Carbohydrate Research, 184 (1988) 113–120
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

MALTO-OLIGOSACCHARIDE HOMOLOGUES OF 3,7-ANHYDRO-2-AZI-1,2-DIDEOXY-D-*glycero*-D-*gulo*-OCTITOL: IMPROVED PHOTOAFFINITY REAGENTS FOR LABELLING THE MALTO-OLIGOSACCHARIDE-BINDING PROTEIN OF *Escherichia coli*

JOCHEN LEHMANN, JÜRGEN STECK, WOLFGANG WEISER,
Institut für Organische Chemie und Biochemie der Universität, Freiburg i. Br., Albertstr. 21, D-7800 Freiburg i. Br. (F.R.G.)

WINFRIED BOOS, AND SABINE WRISSENBERG
Fachbereich Biologie, Universität Konstanz, D-7750 Konstanz (F.R.G.)

(Received March 7th, 1988; accepted for publication, June 16th, 1988)

ABSTRACT

3,7-Anhydro-2-azi-1,2-dideoxy-D-*glycero*-D-*gulo*-octitol (**2**) was synthesized as a β-D-glucopyranosyl analogue, which could be converted into a series of malto-oligosaccharide derivatives (**3**–**7**) by cyclodextrinase-catalyzed glucosyl transfer from α-cyclodextrin (cyclomaltohexaose). The pure analogues **3**–**7** containing 1–5 (1→4)-linked α-D-glucose residues inhibited the uptake of maltose *via* the maltose-binding protein-dependent transport system in *Escherichia coli*. The concentration of half-maximal inhibition of maltose transport at 60nM decreases with increasing chain-length of the analogue, reaching a minimum at 0.02mM for **6** (4 glucose residues). ^{3}H-Labelled α-cyclodextrin was prepared by partial oxidation and reduction of the aldehyde groups with NaB^3H_4. Radiolabelled **5a** was used to photolabel the binding site of the maltose-binding protein.

INTRODUCTION

E. coli possesses a high-affinity system for transport and metabolism of maltose and malto-oligosaccharides. Five proteins are involved[1] in the transport of the saccharides through the outer membrane of the cell into the cytoplasm. Maltoporin, a protein which spans the outer membrane, enables the entrance of malto-oligosaccharides into the periplasm[2]. The periplasmic maltose-binding protein (MBP), which is the recognition site of the transport system[3], is located here and its primary structure[4] and the crystal structure[5] are known. MBP binds maltose and malto-oligosaccharides of all lengths with a K_d in the μM range[6]. Substrate-loaded MBP reacts with the inner membrane components Mal F, G, and K, which control the admittance of the sugar into the cytoplasm[1]. Malto-oligosaccharides longer than maltohexaose cannot pass the inner membrane components[7]. All of the proteins of the system have been shown, or are expected, to

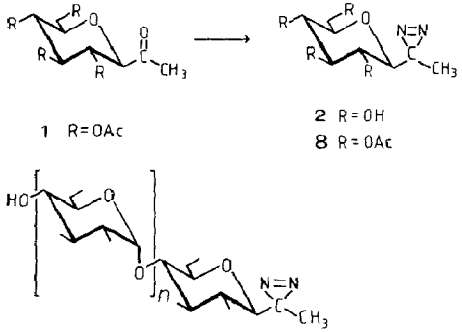
0008-6215/88/$ 03.50

possess binding sites for malto-oligosaccharides. We have described syntheses of 3-azi-1-methoxybutyl D-malto-oligosaccharides which can function as specific photoaffinity labels for these binding sites. The substrates inhibit the uptake of maltose in *E. coli* with rising efficiency as the chain length increases, building up to a level of 40μM for α-substrates longer than maltotetraoside. We now describe syntheses and applications of malto-oligosaccharide derivatives of 3,7-anhydro-2-azi-1,2-dideoxy-D-*glycero*-D-*gulo*-octitol (**2**) as photoaffinity labels for malto-oligosaccharide binding sites. These compounds are expected to be more resistant to hydrolysis than the acetalic 3-azi-1-methoxybutyl glycosides[8].

RESULTS AND DISCUSSION

3,7-Anhydro-2-azi-1,2-dideoxy-D-*glycero*-D-*gulo*-octitol (**2**) was synthesized from 4,5,6,8-tetra-*O*-acetyl-3,7-anhydro-1-deoxy-D-*glycero*-D-*gulo*-octulose[9] (**1**) according to a published method[10]. Malto-oligosaccharide analogues of **2** with 2–6 residues, namely, *O*-α-D-glucosyl- (**3**), *O*-α-maltosyl- (**4**), *O*-α-maltotriosyl- (**5**), *O*-α-maltotetraosyl- (**6**), and *O*-α-maltopentaosyl-(1→6)-3,7-anhydro-2-azi-1,2-dideoxy-D-*glycero*-D-*gulo*-octitol (**7**) were produced by cyclodextrinase-catalyzed transfer of glucosyl residues using α-cyclodextrin (cyclomaltohexaose) as donor and **2** as acceptor. Radioactively labelled malto-oligosaccharides were prepared with α-cyclodextrin-*a* (*a* indicates 6-[^{3}H]-labelling of the glucosyl moieties), which can be obtained by selective oxidation of α-cyclodextrin, using methyl sulfoxide and dicyclohexylcarbodi-imide[11], and subsequent reduction with NaB^3H_4.

The inhibition of the uptake of maltose by increasing concentrations of **3–7** in fully induced cells of *E. coli* strain MC4100 was determined[8] by transport assays. The concentrations of **3–7** that result in the half-maximal inhibition of maltose uptake at 60nM are listed in Table I, and, as an example, Fig. 1 presents the maltose uptake at rising concentrations of **6** (Fig. 1A) and the determination of the half-maximal inhibition (Fig. 1B).



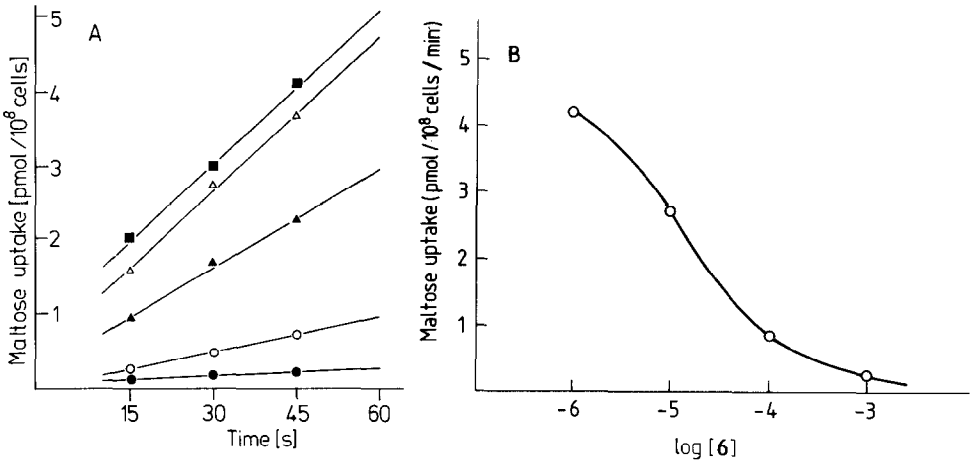


Fig. 1. Uptake of maltose at 60nM external concentration in the presence of increasing concentrations of **6**. *E. coli* strain MC4100 $(Mal)^+$ was induced by growth on maltose. Uptake of 60nM (U-^{14}C)maltose was measured in washed cells at an absorbance of 0.2 at 578 nm by filtering 0.5-mL samples through Millipore filters at the indicated time intervals. Various concentrations of **6** were mixed with the radioactive maltose prior to the addition of cells. The final concentration of the inhibitor was in increasing order: 0 (—■—), μM (—△—), 10μM (—▲—), 0.10mM (—○—), mM (—●—): A, uptake of maltose; B, rate of maltose uptake plotted against the log of the concentration of inhibitor.

The half-maximal inhibitory concentration of each of the analogues used was higher by one or two orders of magnitude than the K_d for maltose (2μM), but almost half as low as measured for the best-binding malto-oligosaccharide with a 3-azi-1-methoxybutyl end-group[8]. This finding indicates that "non-physiological" substituents at the reducing end interfere with, but do not prevent, the binding. At the non-reducing end, as shown with a series of 4,6-*O*-ethylidenemalto-oligosaccharides, chemical modification is ineffective when at least three unmodified glucopyranosyl residues are present in a malto-oligosaccharide chain[12]. Thus, 4,6-*O*-ethylidenemaltotetraose has the same affinity for MBP as does maltose[12].

Transport assays of the malto-oligosaccharide derivatives, made with the same *E. coli* strain (MC4100) as used for inhibition kinetics, show that the homologues of **2** are able to inhibit the uptake of maltose but cannot be transported. For covalent modification of its binding site, purified MBP was irradiated in the presence of 10^{-4}M **5a** with and without 10^{-2}M maltotetraose for protection. As shown in Fig. 2, the MBP was labelled by **5a**, the modification being suppressible to ~70% in the presence of maltotetraose which demonstrates specific labelling in the malto-oligosaccharide binding site. The 30% non-specific labelling may be due to the excessively high concentration of **5a**. At present, because of the limited specific radioactivity available (35 μCi/mmol), the molarity input cannot be decreased. Lower concentrations (10^{-5}M) and high specific radioactivity (2.4 Ci/mmol) caused highly specific modification (~95%) of the MBP by 3-azi-1-

TABLE I

HALF-MAXIMAL CONCENTRATIONS OF MALTO-OLIGOSACCHARIDE ANALOGUES OF 3,7-ANHYDRO-2-AZI-1,2-DIDEOXY-D-*glycero*-D-*gulo*-OCTITOL TO INHIBIT [^{14}C]MALTOSE TRANSPORT AT 60nM CONCENTRATION

Compound	K_i *(mM)*	*Compound*	K_i *(mM)*
3	0.71	**6**	0.02
4	0.07	**7**	0.03
5	0.03	Maltose	0.002

TABLE II

^{1}H-N.M.R. DATA FOR **2** (D_2O, 250 MHz) AND **8** ($CDCl_3$, 400 MHz)

Proton	*Chemical shift (δ)*		*Coupled protons*	$J_{H,H}$ *(Hz)*	
	2	**8**		**2**	**8**
H-1	1.12	1.07	1,2		
H-2			2,3		
H-3	3.05	2.74[a]	3,4	9.7	9.5
H-4	3.12	5.05[a]	4,5	7.8	
H-5	3.4	5.11[a]	5,6	8.2	
H-6	3.26	5.03[a]	6,7	9.3	9.5
H-7	3.27	3.60	7,8	5.5	4.9
H-8	3.87	4.24	7,8′	2.4	2.4
H-8′	3.68	4.15	8,8′	12.5	12.5
$COCH_3$		2.01			
		2.02			
		2.10			
		2.11			

[a]Second-order effects.

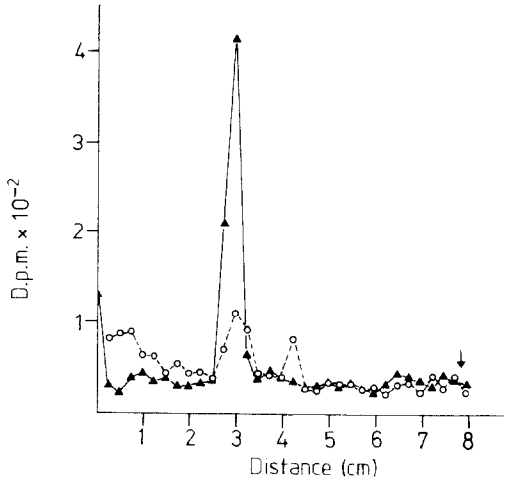


Fig. 2. Labelling of maltose-binding protein by **5a**. Pure MBP was incubated with the photoaffinity label (0.1mM) in the presence (—○—) and absence (—▲—) of maltotetraose (10mM) and irradiated at 350 nm prior to polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS). The gel strips were cut into 2-mm pieces and counted for radioactivity.

methoxybutyl α-D-[^{3}H]maltotrioside[8]. A similar efficiency can be expected for the corresponding and higher homologues of **2**.

The homologues of compound **2** with high specific radioactivity will be useful to modify MBP specifically in the vicinity of the binding site.

EXPERIMENTAL

General methods. — All reactions were monitored by t.l.c. on silica gel F_{254} (Merck) and radioactivity was detected with a Berthold LP 282 linear analyzer or by autoradiography using X-ray film. Column chromatography was carried out on silica gel (230–400 mesh, Merck) or on a column (1.6 × 100 cm) of Biogel P2 (−400 mesh, Bio Rad Laboratories) with distilled water. Radioactivity in solution was measured in a Berthold BF 815 liquid scintillation counter, using Scintigel (Roth, Karlsruhe). ^{1}H-N.m.r. spectra were recorded with Bruker WM 400 and WM 250 instruments, as stated in Tables II and III for solutions in $CDCl_3$ (acetylated compounds; internal Me_4Si) and D_2O [non-acetylated; internal sodium 3-(trimethylsilyl)-1-propanesulfonate]. The ^{13}C-n.m.r. spectrum (internal Me_4Si) was recorded with a Bruker HX 400 instrument at 100.614 MHz. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. Photolysis was carried out in a Rayonet RPR-100 reactor equipped with 16 RPR 3500 Å lamps and monitored using a Perkin–Elmer 555 u.v.-spectrometer. Melting points are uncorrected. Light petroleum refers to the fraction b.p. 60–70°.

α-Cyclodextrin-a. — A solution of α-cyclodextrin (100 mg, 0.3 mmol), dicyclohexylcarbodi-imide (130 mg, 0.3 mmol), and dichloroacetic acid (10 μL) in Me_2SO (2.5 mL) was stirred at room temperature for 2 h, then filtered, and concentrated to dryness. A solution of the residue in water (500 μL) was added to NaB^3H_4 (100 mCi, 62 Ci/mmol) in an ampoule and allowed to react overnight. NaB^3H_4 (50 mg, 1.4 mmol) was then added and, after 10 h, the excess of reductant was destroyed with acetic acid. Following treatment with Amberlite (H^+) resin, the solution was concentrated. The residue was taken up in MeOH (5 mL) and the

TABLE III

^{13}C-N.M.R. DATA[a] FOR **8**

Atom	δ	*Atom*	δ
C-1	15.21	$COCH_3$	170.64
C-2	25.37		170.23
C-3	79.58		169.61
C-4	68.36		169.40
C-5	73.73	$COCH_3$	20.73
C-6	68.10		20.64
C-7	75.72		20.59
C-8	62.08		20.56

[a]Recorded for solutions in $CDCl_3$ at 100.614 MHz; δ relative to Me_4Si.

solution concentrated to dryness. This process was repeated 3 times, yielding solid α-cyclodextrin-*a* (75.7 mg; ~32 mCi/mmol as determined by liquid scintillation counting using an internal standard).

4,5,6,8-Tetra-O-acetyl-3,7-anhydro-2-azi-1,2-dideoxy-D-glycero-D-gulo-*octitol* (**8**). — A solution of 4,5,6,8-tetra-*O*-acetyl-3,7-anhydro-1-deoxy-D-*glycero*-D-*gulo*-octulose[8] (3.0 g, 7.9 mmol) in dry MeOH (100 mL) was saturated with dry NH_3 (~60 mL) for 2 h at −20°. A solution of hydroxylamine-*O*-sulfonic acid (1.6 g, 14.1 mmol) in dry MeOH (40 mL) was added dropwise during 45 min to the vigorously stirred mixture. The solution was stirred for 3 h at −20°, then allowed to attain room temperature overnight, filtered, and concentrated to dryness. A solution of the residue in dry MeOH (60 mL) containing Et_3N (15 mL) was cooled to 0° and iodine was added until the red colour persisted. The solution was then concentrated, and the red syrupy residue was purified by flash chromatography[13] (10:1 EtOAc–MeOH). Acetylation of the syrupy product in C_5H_5N (30 mL) and Ac_2O (18 mL) at room temperature was complete after 18 h (R_F 0.55, 2:1 EtOAc–light petroleum). The mixture was processed as usual to yield a syrup, chromatography (1:15 EtOAc–light petroleum) of which, with recrystallization of the product from EtOAc–light petroleum, gave **8** (2.0 g, 65.4%), m.p. 86°, $[\alpha]_D^{23}$ +48° (*c* 1, chloroform). For the ^{1}H-n.m.r. and ^{13}C-n.m.r. data, see Tables II and III.

Anal. Calc. for $C_{16}H_{22}N_2O_9$: C, 49.74; H, 5.74; N, 7.25. Found: C, 49.46; H, 5.47; N, 7.20.

3,7-Anhydro-2-azi-1,2-dideoxy-D-glycero-D-gulo-*octitol* (**2**). — Compound **8** was deacetylated (Zemplén) (R_F 0.53; 7:2:1 EtOAc–MeOH–H_2O) to yield hygroscopic **2** (212 mg, 72.8%), which was crystallized from EtOH–Et_2O to give material with m.p. 82°, $[\alpha]_D^{23}$ +28° (*c* 0.95, water). For the ^{1}H-n.m.r. data, see Table II.

Anal. Calc. for $C_8H_{14}N_2O_5 \cdot 0.5\ H_2O$*: C, 42.28; H, 6.65; N, 12.32. Found: C, 42.63; H, 6.58; N, 12.08.

Anal. Calc. for $C_8H_{14}N_2O_5 \cdot 0.25\ H_2O$*: C, 43.14; H, 6.56; N, 12.57. Found: C, 43.34; H, 6.78; N, 12.22.

Photolysis of **2** (7.3mM, MeOH) was performed using RPR 3500 lamps (emission maximum, 350 nm). Decrease of the u.v. absorption of the diazirino group at 400–280 nm was recorded after irradiation for 0, 1, 2, 3, 4, 5, 6, 7, and 9 min. Log $E \cdot 10$ at 334 nm (ε_{334} 62) was plotted against irradiation (min). The half life ($t_{1/2}$) of **2** was 5.45 min.

Preparation of O-α-D-*glucosyl*- (**3**), O-α-*maltosyl*- (**4**), O-α-*maltotriosyl*- (**5**), O-α-*maltotetraosyl*- (**6**), *and* O-α-*maltopentaosyl-(1→6)-3,7-anhydro-2-azi-1,2-dideoxy*-D-glycero-D-gulo-*octitol* (**7**). — Glucosyl transfer was carried out at room temperature using **2** (100 mg, 0.46 mmol), α-cyclodextrin (100 mg, 0.1 mmol), cyclodextrinase [(1→4)-α-D-glucan 4-α-D-glucano-transferase, cyclizing, from *Bacillus macerans*; 5 U] in distilled water (1.5 mL). Equilibrium was reached after 2 h as indicated by t.l.c. (7:2:1 EtOAc–MeOH–H_2O or 7:3:3:2:3:2 1-propanol–EtOH–EtOAc–C_5H_5N–H_2O–AcOH). Higher-molecular-weight material, salts,

*Water content was determined by weight decrease.

and excess of enzyme were removed by chromatography of the freeze-dried reaction mixture on a column (1.5 × 3.5 cm) of silica gel, using 7:2:1 EtOAc–MeOH–H_2O. After concentration to dryness, the syrupy residue containing **3–7**, as well as higher homologues and α-cyclodextrin, was taken up in a little water and eluted at room temperature from a column (1.6 × 100 cm) of Biogel P2 with water at 10 mL/h. Lyophilization yielded amorphous **3–7**.

Compound	R_F[a]	*Yield (mg)*
2	0.75	
3	0.52	30 (78.9 μmol)
4	0.35	23 (42.2)
5	0.21	19 (27.0)
6	0.12	16 (18.5)
7	0.06	14 (13.6)

[a]1-Propanol–EtOAc–EtOH–C_5H_5N–water–AcOH (7:3:3:2:3:2).

Transport assays of [^{14}C]maltose in the presence and absence of **3–7** were carried out in *E. coli* strain MC4100 as described.

Photoaffinity labelling of MBP with **5a**, including its analysis by polyacrylamide gel electrophoresis, was performed as described[7].

Cleavage of the homologues **3–7** (1 mg) with α-D-glucosidase from yeast (maltase, α-D-glucoside glucohydrolase) (50 μL of a suspension, 5 mg/mL; Boehringer Mannheim) in sodium phosphate buffer (pH 6.8, 250 μL) was carried out overnight at room temperature.

Preparation of radiolabelled malto-oligosaccharides **3a–6a**. — A solution of **2** (21 mg, 0.115 mmol) and α-cyclodextrin-*a* (21 mg, 0.026 mmol) in distilled water (100 μL) was incubated with cyclodextrinase (2 U). After 2 h, the reaction was complete and the mixture was freeze-dried. The homologues **3a–6a** were isolated as described for the unlabelled compounds **3–7**.

Compound	*Yield (mg)*	*Activity (μCi/mmol)*
3a	8 (21 μmol)	7.5
4a	7 (12.9)	27.6
5a	6 (8.5)	34.6
6a	4 (4.6)	14.6

Co-chromatography (t.l.c.) with authentic **3–6** was carried out to prove the identity of **3a–6a**.

ACKNOWLEDGMENTS

We thank Professor Dr. K. Wallenfels for the donation of preparative m.p.l.c.

equipment, Boehringer Mannheim for a generous gift of cyclodextrinase, and the Deutsche Forschungsgemeinschaft e.V. for financial support.

REFERENCES

1 R. HENGGE AND W. BOOS, *Biochim. Biophys. Acta*, 737 (1983) 443–478.
2 S. SMELCMAN, M. SCHWARTZ, T. J. SILHAVY, AND W. BOOS, *Eur. J. Biochem.*, 65 (1976) 13–19; Z. FERENCI AND K.-S. LEE, *J. Mol. Biol.*, 160 (1982) 431–444; H. NIKAIDO AND M. VAARA, *Microbiol. Rev.*, 49 (1985) 1–32.
3 O. KELLERMANN AND S. SMELCMAN, *Eur. J. Biochem.*, 47 (1974) 139–149.
4 P. DUPLAY, H. BEDOUELLE, A. FOWLER, B. ZABIN, W. SAURIN, AND M. HOFUNG, *J. Biol. Chem.*, 259 (1984) 10606–10613.
5 F. QUICHO, W. E. MEADOR, AND J. W. PFLUGRATH, *J. Bacteriol.*, 133 (1979) 181–184.
6 C. WANDERSMAN, M. SCHWARTZ, AND T. FERENCI, *J. Bacteriol.*, 140 (1979) 1–73.
7 T. FERENCI, *Eur. J. Biochem.*, 108 (1980) 631–636.
8 R. THIEME, H. LAY, A. OSER, J. LEHMANN, S. WRISSENBERG, AND W. BOOS, *Eur. J. Biochem.*, 160 (1986) 83–91.
9 W. WEISER, J. LEHMANN, C. F. BREWER, AND E. J. HEHRE, *Carbohydr. Res.*, in press.
10 R. THIEME, G. KURZ, AND J. LEHMANN, *Carbohydr. Res.*, 136 (1985) 125–133.
11 K. OMICHI AND T. IKENAKA, *J. Biochem. (Tokyo)*, 92 (1983) 1055–1060.
12 J. STECK, S. WRISSENBERG, M. SCHMIDT-SCHUCHARDT, J. LEHMANN, AND W. BOOS, unpublished results.
13 W. C. STILL, M. KAHN, AND A. MITRA, *J. Org. Chem.*, 43 (1978) 2923–2925.