Title; Syntheses and structure-membrane active antimicrobial activity relationship of alkylamino-modified glucose, maltooligosaccharide, and amylose

Short running title; antimicrobial alkylamino-modified sugars

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Abstract
Antimicrobial alkylamine-modified sugars were prepared. Microwave-assisted click reaction efficiently achieved poly-functionalization of oligo- and polysaccharides. The sugars exhibited a unique relationship of their bacterial membrane permeabilization and antimicrobial activity with the number of functional groups and the structure of the molecular scaffold. It shows that the assembly of the functional groups is necessary for being antimicrobial. The amylose derivatives also exhibited synergy to minimize the necessary dose of conventional antibiotics and increase their antimicrobial potency.

Keywords:
maltooligosaccharide
amylose
microwave-assisted click reaction
antimicrobial activity
synergistic activity

Antimicrobial resistance in bacteria is one of the most serious threats in global public health.[1] As conventional drugs are being lost, demand for novel antibiotics has pointed out membrane active antimicrobial peptides. The peptides exhibit broader-spectrum activity because they disrupt the bacterial membrane with physical interaction, and they are believed to develop slower resistance than conventional medicine do because the cell membrane alteration may be metabolically expensive.[2-4] Polymyxin B is one such antimicrobial cyclic peptide that has been recognized as a promising agent against multidrug-resistant bacteria (Fig. 1).[5,6] It contains positively charged five diaminobutanoic acid residues those interact with the anionic phosphate moiety of the bacterial cell membrane, and hydrophobic groups those interact with the fatty acid moieties in the lipid membrane, to permeabilize both outer and cytoplasmic membranes of gram-negative bacteria.[7] From polymyxin B and other membrane active peptides,[8-10] we drew the inspiration to design simpler compounds possessing the biological activity of antimicrobial peptides. The molecular scaffold used for this purpose are cyclic oligosaccharides called cyclodextrins (CDs). Note that γ-CD comprises eight D-glucose residues with a truncated cone structure of diameter ca. 1 nm, which is almost the same as that of polymyxin B. The cone is rimmed by hydroxy groups in order to be chemically modified. As a membrane active functionality to interact with bacterial membrane we chose an alkyl moiety-modified amino group that unite the hydrophobic and cationic. The groups were introduced onto a CD molecule by copper-catalyzed azide-alkyne cycloaddition,[11-13] which has been adopted for the modification of CD molecules.[14-16] Click chemistry yielded a new series of antimicrobial γ-CD derivatives which possessed eight alkylamino groups per molecule.[17] These derivatives strongly disrupted the bacterial membrane and inhibited bacterial proliferation including methicillin-resistant *Staphylococcus aureus* and other drug-resistant bacteria.[18] These results evidently demonstrate that the chemistry to develop antimicrobial agents using CDs is promising. However, the results prompt the question: Why is CD highly effective? Is the other scaffold attaching the membrane active functionalities antimicrobial? In order to address the questions, we will discuss the followings; antimicrobial activity of 1) the linear oligosaccharide

that possesses the same number of groups as those of CD, 2) the mono- and disaccharides possessing one and two alkylamino substituents, and 3) the polysaccharides that possess a large number of alkylamino groups. The discussion will draw insight into roles of a CD molecule for the antimicrobial scaffold. And also it may open possibility to extend our antimicrobial design from CD molecule to other sugars, which lead novel antimicrobial drug and material made of the sugars. This paper will present the preparation of alkylamino-modified glucose **1**, maltose **2**, maltooctaose **3** and amyloses **4-6** and their unique relationship of bacterial membrane permeabilization and antimicrobial activity with the number of functional groups and the size and shape of the molecular scaffold.

To examine the effect of the molecular scaffold, a pentylamino group was introduced into glucose, maltose, maltooctaose and amyloses as the antimicrobial group on the γ-CD disrupted bacterial membrane.[18] In order to attach the pentylamino groups onto each sugar, a microwave (MW)-assisted Huisgen 1,3-dipolar cycloaddition reaction, known as the click chemistry reaction was adopted, which enabled polyfunctionalization of the CD molecule.[18,19] The maltooctaose derivative **3** was synthesized as shown in Scheme 1.[a] The 1-*O*-methylation of maltooctaose was done in order to avoid undesired side reactions on the relatively reactive 1-OH group of the reducing end. Firstly, the 1-OAc moiety of the maltooctaose per-acetate **7** was selectively deacetylated by $(NH_4)_2CO_3$ to give **8** where loss of Ac group was confirmed by ^{1}H-NMR and MALDI-MS (m/z, 2388, M+Na^+).[20] Next, the 1-OH group of **8** was converted to give an imidate **9** which showed a characteristic imidate NH signal (δ 8.88) on its ^{1}H-NMR spectrum. The reaction of the imidate **9** with methanol gave the desired methoxide **10**, which was determined by its MS spectrum (m/z 2402, M+Na^+) and NMR spectrum (δ 3.49, CH_3O-). In order to get the octa-6-chlorinated **13**, all the acetyl groups of the **10** was firstly deprotected by $NaOCH_3$-CH_3OH treatment (**11**), which was determined by disappearance of Ac signals on the ^{1}H-NMR spectrum. All the C6s of **11** were chlorinated by Vilsmeier type halogenation[21] (**12**) and the remaining hydroxyl groups were acetylated (**13**). Nucleophilic substitution of the Cl groups by azide ion gave the octa-azide **14**. MW-assisted Huisgen reaction of the azide **14** with Boc-propargyl-pentylamine yielded a click product **15**, followed by the deprotection of

acetyl groups and Boc groups to give the desired maltooctatose **3** (60.1% yield, three steps from azide **14,** as shown in Scheme 1; ^{1}HNMR (400 MHz, DMSO-d_6) δ 0.84 (24H, CH_3), 1.26 (32H, $\underline{(CH_2)_2}$-CH_3), 1.57 (8H, N-CH_2-$\underline{CH_2}$-), 2.88 (16H, N-$\underline{CH_2}$-CH_2-), 3.15-3.58 (27H, CH_3O-, H2,3,4-octaose), 3.90-4.46 (40H, triazole-$\underline{CH_2}$-N, H5,6-octaose), 5.13-5.21 (8H, H1-octaose), 8.02-8.11 (8H, triazole); ^{13}CNMR (125 MHz, DMSO-d_6) δ 13.55 (CH_3), 21.48, 24.80, 27.78 (C2,3,4-pentylamino group), 40.62 (triazole-$\underline{CH_2}$-N), 46.02 (C1-pentylamino group), 49.98, 51.16, 51.74 (C6-octaose), 55.87 (CH_3O), 68.82 (C5-octaose), 68.82, 69.66, 70.90, 71.21, 72.19, 72.51 (C2,3-octaose), 80.40 (C4-octaose), 99.70 (C1-octaose), 126.78 (CH-triazole), 137.84 (C-triazole); MS (MALDI): calcd for $C_{113}H_{196}N_{32}NaO_{33}$ $(M+Na)^+$ 2552.4540, found 2552.6072; Found: C, 38.70; H, 4.81; N, 9.75%. Calcd for $C_{113}H_{196}N_{32}O_{33}$+18.0$CF_3COOH$+4.0$H_2O$: C, 38.44; H, 4.81; N, 9.63%). The MW-assisted click reaction efficiently completed the introduction of the eight alkylamino groups into the oligosaccharide molecule, as observed in the case of CD.[17-19] Similar to the maltooctaose derivative **3,** the glucose derivative **1** and the maltose derivative **2** were prepared from the corresponding 6-azido per-acetates **16**[22] and **18**[23] (yields: **1** 78.1% and **2** 45.4%, three steps from the azides) (Scheme 2). The pentylamino-modified amylose derivative **4** (number of glucoses, n = ca. 90) was prepared from the corresponding 6-azido-2,3-per-acetate **20**[19] (37.2% yield, three steps) (Scheme 2). Azido-per-acetate (n = ca. 1000) **22**[19] yielded amylose **5** (52.2% yield, three steps). Also, hexylamino groups were introduced on an amylose (number of glucoses, n = ca. 90) to give the derivative **6**. Polymodification of amylose (reaction points 90~1000) was well achieved via the MW-assisted click reaction, as in the case of the mono- and oligosaccharides mentioned above and reported previously.[17-19]

The antimicrobial activity of the prepared pentylamino-modified sugars **1-6** was examined to determine their minimum inhibitory concentration (MIC) against the gram-positive strains, i.e. *Bacillus subtilis and Streptococcus aureus*, and the gram-negative strains, i.e. *Escherichia coli, Salmonella typhimurium* and *Pseudomonas aeruginosa* (Table 1). The MIC values of glucose **1** and maltose **2** against the gram-positive and gram-negative bacteria are larger than 256 μg cm^{-3}. This shows that one or two substituents in the molecule are not enough to be antimicrobial. The mono- and di-

saccharides **1** and **2** contains amphipathic structures containing hydrophilic and hydrophobic moieties but they may not form a molecular assembly (or a supramolecular structure) to present eight or more pentylamino groups for being antimicrobial. Conversely, the eight pentylamino group-possessing maltooctaose **3** showed significant antimicrobial activity against gram-positive bacteria. The MIC values are almost the same as those of the γ-CD derivative **25**[18] (Fig. 2) which possesses the same number of pentylamino groups in the cyclic structure. Preliminary experiments using β- and α-CD derivatives (**26** and **27**) modified by seven and six pentylamino groups demonstrated that their antimicrobial activity (MIC, *S. aureus*, **26**; 8, **27**; 4, *E.coli*, **26**; 128, **27**; 128 μg cm^{-3}) is almost the same as that of the γ-CD analogue **25**. It is noteworthy to describe here that assembling the appropriate number of substituents is necessary for the compound being antimicrobial and it is irrespective of those on a cyclic scaffold or a linear one. The MIC value of octaose **3** against gram-negative *E.coli* was 256 > μg cm^{-3} and much larger than that of γ-CD **25** (32 μg cm^{-3}). The difference might be attributed to the arrangement of the antimicrobial groups on the cyclic or linear molecular scaffold, and it may be a better point of the CD molecule scaffold. Amylose **4,** which possesses pentylamino groups (n = ca. 90), showed an antimicrobial activity a little less to that of the octaose **3** against *B. subtilis* and *S. aureus*, while the MIC value of **4** against *E.coli* (64 μg cm^{-3}) was much smaller than that of **3**. This suggests that the structure possessing many substituents (n = ca. 90) is superior to the octa-substituted structure in terms of interacting with the *E. coli* membrane. However, amylose **5,** which comprises even a larger number of pentylamino glucoses than **4** (n = ca. 1000), was less antibacterial than **3** and **4**. Antimicrobial activity depends on the number of substituents and/or the molecular size, and the presence of an optimum number of substituents and/or molecular size is noteworthy. From the view point of difference in alkyl moieties on the same amylose molecular scaffold (n = ca. 90) the hexyl amine-modified derivative **6** was less active than the pentyl **4** against *B. subtilis, S. aureus* and *E.coli.* All the amyloses here exhibited low antimicrobial activity against gram-negative *S. typhimurium* and *P. aeruginosa.* Thus, the octaose **3** and amylose **4** exhibited gramicidin S peptide-like antimicrobial activity which is high against gram-positive bacteria and low against gram-negative bacteria.[10]

The relatively lower antimicrobial activity of the amyloses against gram-negative bacteria in comparison with gram-positive bacteria may be due to the differences between the membrane structures of these bacteria. Presumably, the outer membrane in a gram-negative strain may obstruct the amyloses, as in the case of peptides[10] and CDs.[17] However, the amyloses possess poly-cationic amino moieties and therefore they may interact with polyanionic lipopolysaccharides of the outer membrane as polymyxin B nonapeptide do.[24] In order to determine whether the amylose interacts with the gram-negative bacterial outer membrane or not, the synergistic antimicrobial activity was examined by combining the amylose with a probe antibiotic (Table 2). The probe, novobiocin (Fig. 3), exhibited low activity (MIC, *S. Typhimurium* 512, *E.coli* 128, and *P. aeruginosa* 1024 μg cm^{-3}) when dosed alone because of the outer membrane acting as a barrier.[17] In contrast, upon addition of the amyloses, the antibiotic inhibited the growth of *S. Typhimurium*. The most evident is the case of combining novobiocin with the pentylamine-modified amylose **4** (n = ca. 90). At a dose of 32 μg cm^{-3}, **4** diminished the MIC of novobiocin to <1 μg cm^{-3} (less than 1/512 of that dosed alone), whereas MIC of hexyl **6** with the same number of glucose residues remained constant at 512 μg cm^{-3}. In the case of *E.coli* similar relationship was observed between **4** and **6**. This suggests that the larger (more hydrophobic) alkyl moieties may reduce the synergistic effect. The pentylamine-modified longer amylose **5** (n = ca. 1000) showed an effect similar to that of **4** (n = ca. 90). In the case of *P. aeruginosa*, none of the amyloses exhibited any synergistic effects (data not shown). The observed effects clearly demonstrate that the amyloses **4** and **5** can permeabilize the outer membrane of the bacteria. The *E.coli* outer membrane permeabilization of the sugars was examined by the experiments using an *N*-phenyl-1-naphtylamine (NPN) fluorescent probe.[25] When the outer membrane of the bacteria is damaged, the hydrophobic NPN partitions into the membrane and exhibits increased fluorescence. As shown in Fig. 4 the sugars caused significant increase in fluorescence as observed in the case of polymixin B (PMB), a peptide antibiotic known for its outer membrane permeabilization activity.[26] The observed fluorescent signal was **4** (pentyl, n = ca. 90) > **5** (pentyl, n = ca. 1000) > **6** (hexyl, n = ca. 90), which reflects outer membrane permeabilization ability of the sugars, and the intensity is parallel to the observed synergistic effect (Table 2). The results and those of the stand-alone

antimicrobial activity indicate a membrane-disrupting antimicrobial mechanism of amylose. The pentyl amylose **4** (n = ca. 90) interacts with the outer membrane of *E. coli* and *S. Typhimurium* and permeabilize them, whereas the more hydrophobic hexyl **6** (n = ca. 90) does not disrupt the outer membranes. In the *E.coli* cell, the amylose **4** subsequently approaches an inner cytoplasmic membrane of the bacteria and disrupt it to inhibit the bacterial growth. Conversely, the antimicrobial activity of **4** against *S. Typhimurium* was significantly low, suggesting that they may not interact with its cytoplasmic membrane strongly. The larger pentyl amylose **5** (n = ca. 1000) may equally interact with the membranes as **4**. In the case of *P. aeruginosa*, interaction of amyloses with the outer membrane is not enough to disrupt it. These may reflect the characteristics of each bacterial cell membrane (and the lipid molecules). The sugars discriminate the membranes, thereby exhibiting their unique membrane and antimicrobial activity.

The hemolysis of rabbit red blood cells by the sugars was examined where the concentration of sugar was 50 μg cm^{-3}. Lysolecithin (50 μM) was used to determine the 100% level. The pentyl amyloses **4** and **5** whose number of the alkylamino moieties are ca. 90 and ca.1000 exhibited moderate hemolysis (**4**, 52.3%, **5**, 41.0 %) while activity of the octaoses **3** and **25** possessing eight the pentylamino groups was very less (< 4.3%). The activity toward red blood cells may depend on number of the substituents and/or the molecular size of the sugars. It should be noteworthy that the least hemolytic linear and cyclic octaoses (**3** and 2**5**) are the most antimicrobial among the sugars and therefore they are specific against bacteria. The hexyl amylose **6** is more hemolytically-active (97.1%) than the pentyl amylose **4**. Presumably a more hydrophobic hexyl group may cause stronger interaction with the red blood cell membrane than the pentyl group.

In conclusion, we studied the antimicrobial functionalities of sugars possessing alkylamino groups. We established a molecular rationale to express the antimicrobial activity. The observed dependence of the number of alkylamino groups in the molecule clearly shows that the assembly of the functional groups is necessary for generating antimicrobial effects. However, a CD molecule appeared to be the best scaffold for obtaining such an organized structure. The reasons are the follows; first, the CD molecule

yielded the most antimicrobial compound with less hemolytic activity. Second, CD is a more common and easily available sugar compared with other oligosaccharides. Third, it is obtainable as a chemically pure material and gives a pure derivative. For constructing a polymer with such an organized structure, an amylose comprising glucose residues (n = ca. 90) is very useful. Our synthetic methods via a MW-assisted click reaction enabled a rationally designed and poly-functionalized structure for studying the structure–activity relationships and achieving the desired antimicrobial activity. The amylose derivatives exhibited good antimicrobial activity against gram-positive bacteria. It is also noteworthy that the sugars such as **4** and **5** can permeabilize the bacterial membrane, minimize the necessary dose of conventional antibiotics and increase their antimicrobial potency. It can potentially revitalize the existing antibiotics to treat drug-resistant bacterial infections that are untreatable using the existing antibiotics. This study is currently ongoing.

Acknowledgements

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Conflict of Interest

Authors have no conflict of interest.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

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Note

[a] For the preparation of the octaose derivative **3** conventional method for carbohydrate synthesis was adopted. Each the intermediates to **3** was properly purified, if necessary by silica-gel chromatography, to show a single spot on TLC analysis. All the intermediate structure was not completely characterized but characteristic spectra was obtained to show that the corresponding chemical conversion was properly achieved. The structure and purity of the final octaose derivative **3** to be applied for antimicrobial activity examination was fully determined by ^{1}H- and ^{13}C-NMR, MS, and elemental analyses.

Figure captions

Fig. 1 Structure of polymyxin B.

Fig. 2 Structures of γ-, β-, and α-CD derivatives **25**, **26**, and **27**.

Fig. 3 Structure of novobiocin.

Fig. 4 Uptake of *N*-phenyl-1-naphtylamine (NPN) by *E.coli* through outer membrane permeabilization of the sugars: black bars, with *E.coli*, white bars, without *E.coli*, PMB, polymixin B.

Scheme 1[a]

[a] Reagents and Conditions: (a) $(NH_4)_2CO_3$, DMF, 22 h, (b) CCl_3CN, DBU, CH_2Cl_2, 18 h, (c) CH_3OH,TMSOTf,CH_2Cl_2, -20 ◦C, 30 min, (d) $NaOCH_3$, CH_3OH (e) MsCl, DMF, 60 ◦C, 17 h, (f) Ac_2O, pyridine, (g) NaN_3, DMF, 70 ◦C, 5 days, (h) *N*-Boc-*N*-pentyl-*N*-2-propynylamine, sodium ascorbate, DMSO-H_2O, 70 ◦C (microwave heating), 2 h, (i) $NaOCH_3$, CH_3OH, (j) TFA.

Scheme 2[a]

[a] Reagents and Conditions: (a) *N*-Boc-*N*-pentyl-*N*-2-propynylamine,$CuSO_4$, sodium ascorbate, DMSO-H_2O, 70 ◦C, 35 min (**16**), 70 ◦C, 90 min (**18**), or 120 ◦C, 10 min (**14** and **15**) (microwave heating), (b) $NaOCH_3$, CH_3OH, (c) TFA.

Table 1 MIC (μg cm^{-3}) values of the sugars.

Table 2 Synergistic antimicrobial activity of the combination of amyloses **4–6** with novobiocin against *S. Typhimurium* and *E.coli*.

Fig. 1 Structure of polymyxin B

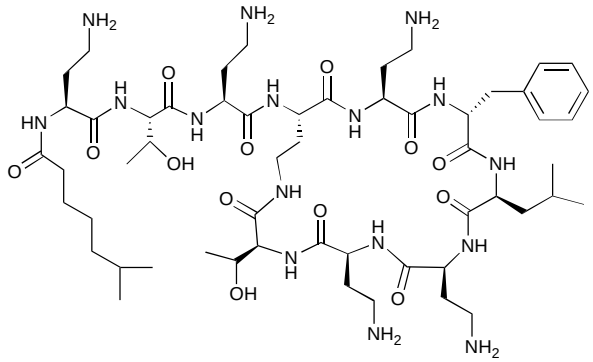
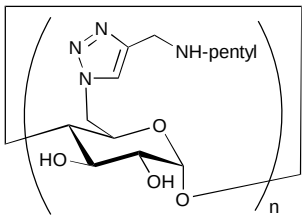


Fig. 2. Structure of γ-, β-, and α-CD deivatives **25**, **26**, and **27**.



25 n = 8
26 n = 7
27 n = 6

Fig. 3. Structure of novobiocin

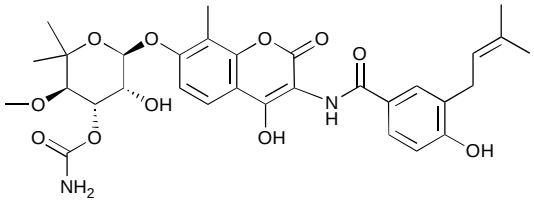
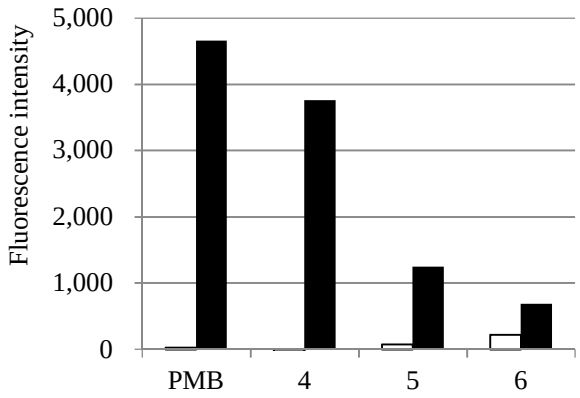
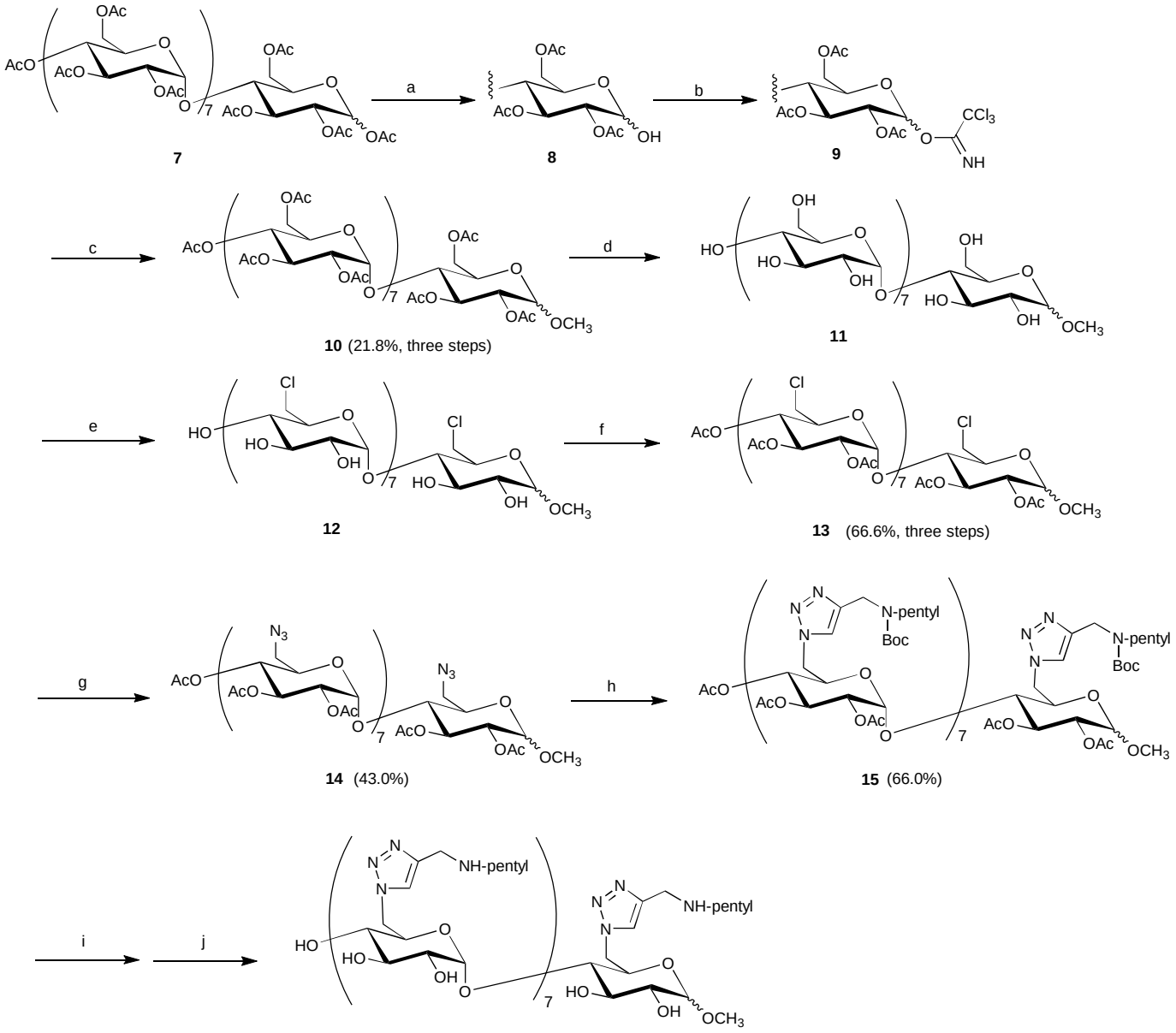
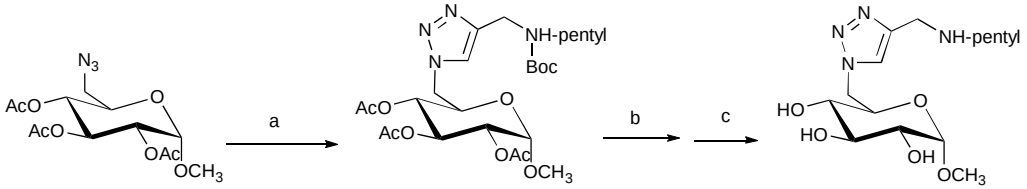


Figure 4 Uptake of NPN by *E.coli* through outer membrane permeabilization of the sugars: black bars, with *E.coli*, white bars, without *E.coli*, PMB, polymixin B.

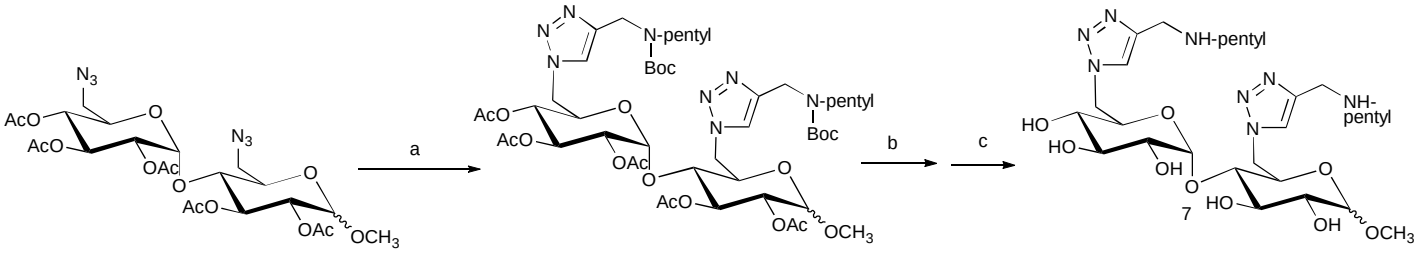



7
a
8
b
9
c
d
10 (21.8%, three steps)
11
e
f
12
13 (66.6%, three steps)
g
h
14 (43.0%)
15 (66.0%)
i
j
3 (91.1%, two steps)

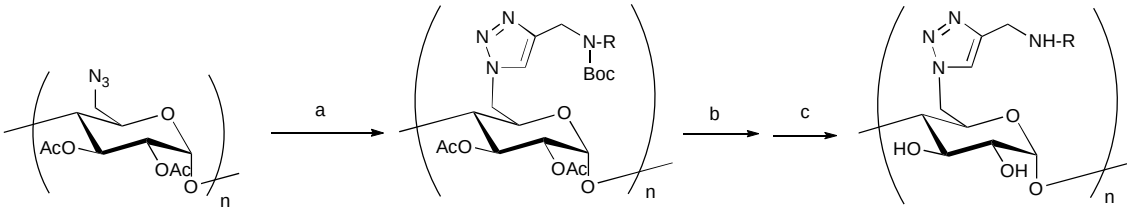
Scheme 1



16 **17** **1** (78.1%, three steps)



18 **19** **2** (45.4%, three steps)



20 n = ca. 90
22 n = ca. 1000

21 R = pentyl, n = ca.90
23 R = pentyl, n = ca.1000
24 R = hexyl, n = ca.90

4 R = pentyl, n = ca.90, 63.5 %, three steps
5 R = pentyl, n = ca.1000, 52.2 %, three steps
6 R = hexyl, n = ca.90, 68.6 %, three steps

Scheme 2

Table 1. MIC (μg cm^{-3}) values of the sugars.

compounds		gram positive bacteria		gram negative bacteria		
	number of alkylamino moieties	*B. subtilis*	*S. aureus*	*E.coli*	*S. Typhimurium*	*P. aeruginosa*
1	1	256>	256>	256>	256>	256>
2	2	256>	256>	256>	256>	256>
3	8	8	16	256>	256>	256>
4	ca.90	32	32	64	256	256>
5	ca.1000	64	256	128	256>	256>
6	ca.90	128	128	256	256	256>
25	8	8	4	32	256	256>
polymyxin B	-	8	32	1	2	2
Gramicidin S[a]	-	-	4	32	-	-

[a] See ref.10.

Table 2. Synergistic antimicrobial activity of the combination of amyloses 4–6 with novobiocin against *S. Typhimurium* and *E.coli*.

Amylose				MIC of novobiocin (μg cm^{-3}) bacteria	
sugars	number of glucoses	alkyl group	dose (μg cm^{-3})	*S. Typhimurium*	*E.coli*
			0	512	128
4	90	pentyl	16	512	128
			32	<1	32
5	1000	pentyl	16	512	128
			32	16	32
6	90	hexyl	16	512	128
			32	512	128