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TETRAHEDRON: *ASYMMETRY*

Chemo-enzymatic synthesis of TDP-β-L-ascarylose

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Abstract

TDP-3,6-dideoxy-β-L-*arabino*-hexopyranose (TDP-β-L-ascarylose) has been produced on a 200 mg-scale from TDP-3-deoxy-α-D-glucose by the L-rhamnose synthesising enzyme system which was isolated from *Salmonella entericum* LT2. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

The immunological diversity of gram-negative bacteria is to a large extent attributed to the *O*-antigen, which comprises a part of the lipopolysaccharide. The role played by 3,6-dideoxy sugars in the *O*-antigenic structure is intriguing in that they are major antigenic determinants.[1] Ascarylose has been identified together with another four out of the eight possible diastereoisomers in serotypes of *Yersinia pseudotuberculosis*.[2,3] Thus, a better access to these structures could facilitate QSAR-studies aimed at the development of synthetic vaccines. Since deoxy sugar glycosides are secondary gene products, which are synthesised from the respective activated nucleoside diphosphate sugar by co-factor dependent glycosyl transferases, a preparative method yielding nucleoside diphospho deoxy sugars could satisfy the demand of such structures for further research.

Work from this laboratory has therefore been focused on following the bacterial pathway by starting off from TDP-α-D-glucose **1** and derivatives thereof. This method has the advantage of having a very stable starting material, accessible from D-glucose through chemical synthesis or biochemical transformation (Scheme 1).

The successful synthesis of TDP-6-deoxy-α-D-*xylo*-hexopyranos-4-ulose **2**,[4,5] and the generation of TDP-β-L-rhamnose **4** by an overexpressed enzyme system isolated from *Salmonella entericum* LT2,[6] stimulated this study into the use of enzymes for the synthesis of an activated L-configured 3,6-dideoxy sugar.

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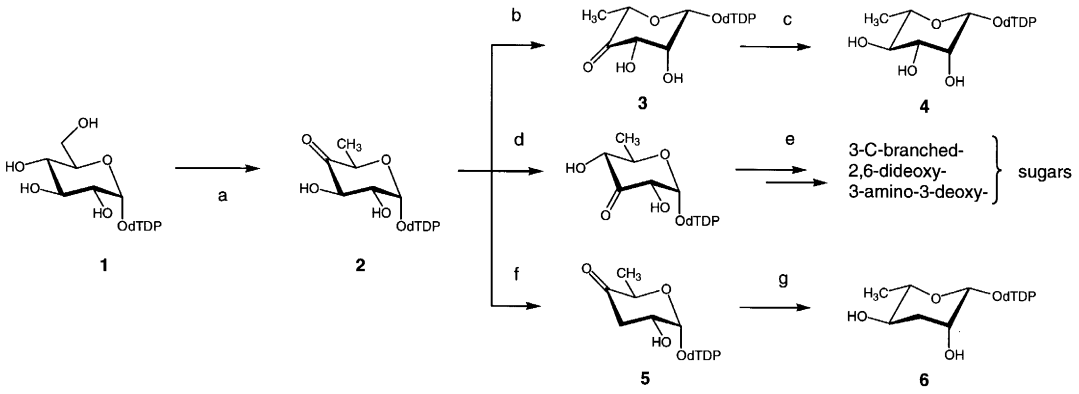


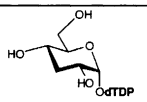
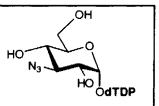
Scheme 1. (a) TDP-glucose-4,6-dehydratase; (b) 3,5-epimerase; (c) 4-reductase; (d) ketoisomerase; (e) various enzymes; (f) PMP-transferase and 3-dehydratase; (g) 5-epimerase and 4-reductase

2. Results and discussion

Both cultures, *E. coli* p4439 and p3378, were kindly provided by the laboratory of Prof. P. Reeves.[6,7] The p4439 harboured the *rfb B*-gene from *Salmonella entericum* LT2 coding for the 4,6-dehydratase controlled by a T7-promoter, and complementarily p3378 harboured the first seven genes of the entire *rfb*-cluster.

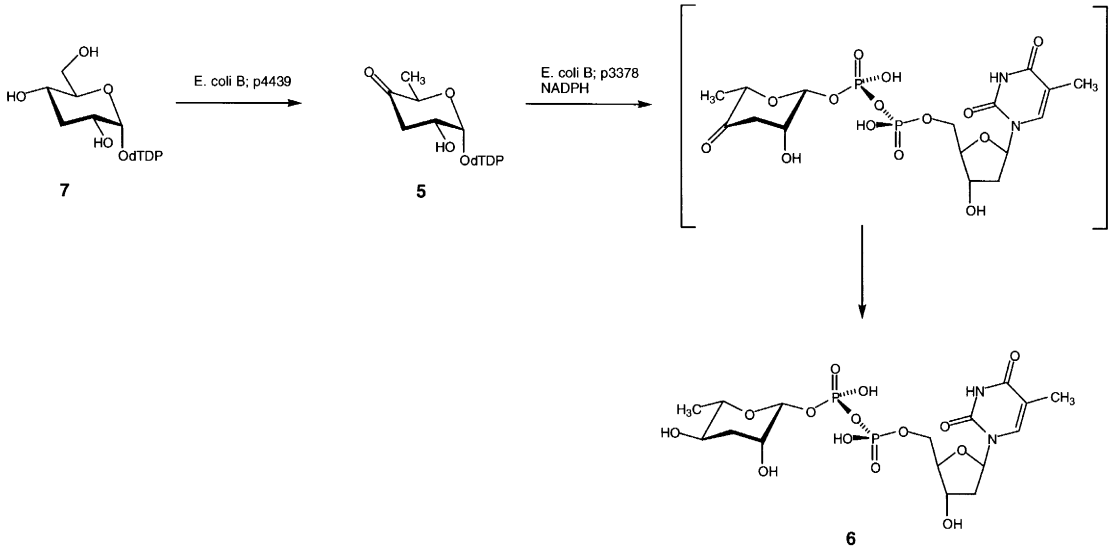
The underlying idea was to circumvent the endogenous pathway, which for the 3,6-dideoxy sugar involves a PMP-dependent transferase and a 3-dehydratase as has been described by Liu and co-workers for the CDP-sugar series.[8–10] Previous work has shown that **7** and **8** are both substrates for the TDP-glucose-4,6-dehydratase from *E. coli* B (EC 4.2.1.46, cf. Table 1).[5] If this broad substrate specificity would persist for all the following enzymes in the L-rhamnose pathway, the enzymatic epimerisation at C-5, followed by stereoselective reduction of C-4, should yield the respective L-*arabino*-configured 3,6-dideoxy sugars.

Table 1
Substrate specificities for the 3-deoxy sugars **7** and **8**

		
	7	**8**
K_m {µM}	200 ±35	350 ±60
V_{max} {µmol/h×mg}	130 ±22	90 ±15
V_{max}/K_m {ml/s×mg}	≈0.18	≈0.07

According to the procedure described by Marumo et al.[7] for the synthesis of TDP-β-L-rhamnose, chemically synthesised dTDP-3-deoxy-α-D-glucose **7** was incubated with a raw extract from a 200 ml-fermentation of *E. coli* strain p4439. NAD^+ was added as a cofactor and the reaction was followed by TLC which indicated complete conversion after 6 h. The entire mixture was then passed through an Amicon cell (10 kD) and the filtrate was lyophilised. Since, in our hands, the enzyme preparation was approx. 20× less active than described, the rifampicin-addition was omitted and the strains were shaken for 10 h at 42°C, and disrupted by passing through a French press.

The raw lyophilisate containing 4-ulsoe **2** was re-dissolved in a TRIS/HCl buffer, supplemented with NADPH, and incubated with the p3378 extract isolated from a 100 ml fermentation. After 12 h at 37°C, the mixture was worked up according to our standard procedure by heat denaturation of the proteins, lyophilisation and G-10 gel permeation chromatography. The sugar nucleotide was purified by anion exchange chromatography to yield 210 mg of an off-white, hygroscopic product, which was sensitive to decomposition in solution at room temperature (Scheme 2).



Scheme 2.

The $t_{1/2} \approx 180$ min in D_2O was judged from the decay of the signal at 4.84 ppm, which was assigned to H-1′′ due to a small $^3J_{1,2}$-coupling of 3.0 Hz, indicating a β-glycosidic linkage and a $^3J_{1,P}$-*hetero*-coupling underlining the presence of an anomeric phosphate group. The reduction of C-4 was assumed from the dq-signal for H-5′′. The absolute configuration of the sugar cannot be directly proven from these experiments, since it may theoretically be either α-D or β-L. However, it is safe to assume that a β-L-4-ulose reductase, although having a broad substrate specificity, would be inactive on an α-D-4-ulose and hence the drawn configuration (β-$^1C_4$(L)) may be presumed. Although it was not possible to extract useful information from the ^{31}P-spectra, the presence of both C,P-couplings in the ^{13}C-spectra for C-1′′ and C-5′ strongly suggested the presence of an intact pyrophosphate bridge between the ribonucleotide and the L-hexose.

3. Conclusion

It has been shown that both enzymes, PMP-transferase and 3-dehydratase, in the biosynthetic pathway of 3,6-deoxy sugars can be circumvented by using a suitable, deoxygenated starting material. Whereas **7** was smoothly converted into TDP-ascarylose **6**, the corresponding TDP-3-azido-3-deoxy-glucose was not a substrate and remained unchanged.

Further research is now directed towards the enzymatic deoxygenation of 3-*C*-methyl-branched hexoses.

4. Experimental

4.1. General methods

^{1}H NMR spectra were recorded on a Bruker AM-400 spectrometer at the frequencies indicated, employing standard pulse angles. Where necessary, coupled protons were assigned by ^{1}H, ^{1}H-COSY and carbons by ^{1}H, ^{13}C-correlated spectroscopy. All reactions were monitored on silica gel plates (GF_{254}, E. Merck) and detected by either UV-absorption or charring with 5% H_2SO_4 in EtOH and subsequent heating to 500°C. Diphosphates were purified by chromatography over Dowex 2×8 (Cl^--form) and eluted by a linear gradient 0→0.8 M LiCl. Product-containing fractions were pooled, lyophilised and desalted by passing twice over Sephadex G10.

4.2. Isolation of TDP-rhamnose-synthesising enzymes from E. coli

The strains containing plasmid p4439 (dTDP-D-glucose-4,6-dehydratase) and p3778 (*S. enterica* rfB cluster, 3,5-epimerase and reductase) were grown analogously as described in the literature.[7]

The following solutions were prepared and used sterile: solution A (2% peptone, 1% yeast extract, 0.5% NaCl, 0.2% glucose, 100 mg/l ampicillin, adjusted to pH 7.4); buffer A (50 mM TRIS/HCl, adjusted to pH 7.6, 10 mM $MgCl_2$,1 mM EDTA).

4.2.1. Strain p4439

The strain was grown in solution A and aeration at 30°C overnight, until A(530) had reached 1.3–1.5. The temperature was then increased to 42°C and growing was continued for 10 h. The culture was centrifuged for 20 min at 15 000×*g* at 4°C, and at that temperature washed twice with buffer A. The preparation could be stored at –20°C until used or worked up further. The pellets were re-suspended in buffer A (*c* 0.2 g wet mass/ml) and broken by a French press. The cell debris was removed by centrifugation at 30 000×*g* for 1 h at 4°C. The supernatant was then used for further reactions with the sugar nucleotides. For storage purposes (at −20°C), this fraction was applied to a G25 column, eluted with buffer A:glycerol, 80:20, and, to the protein fraction, glycerol was added to a final concentration of 50% (vol).

4.2.2. Strain P3778

The strain was grown with shaking in solution A at 37°C to an optical density of A(530)=0.5. The culture was centrifuged for 20 min at 15 000×*g* at 4°C, and at that temperature washed twice with buffer A. The preparation could be stored at −20°C until used or worked up further. The pellets were thawed in buffer A (*c* 1 g wet mass/ml) and sonicated twice, whereby the sample was frozen between both sonication treatments consisting of six individual bursts of 10 s each. During the treatment the sample was constantly chilled on ice. The cell debris was removed by centrifugation at 30 000×*g* for 1 h at 4°C. The supernatant was then applied to a G25 column, eluted with buffer A:glycerol, 80:20. The protein fraction was then used for further reactions with the sugar nucleotides; for storage purposes (at −20°C), glycerol was added to the protein to a final concentration of 50% (vol).

4.3. TDP-3,6-dideoxy-α-L-arabino-*hexopyranose* **6**

In a sterile plastic container, which was constantly flushed with Argon and which was handled under a laminar flow bench, TDP-3-deoxy-α-D-*ribo*-hexopyranose (**7**, 1.0 g, 1.6 mmol), which was prepared from chemically synthesised dilithium salt[11] by passing through a column of Dowex W50

(Na^+) at 4°C and subsequent lyophilisation, was dissolved in a TRIS/HCl buffer (50 mM, 1 ml, pH 7.5, containing 0.5 mM DTT). To this the raw p4439 enzyme extract (containing 240 U of TDP-glucose-4,6-dehydratase activity as estimated from assays described in the lit.[12,13] having TDP–glucose as substrate) was added, and supplemented with NAD^+ (1.42 g, 2.0 mmol). The mixture was shaken at 37°C for 6 h and monitored by TLC (solvent a: 2-propanol:EtOH:H_2O=5:3:2, 5% HOAc, 2% NEt_3 or solvent b: 2-propanol:H_2O:NH_3 in water=7:3:1). The reaction was passed through a filter (cut-off 10 kD) and the filtrate was lyophilised. The obtained material was indistinguishable by TLC from authentic reference material.[5] For analytical purposes, an aliquot was purified by anion exchange chromatography as described in the general methods section. For NMR data see Tables 2 and 3.

Table 2

	7		**5**		**6**	
	^{1}H NMR (ppm)	Mult; ^3J (Hz), [J_{het}(Hz)]	^{1}H NMR (ppm)	mult; ^3J (Hz), [J_{het}(Hz)]	^{1}H NMR (ppm)	mult; ^3J (Hz), [J_{het}(Hz)]
Hexose						
1''	5.54	Dd, 3.1 [6.5]	5.38	dd, 3.1 [6.5]	4.84	dd, 3.0 [6.6]
2''	3.82-3.51	M, 3H	3.74	m_c	3.22	m, 2H
3''	2.34 + 2.15	Ddd / ddd, 12.6 10.5, 5.3	2.47 + 2.25	dd/dd, 12.6 11.6, 5.5	2.28 + 2.05	dd/dd, 12.4 10.0, 4.8
4''	See H-2''		./.		3.30	m
5''	4.00-3.90	M, 2H	4.02	q, 6.6	4.00	≈dq, 10.1, 6.6
6a''	See H-5''		1.09	d	1.06	d
6b''	See H-2''					
Ribose						
1'	6.22	Dd	6.22	dd	6.20	dd
2a'	2.35-2.30	M, 2H	2.35-2.30	m, 2H	2.35	m, 2H
2b'	See H-2a'		see H-2a'		See H-2a'	
3'	4.68	M	4.68	m	4.70	m
4'	4.08-3.90		4.08-3.90	m	4.00-3.90	m
5a'	See H-4'		see H-4'		See H-4'	
5b'	See H-4'		see H-4'		See H-4'	
Base						
6	7.72	S	7.72	s	7.74	s
CH_3	1.84	S	1.84	s	1.77	s
OCH_3						

The raw material lyophilisate was re-suspended in a TRIS/HCl buffer (50 mM, 3 ml, pH 7.5, containing 0.5 mM DTT), supplemented with NADPH (tetrasodium salt, 1.45 mg, 1.6 mmol) and incubated with 45 U (estimated according to the lit.[6,7]) of the p3378 extract to convert the 4-ulose intermediate. The

Table 3

	7		5		6	
	^{13}C NMR (ppm)	J-hetero (Hz)	^{13}C NMR (ppm)	J-hetero (Hz)	^{13}C NMR (ppm)	J-hetero (Hz)
Hexose						
1''	96.0	6.4	95.7	6.4	92.7	6.4
2''	86.8	8.4	69.1	8.5	85.3	8.0
3''	28.1		32.4		27.5	
4''	68.6		93.2; [203.1[3)]]		67.0	
5''	69.9		69.8		68.0	
6''	n/o		12.2		14.0	
Ribose						
1'	85.3		85.6		82.0	
2'	38.7		38.4		37.0	
3'	71.5		71.0		69.8	
4'	83.2	n/o	81.2	n/o	80.1	n/o
5a'	65.7	4.6	65.9	4.5	65.0	4.8
Base						
2	151.8		153.1		150.0	
4	166.9		167.7		167.3	
5	112.0		112.4		112.7	
6	136.7		138.4		137.6	
CH_3	11.8		11.6		11.9	

incubation mixture was shaken at 37°C and the mixture was worked up according to reported standard procedures for nucleoside diphosphate sugars[5] by heat denaturation of the proteins, lyophilisation and G-10 gel permeation chromatography. The product was purified by anion exchange chromatography to yield **6** (210 mg, 0.39 mmol, 24%) as an off-white, hygroscopic product, which was sensitive to decomposition in solution at room temperature. For NMR data see Tables 2 and 3. For mass spectrometry of the product, the dilithium salt was passed through a bed of Dowex W50 (H^+) at 4°C and lyophilised prior to analysis. DCI (NH_4^+), *m/z* (%): 550 [M^++NH_4^+].

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