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Enzymatically oxidized lactose and derivatives thereof as potential protein cross-linkers

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Abstract—The enzyme galactose oxidase [EC 1.1.3.9] was applied to convert lactose, lactylamine and lactobionic acid into their corresponding 6′-aldehyde compounds. The potential protein cross-linking ability of these oxidized lactose and derivatives thereof was investigated using *n*-butylamine as the model compound. First, oxidized lactose gave double Maillard reaction products that were stable under mild alkaline conditions. Second, reductive amination of lactose followed by enzymatic oxidation gave cross-links that were stable under both neutral and alkaline conditions. Third, stable cross-links were obtained through enzymatic oxidation and amidation of lactobionic acid.

Keywords: Protein cross-linking; Lactose; Lactose dialdehyde; Galactose oxidase; Maillard reaction

1. Introduction

Cross-linking of proteins is an important method to modify their chemical, physical and functional properties.[1,2] Currently, protein cross-linking is generally achieved by: (i) chemical cross-linking with glutaraldehyde or formaldehyde and (ii) enzymatic cross-linking using transglutaminase.[3,4] The first method is rapid and inexpensive, but not allowed for food applications, whereas the second food-grade method is rather expensive and requires long reaction times or unfolding of the native proteins.[5] Therefore, a novel rapid and inexpensive enzyme mediated process for the preparation of a broad spectrum of cross-linked proteins for both food and non-food applications is highly desirable.

Carbohydrates are linked to proteins upon heating via the Maillard reaction, a common reaction in food processing.[6] The first step in the Maillard reaction is a condensation reaction between the aldehyde group of reducing sugars and the amino group of lysine residues of the protein, leading to glycosylation of the protein.[7] Oxidized carbohydrates containing two or more aldehyde groups can react with lysine residues of different proteins, leading to cross-linking. In recent studies, the proof of principle of galactose dialdehyde[8] and a range of other sugars[9] as a protein cross-linker has been described. Our attention is now focused on the use of lactose dialdehyde (LACTA; β-D-galacto-hexodialdo-1,5-pyranosyl-(1→4)-D-glucose) and other oxidized lactose derivatives as protein cross-linkers. 6′-Aldehydes of lactose derivatives can be conveniently generated by an oxidation with molecular oxygen catalyzed by the enzyme galactose oxidase.[10]

Cross-linking by LACTA and other lactose derivatives is especially interesting for the valorization of industrial whey protein mixtures, as they already contain lactose that can act as cross-linking agent upon oxidative enzymatic treatment in a cascade reaction.[11]

We investigated the potential of LACTA and other lactose derivatives as protein cross-linkers, using *n*-butylamine (H_2N–Bu) as the model compound, as it has the same structure as the lysine side chain. The structural and functional analysis of the model

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compounds gave more insight in the reaction mechanism, reaction conditions and product properties, which is essential for further investigation of protein cross-linking by lactose derivatives.

2. Results and discussion

2.1. Enzymatic oxidation and double Maillard reaction: lactose-based cross-linking

LACTA (**2**) was obtained by the selective oxidation of lactose (**1**) at the C-6′ position using the galactose oxidase/catalase [EC 1.11.1.6] enzyme system.[10,12] The reaction with *n*-butylamine at both aldehyde groups was followed by spontaneous dehydration at C-4′ and C-5′ to give di-imine **3** (Scheme 1). The straightforward enzymatic preparation of LACTA (**2**) and formation of its double Maillard product (**3**) with *n*-butylamine indicates its potential for protein cross-linking.

The ^{13}C NMR analysis of the reaction of LACTA with *n*-butylamine gave data that are characteristic for linkage to amines (Table 1). These characteristic NMR signals can be used as references to monitor protein coupling, especially when ^{13}C-enriched compounds are used.[15] The ^{13}C NMR spectra of lactose (**1**), LACTA (**2**) and di-imine **3** were assigned based on literature data of similar structures.[13]

The signal of C-6′ shifts from 61.2 to 88.6 ppm when lactose (**1**) is oxidized to LACTA (**2**, the aldehyde at C-6′ is in the hydrate form in water). The double reaction of LACTA (**2**) with butylamine leads to a shift of C-1 (β) from 96.2 to 91.7 ppm. The signal of the imine at C-6′ of **3** was found at 158.2 ppm.[8,14] In addition, due to the dehydration at C-4′/C-5′ signals appear in the vinylic region of **3** (115 and 148 ppm).

As shown by in situ ^{13}C NMR, the double linkage of *n*-butylamine to LACTA (**2**) proceeds in 2 h at 60 °C when excess *n*-butylamine is used. After evaporation of excess *n*-butylamine, pure di-imine **3** is obtained. The use of an organic solvent (methanol or ethylene glycol) and only 2 mol of *n*-butylamine gave similar results.

Table 1. Key ^{13}C NMR data of compounds **1**, **2**, **3**, **6**, **7**, **10** and **13**[a]

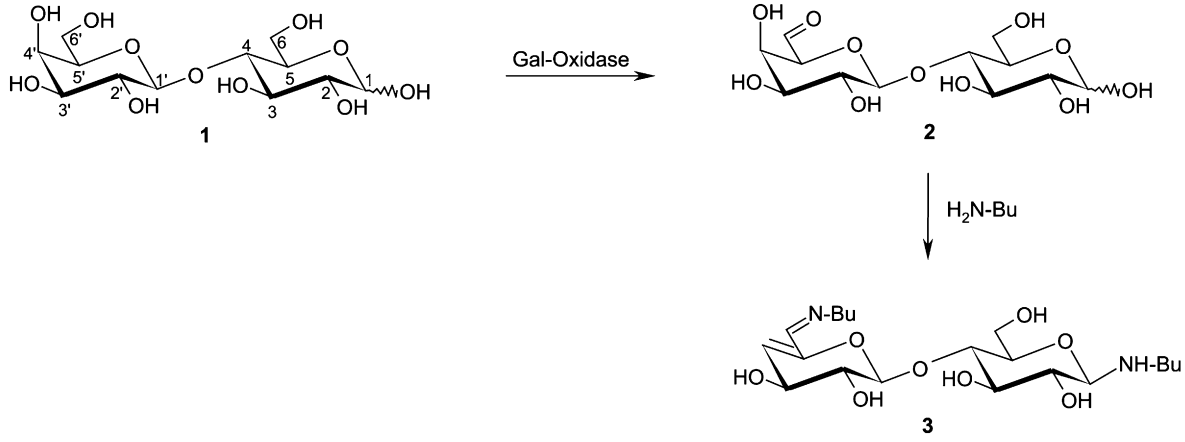
Compound	C-1	C-4′	C-5′	C-6′
1[b]	α: 92.0 (~40%) β: 95.9 (~60%)	68.7	75.5	61.2
2[b]	α: 92.3 (~40%) β: 96.2 (~60%)	68.5	77.5	88.6
3	α: not observed β: 91.7	114.8	148.1	158.2
6	50.5	114.8	147.8	159.7
7	50.1	106.5	146.0	56.6
10	175.0	114.1	147.7	158.8
13	175.1	114.2	147.6	159.0

[a] Data may vary slightly at different concentrations. Typical values were 298 K, 100 mg compound/mL CD_3OD.
[b] Dissolved in 20% D_2O/H_2O. Methanol was used as an internal reference ($\delta = 49.0$ ppm).

^{13}C NMR analysis also showed that the reaction at C-6′ is faster than at C-1 and that the dehydration at C-4′/C-5′ occurs readily after the formation of the imine at C-6′, similar to the dehydration of galactose dialdehyde with L-proline as described by Schoevaart and Kieboom.[15,16] The rate-limiting step in the formation of di-imine **3** is the amination reaction at C-1.

As mixtures of whey protein and lactose are obtained as aqueous solutions, the applicability of LACTA as cross-linker in water is of great interest. When 2.5 mol *n*-butylamine was added to an aqueous solution of LACTA, the formation of the imine at C-6′ was complete in 1 h at rt. The dehydration at C-4′/C-5′ and imine-formation at C-1 were found to proceed at a slower rate in water than in organic solvents, but were both completed after 24 h. These results show that the double Maillard reaction of LACTA can be performed in aqueous solutions, which enhances its practical applicability.

As glycosylamines are sensitive to hydrolysis in water,[17] the stability of di-imine **3** was determined.



Scheme 1. Formation of di-imine **3** by the double Maillard reaction of lactose dialdehyde with *n*-butylamine.

The product appeared to be stable in water under mild alkaline conditions. At neutral pH fast hydrolysis occurred at C-1, whereas the imine at C-6′ was not hydrolyzed. Apparently, the conjugated double bond at C-4′/C-5′ gives an extra stability to the imine at C-6′.

This difference in both reactivity and stability of the aldehyde functions at C-1 and C-6′ in LACTA allows manipulation of single or double Maillard products as desired.

As basic conditions are often undesired, the more stable amide- and amine-derivates of lactose were investigated for protein cross-linking under neutral or mildly acidic conditions.

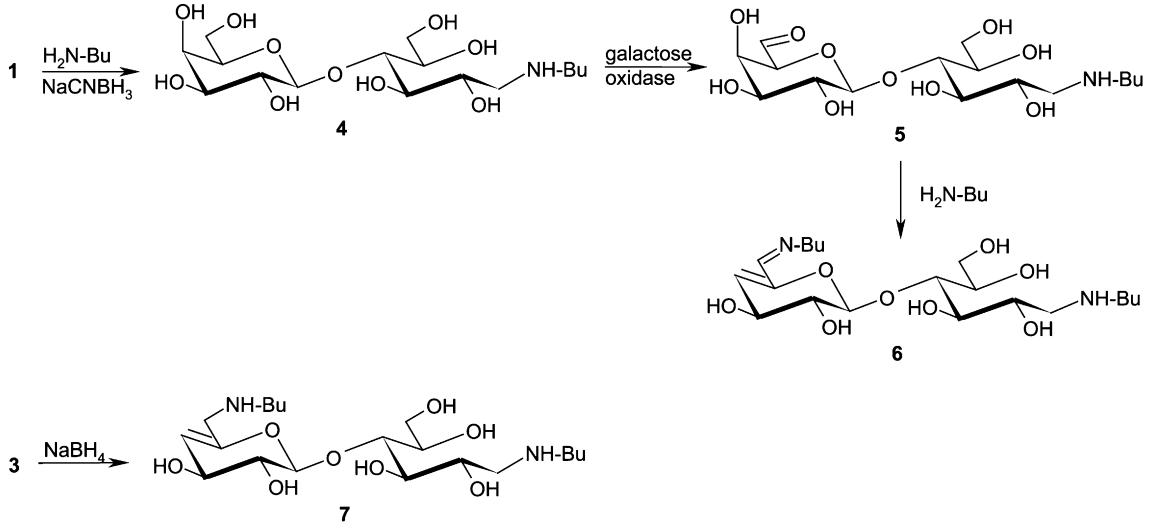
2.2. Reductive amination, enzymatic oxidation and Maillard reaction: lactylamine based cross-linking

Reductive amination[18,19] of lactose and *n*-butylamine with sodium cyanoborohydride yielded 1-butylamino-1-deoxylactitol **4** (β-D-galactopyranosyl-(1→4)-1-butylamino-1-deoxy-D-glucitol).[19] Subsequent enzymatic oxidation and reaction with *n*-butylamine gave imine **6** (Scheme 2), which is stable in aqueous solutions at neutral pH. Alternatively, the treatment of di-imine **3** with sodium borohydride gave diamine **7**, which is not sensitive to hydrolysis. These chemo-enzymatically obtained lactose derivatives may be useful alternatives for LACTA as protein cross-linker, especially for applications for which stability at neutral pH is desired.

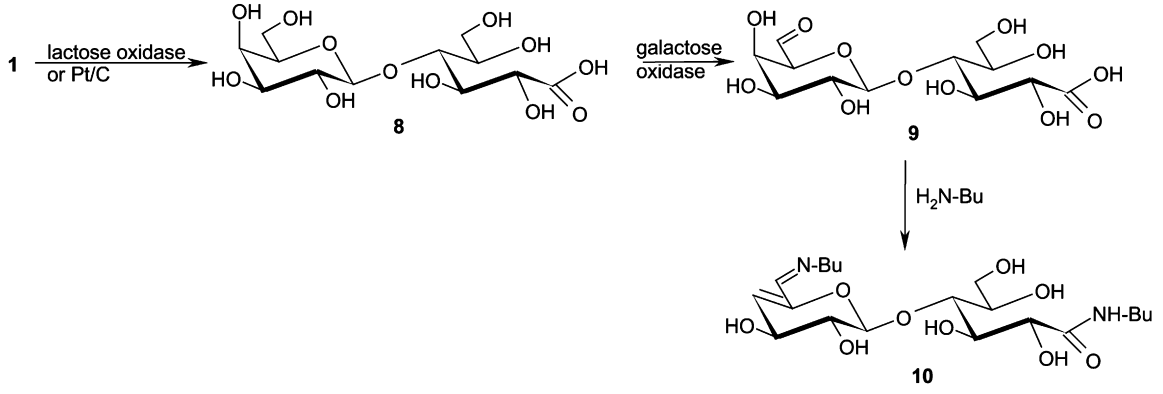
The NMR data of the characteristic positions of cross-linking products **6** and **7** is given in Table 1. The NMR signal of C-1 shifts to ~50 ppm upon reductive amination, the chemical shift value of the amine at C-6′ is slightly higher.[19,20]

2.3. Amidation, enzymatic oxidation and Maillard reaction: lactobionic acid based cross-linking

As amide bonds are stable in water under both basic and acidic conditions, the potential of lactobionic acid



Scheme 2. Chemo-enzymatic formation of lactylamine-based cross-linkers.



Scheme 3. Enzymatically oxidized lactobionic acid as a potential protein cross-linker.

(LBA; β-D-galactopyranosyl-(1→4)-D-gluconic acid, **8**) derivatives as protein cross-linkers was explored (Scheme 3). LBA can be obtained by enzymatic[21,22] or chemical[23,24] oxidation of lactose. The enzymatic oxidation of LBA gave lactobionic acid aldehyde (LBAA; β-D-galacto-hexodialdo-1,5-pyranosyl-(1→4)-D-gluconic acid, **9**),[10] which reacted with 2 equiv *n*-butylamine to give the double linked product **10**, containing the stable, conjugated imine at C-6′ and a stable amide bond at C-1.[25] The ^{13}C NMR shift of C-1 of amide (**10**) is 175 ppm, com- pared to 179 ppm for C-1 of lactobionic acid (Table 1).[25]

Longer cross-linking agents were obtained by starting with the reaction products from two LBA's with both 1,3-diaminopropane and 1,6-diaminohexane, using the procedure of Garelli-Calvet et al.[25] (Scheme 4). The obtained diamides (**11**) were enzymatically oxidized using galactose oxidase to give the corresponding C-6′ aldehydes (**12**). These aldehydes were linked to *n*-butylamine, which gave the stable C6′-imines in conjugation with the double bond at C-4′/C-5′(**13**), demonstrating the potential protein cross-linking ability of these oxidized LBA-diamides. In this way, diamines of different chain length can be applied, including functional groups that change the properties of the products, or can be used for further conversions.

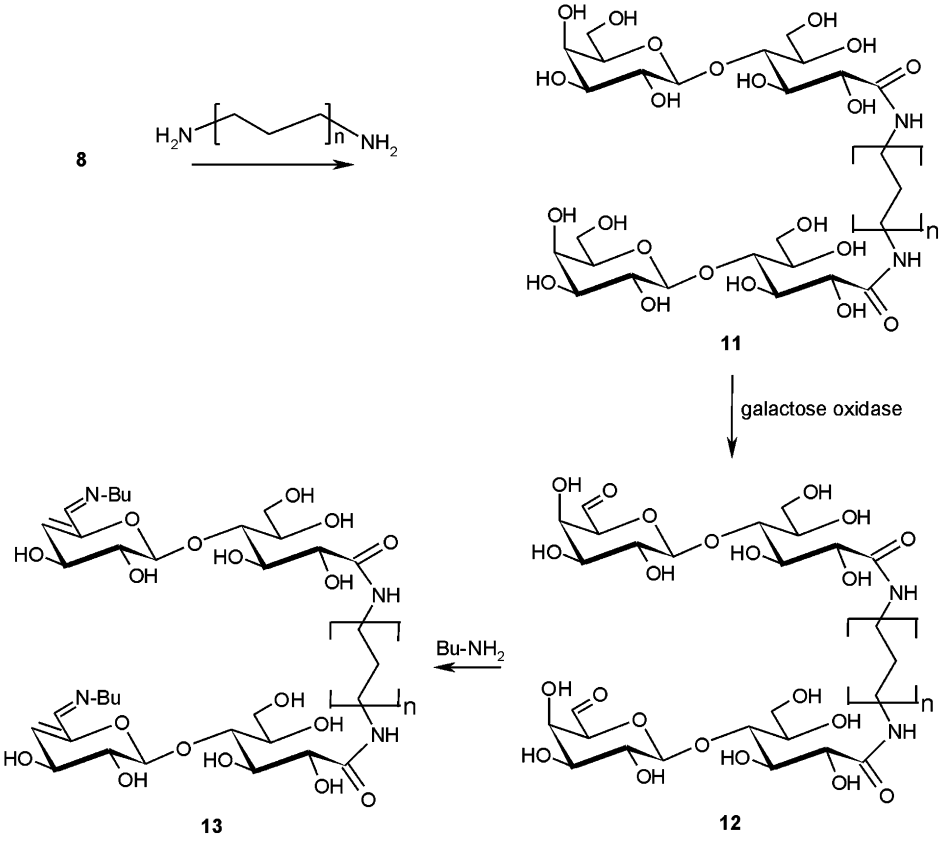
3. Experimental

3.1. General

^{13}C noise-decoupled NMR spectra were recorded with a Bruker DPX-300 spectrometer at 75.5 MHz and a Bruker DPX-400 at 100.7 MHz, using CH_3OH ($\delta = 49$ ppm) as an internal standard. All spectra were recorded in D_2O at 298 K, except where noted otherwise. All commercially available chemicals were purchased from Sigma–Aldrich, Acros or Fluka. All chemicals were used without further purification. Galactose oxidase (26 U/mg, D. dendroides) was a gift from Hercules (Barneveld, The Netherlands) and had the same specificity as the enzyme bought from Sigma. Catalase (2390 U/mg, Bovine liver) was purchased from Sigma–Aldrich. The products were analyzed by ^{13}C NMR without isolation or purification. All reactions showed quantitative conversion of the starting compound into the desired product, unless stated otherwise.

3.2. 6-Butylimino-4,6-dideoxy-β-D-galacto-hex-4-enopyranosyl-(1→4)-1-butylamino-1-deoxy-D-glucose (3)

In a 100 mL vial with a gas-tight Teflon cap lactose (**1**, 0.36 g, 1.0 mmol) was dissolved in 10 mL 0.1 M phos-



Scheme 4. Preparation of double lactobionic acid-derived cross-linkers ($n = 1, 2$).

phate buffer (pH 7.4) and 1 mg catalase and 10 mg galactose oxidase were added. Oxygen was applied for 30 s while the vial was shaken, and this procedure was repeated twice a day. The flask was shaken at rt for 2 days after which water was removed by freeze-drying. ^{13}C NMR showed that lactose dialdehyde (**2**) was obtained quantitatively. A suspension of lactose dialdehyde (**2**) in *n*-butylamine was stirred at 70 °C for 2 h. Subsequently, the excess *n*-butylamine was removed by evaporation in vacuo. ^{13}C NMR (CD_3OD) analysis showed a full conversion of lactose dialdehyde into di-imine **3**.

3.3. 6-Butylimino-4,6-dideoxy-β-D-galacto-hex-4-enopyranosyl-(1→4)-1-butylamino-1-deoxy-D-glucitol (6)

A suspension of lactose (**1**, 3.6 g, 10 mmol), propionic acid (1.45 g, 1.5 mL, 20 mmol) and *n*-butylamine (1 mL, 10 mmol) in 40 mL MeOH was refluxed for 1 h. A solution of sodium cyanoborohydride (0.8 g, 12 mmol) in 15 mL MeOH was slowly added to the resulting white solid. After reflux for 14 h in MeOH, the product was concentrated and washed with acetone. The obtained white powder was dissolved in 10 mL MeOH and 200 mL acetone was added while stirring the solution. The resulting precipitate was collected by filtration, washed with diethyl ether and dried under a nitrogen flow to give the propionic acid salt of β-D-galactopyranosyl-(1→4)-1-butylamino-1-deoxy-D-glucitol (**4**) in a quantitative yield. The product was analyzed with ^{13}C NMR in a DCl/D_2O solution (pH 1). In a 100 mL vial with a gas-tight Teflon cap **4** (0.47 g, 1.0 mmol) was dissolved in 20 mL 0.1 M phosphate buffer (pH 7.4) and 1 mg catalase and 20 mg galactose oxidase were added. Oxygen was applied for 30 s while the vial was shaken, and this procedure was repeated twice a day. The flask was shaken at rt for 2 days after which water was evaporated by freeze-drying, to give the desired product β-D-galacto-hexodialdo-1,5-pyranosyl-(1→4)-1-butylamino-1-deoxy-D-glucitol (**5**). To a solution of **5** in MeOH *n*-butylamine (5 mL) was added. After stirring at 70 °C for 2 h, MeOH and *n*-butylamine were evaporated and the resulting product **6** was analyzed with ^{13}C NMR (CD_3OD).

3.4. 6-Butylamino-4,6-dideoxy-β-D-galacto-hex-4-enopyranosyl-(1→4)-1-butylamino-1-deoxy-D-glucitol (7)

Sodium borohydride (0.1 g, 2.6 mmol) was added to a solution of lactose di-imine **3** (0.59 g, 1.36 mmol) in 30 mL water. The mixture was stirred for 16 h at rt and Dowex H^+ was added to remove the excess hydride. After filtration and evaporation of the filtrate, the product was recrystallized with MeOH/water to remove the borate salts. ^{13}C NMR analysis (CD_3OD) showed that a pure 6-butylamino-4,6-dideoxy-β-D-galacto-hex-4-enopyranosyl-(1→4)-1-butylamino-1-deoxy-D-glucitol (**7**) was obtained.

3.5. 6-Butylimino-4,6-dideoxy-β-D-galacto-hex-4-enopyranosyl-(1→4)-*N*-butyl-D-gluconamide (10)

Lactose (**1**, 0.63 g, 1.75 mmol) was dissolved in 25 mL 0.1 M KOH and 50 mg 10% Pt/C was added. After stirring for 5 h at rt under a nitrogen atmosphere, the solution was neutralized with Dowex H^+. The mixture was filtrated and freeze-dried to give lactobionic acid (β-D-galactopyranosyl-(1→4)-D-gluconic acid, **8**), as confirmed by ^{13}C NMR analysis. In a 100 mL vial with a gas-tight Teflon cap lactobionic acid **8** (0.6 g, 1.7 mmol) was dissolved in 20 mL 0.1 M phosphate buffer (pH 7.4) and 1 mg catalase and 20 mg galactose oxidase were added. Oxygen was applied for 30 s while the vial was shaken, and this procedure was repeated twice a day. The flask was shaken at rt for 2 days after which water was evaporated by freeze-drying, to give lactobionic acid aldehyde **9** (LBAA, β-D-Galacto-hexodialdo-1,5-pyranosyl-(1→4)-D-gluconic acid). A solution of LBAA **9** (0.4 g, 1.1 mmol) and *n*-butylamine (0.21 mL, 2.1 mmol) in 40 mL MeOH was refluxed for 24 h. The solvent was removed by evaporation in vacuo to give 6-butylimino-4,6-dideoxy-β-D-galacto-hex-4-enopyranosyl-(1→4)-*N*-butyl-D-gluconamide (**10**) as a brown solid. ^{13}C NMR analysis (CD_3OD) showed a small degree of degradation products beside the desired product **10**.

3.6. 1,3-Di-(6-butylimino-4,6-dideoxy-β-D-galacto-hex-4-enopyranosyl-(1→4)-*N*-butyl-D-gluconamido)propane (*n* = 1, 13)

A solution of lactobionic acid **8** (2.0 g, 5.6 mmol) and 0.22 mL diaminopropane (2.7 mmol) in 100 mL MeOH was refluxed for 24 h. After evaporation of the solvent and freeze-drying, 1,3-dilactobionamidopropane ($n = 1$, **11**) was collected and analyzed with ^{13}C NMR. In a 100 mL vial with a gas-tight Teflon 1,3-dilactobionamidopropane (**11**, 0.2 g, 0.25 mmol) was dissolved in 10 mL 0.1 M phosphate buffer (pH 7.4) and 1 mg catalase and 20 mg galactose oxidase were added. Oxygen was applied for 30 s while the vial was shaken, and this procedure was repeated twice a day. The flask was shaken at rt for 2 days after which water was evaporated by freeze-drying, to give the corresponding dialdehyde **12** (1,3-di-(β-D-galacto-hexodialdo-1,5-pyranosyl-(1→4)-D-gluconamido)propane, $n = 1$). A solution of **12** ($n = 1$, 0.2 g, 0.25 mmol) and *n*-butylamine (5 mL) in 40 mL MeOH was refluxed for 3 h. The solution was evaporated in vacuo to give 1,3-di-(6-butylimino-4,6-dideoxy-β-D-galacto-hex-4-enopyranosyl-(1→4)-*N*-butyl-D-gluconamido)propane (**13**). The product was characterized by ^{13}C NMR analysis (CD_3OD).

3.7. 1,6-Di-(6-butylimino-4,6-dideoxy-β-D-galacto-hex-4-enopyranosyl-(1→4)-*N*-butyl-D-gluconamido)hexane (*n* = 2, 13)

Using the same procedure as described above, lactobionic acid reacted with 2.1 equiv diaminohexane to give 1,3-dilactobionamidohexane ($n = 2$, **11**). Subsequently, dilactobionamidohexane (**11**, 0.2 g, 2.2 mmol) was converted quantitatively into the corresponding dialdehyde **12** [$n = 2$, 1,6-di-(β-D-galacto-hexodialdo-1,5-pyranosyl-(1→4)-D-gluconamido)hexane] via enzymatic oxidation with galactose oxidase. Using the same method as described above, **12** ($n = 2$, 0.2 g, 0.22 mmol) was converted into the corresponding di-imine **13** ($n = 2$), as confirmed by ^{13}C NMR analysis.

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