A. (1981) Izv. Akad. Nauk. SSSR Ser. Khim. 699–700.