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Carbohydrate Research 337 (2002) 899–904

CARBOHYDRATE RESEARCH

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Galactose dialdehyde as potential protein cross-linker: proof of principle

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Received 24 October 2001; accepted 11 February 2002

Abstract

Combined enzymatic oxidation of D-galactose by D-galactose oxidase [EC 1.1.3.9] in water, amination with butylamine, and oxalic acid catalyzed Amadori rearrangement in methanol yielded 1,6-bis(butylamino)-1,6-dideoxy-*erythro*-hexo-2,5-diulose, demonstrating how in situ formed *galacto*-hexodialdose can be used to cross-link protein residues. The various species formed during this three-step conversion are present as bicyclic structures in solution as established by ^{13}C labeling and in situ NMR spectroscopy of the reaction mixtures. Using protein (gelatin) instead of butylamine, distinct Amadori product formation was observed using 99% enriched D-(1-^{13}C)- and D-(2-^{13}C)-galactose.

Keywords: *galacto*-Hexodialdose; Dialdehyde; Dialdose; Galactose oxidase; Amadori rearrangement; Maillard reaction

1. Introduction

General considerations.—Cross-linking of proteins finds wide application in, e.g., X-ray analysis, operational stability of enzymes in industrial processes (CLEC's[1] or CLEA's[2]), modification of donor heart valves, the preparation of wound dressers,[3] and in the modification of food—like the 'gluing' of pieces of meat with transglutaminase. The most widespread used cross-linker is glutaraldehyde (pentanedial) that reacts with the lysine amino groups of the protein. The bifunctional molecule is unsurpassed in cross-linking capacity, but unfortunately it is toxic. In our search for a potential food-grade analogue, a dialdehyde based on a sugar moiety was taken, with *galacto*-hexodialdose (GALA) as a first example (Scheme 1(2)). Such an analogue offers interesting possibilities since (1) sugars are plentiful and cheap; (2) enzymatically easy to modify; and (3) no toxicity of such an oxidized sugar derivative is expected. GALA can be obtained in one step by enzymatic oxidation of D-galactose (Scheme 1(1)) with molecular oxygen mediated by D-galactose oxidase[4] in the presence of catalase (to decompose hydrogen peroxide). In addition to D-galactose, many D-galactose containing sugars are substrates for D-galactose oxidase and could also serve as potential cross-linkers. These include lactose, raffinose,[5] galactan,[6] D-galactosylated cellulose,[7,8] lipopolysaccharides,[9] cell wall glycoproteins,[10,11] and other polymers.[12–15] The reason for this broad specificity is that the enzyme is able to oxidize terminal D-galactose units, after which the aldehyde group functionality in the polymer can undergo a variety of reactions.[16] This suggests that attachment of the D-galactose moieties to the protein prior to oxidation is a possible option for cross-linking.

In a recent study,[17] we established the solution structure, properties, and reactivity of GALA. We now report its reactivity towards butylamine as a model[18,19] for protein lysine moieties. In addition, D-galactose attachment to butylamine and gelatin (as an important industrial protein example) followed by enzymatic oxidation has been studied.

Mechanistic and analytical considerations.—The reaction of glutaraldehyde with an amine group involves the formation of an imine followed (or preceded) by aldol reactions.[20] Subsequent dehydration leads to stable unsaturated cross-links. The sugar-based counter-

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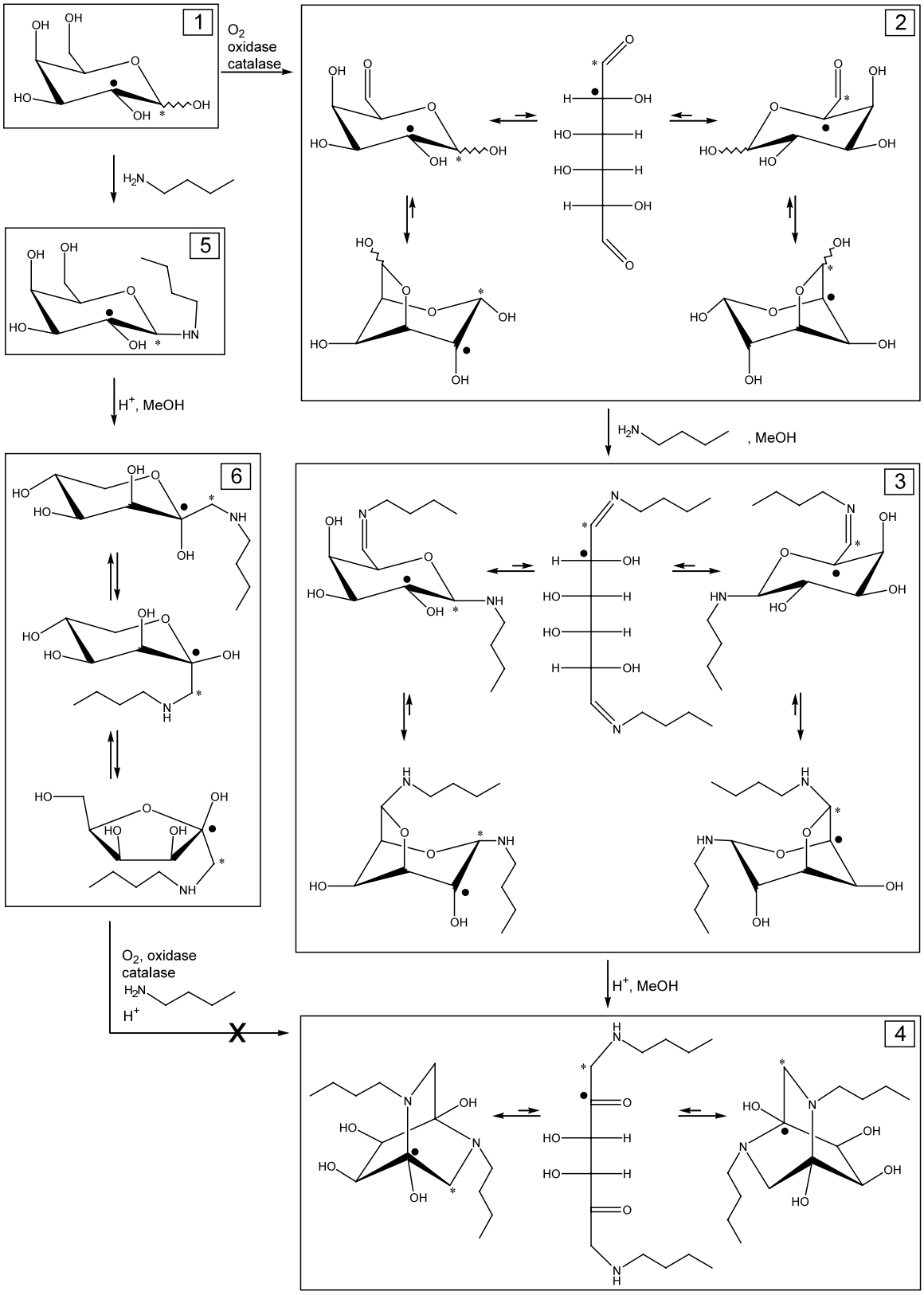
0008-6215/02/$ - see front matter
PII: S0008-6215(02)00051-4



Scheme 1. Combined three-step conversions of D-(1-^{13}C)- and D-(2-^{13}C)-galactose (**1**, ^{13}C labels indicated with * and ●, respectively) towards the racemic bicylic cross-link entities D,L-**4** using butylamine as the model for protein lysine moieties. The interconversion and composition of GALA (D,L-**2**),[17] the glycosylamines (D,L-**3** and D-**5**), and the Amadori products (D,L-**4** and D-**6**) are determined by the ^{13}C labeling composition using NMR spectroscopy (see Table 1).

part is known as the Maillard or browning reaction,[21,22] the reaction between aldose sugars and amino groups. At room temperature and neutral pH it takes place, albeit very slowly. Increase of pH and temperature speeds up the cascade of reactions from weeks to minutes, leading ultimately to advanced glycosylated end products. A variety of products (like those from glutaraldehyde reactions) is found because of a chain of successive reactions. This cascade can be avoided by controlled enzymatic attachment of the sugar to the protein, which is also referred to as glycation, which is well known and described.[23–25] The first step in the Maillard reaction is the reversible formation of a glycosylamine that can undergo a so-called Amadori rearrangement[26] to a 1-amino-1-deoxy-2-ketose, which will not hydrolyze under ambient conditions. A sugar dialdehyde like GALA therefore can, in principle, act as a protein cross-linker through double glycation and Amadori rearrangement reactions. To the best of our knowledge, dialdehydes have never been studied in Amadori rearrangements or Maillard reactions.

In a cross-linked protein the weight amount of GALA cross-linker would be small compared to that of the protein. To detect and to unravel the chemical structure of the cross-link ^{13}C D-galactose and NMR spectroscopy were used. With the use of both 99% enriched D-(1-^{13}C)- and D-(2-^{13}C)-galactose, information about four of the six carbon atoms is obtained because the open-chain structure of GALA is symmetrical (Scheme 1(2)); hence, the aqueous solution consists of equal amounts of D-(1-^{13}C)-GALA and L-(6-^{13}C)-GALA or D-(2-^{13}C)-GALA and L-(5-^{13}C)-GALA (denoted as (1,6-^{13}C)- and (2,5-^{13}C)-GALA, respectively). Reactions of GALA with amino groups have been studied with butylamine as a model for an elementary protein (resembling the side chain of lysine), which also allowed for the unlabeled positions C-3 and C-4 to be characterized. On the basis of the ^{13}C NMR chemical shifts of these simplified products, the detection and assignment of signals in galactose–protein reaction products is feasible. Although butylamine might at first sight seem rather different than a lysine residue or derivatives thereof, only the sugar carbons are observed, and since the electron density at the nitrogen atom is the same, good results can be obtained. Shorter alkylamines show distinct deviation in chemical shift. For the protein cross-link reaction, gelatin[27] was used that contained 4.4 wt% of lysine, i.e., approximately 20 mmol cross-linkable residues per 100 g of gelatin is available. Therefore, the cross-link 'concentration' is about 20 mM. This is sufficient to be detected by routine NMR analysis[28,29] when using 99% enriched D-(1-^{13}C)- or D-(2-^{13}C)-galactose.

2. Results and discussion

Formation of glycosylamines.—Although GALA is stable for several weeks in water at room temperature and neutral pH, increasing the pH to 10 causes serious degradation reactions. No structures can be identified in this mixture, not even with the help of ^{13}C-labeled sugars. This behavior is comparable with that of glutaraldehyde. Clearly, to prepare glycosylamines and Amadori products with GALA, non-aqueous conditions must be used. Alcoholic solvents are generally used to this purpose.[30] As GALA dissolves in too low a concentration in methanol or ethanol for ordinary NMR spectroscopy, the reaction with butylamine was initially carried out in ethylene glycol. Although ^{13}C NMR showed, as discerned by the abundance of signals compared to those in aqueous solutions, that GALA in ethylene glycol exists as oligomers, addition of butylamine clearly gave the expected glycosyldiamine **3** (Scheme 1(3)). This compound again has a bicyclic structure, as is evident from the L-(6-^{13}C) NMR signal, which is close to the D-(1-^{13}C) signal (**3**, GALA-(NHBu)$_2$, Table 1). In addition, even a minor amount of the imine form of **3** was observed as evidenced by the 162 ppm ^{13}C signals in the NMR spectrum. However, such a preparation is not entirely stable. After one day at room temperature, a substantial drop in signal intensity was observed, and after three days the compound had completely disappeared. Although ethylene glycol helped to dissolve the structure of the glycosyldiamine, possible acetal formation with the solvent after addition of oxalic acid discouraged its further use.

For D-galactose aqueous basic conditions did not pose problems for the preparation of *N*-butyl-D-galactosylamine (**5**, GALNHBu, Scheme 1(5)) which completely occurs in the β-pyranose structure. The glycosylamine could also be prepared in methanol or ethanol since the solubility of D-galactose was much better than that of GALA. With the help of D-(1-^{13}C)- and D-(2-^{13}C)-galactose, the formation of the glycosylamine with gelatin (**7**, β-Gal-gelatin, Table 1) in water can be observed under alkaline conditions (pH 10). The amount of cross-linkable lysine residues present in gelatin was found to be about 30%, based on the proportion of D-(1-^{13}C)-galactose and D-(1-^{13}C)-galactosylamine signals.

Formation of Amadori products.—The use of standard Amadori conditions for aliphatic amines (methanol in the presence of oxalic acid at 50 °C for two days) resulted in complete degradation of the glycosyldiamine of GALA (**3**, Scheme 1(3)). At room temperature for one day, a significant amount of double Amadori product **4** had already formed (Scheme 1(4)). It turned out to have a complete symmetrical configuration, as only one ^{13}C NMR signal per label was found in the D,L-mixture (**4**, GALU-(NHBu)$_2$,

Table 1). This bicyclic molecule reflects the structure of the cross-links formed by GALA with lysine moieties of proteins under ambient conditions, and ultimately serves as proof of principle for dialdose based cross-linking. Since the yield was still low ($\sim 5\%$), it was impossible to determine the C-3 and C-4 chemical shifts (natural abundance) or to repeat the experiment with gelatin, given that the cross-link concentration would be 1 mM, which is below the detection limit.

Heating of *N*-butyl-D-(1- and 2-^{13}C)-galactosylamine (**5**) under the above-mentioned standard conditions gave complete conversion to its Amadori product *N*-butylamino-1-deoxy-D-tagatose (**6**, Scheme 1(6)). The chemical shifts (**6**, Tag-NHBu, Table 1) are in excellent agreement with those of the product formed from D-(1-^{13}C)- or D-(2-^{13}C)-galactose and gelatin (**8**) under comparable conditions. The structural information obtained from reactions with butylamine can therefore be used to describe the reaction products with gelatin. In order to prepare gelatin Amadori products, the glycosylamine had to be precipitated from a basic solution and suspended in alcohol for acidic treatment.

Oxidation of Amadori products.—As D-galactose forms Amadori products with gelatin (**8**, α,β-Tag-gelatin, Table 1) and D-galactose oxidase also oxidizes terminal D-galactose units of polymers, first reacting D-galactose with the protein (towards Amadori moieties of type **6**), followed by enzymatic oxidation of the 6-CH_2OH, would be an alternative cross-linking method (towards moieties of type **4**). However, *N*-butylamino-1-deoxy-D-tagatose (**6**) did not show any detectable conversion by D-galactose oxidase. The Amadori products of D-galactose are apparently no substrates for D-galactose oxidase. The favorable pyranose forms of **6** make the 6-CH_2OH unavailable for D-galactose oxidase, while the available 6-CH_2OH in the furanose form of **6** (estimated 13% from NMR spectroscopy, Table 1) apparently has a too low a reactivity due to the butylamino moiety and the decreased ring size. A parallel can be drawn with D-tagatose. This ketose derivative of D-galactose has only 0.5% of the activity[31] of the natural substrate for D-galactose oxidase. Obviously, the extra steric hindrance of the alkyl tail is detrimental for the remainder of the enzyme activity. So, only O-galactosylation of the protein remains for this approach in order to safeguard the right galactose configuration for good enzymatic activity.

3. Conclusions

Labeling with ^{13}C and the use of NMR spectroscopy proved to be a viable method to in situ analyze the three-step conversion of the D-galactose. Galactose dialdehyde (GALA) and its reaction products had all bicyclic structures in solution. The synthesis of the bicyclic double Amadori product from GALA and butylamine is the proof of principle of dialdose-based cross-linking of proteins. Further support is obtained by the formation of Amadori products from the reactions of D-galactose with both butylamine and gelatin. As the mono Amadori product from D-galactose and butylamine could not be oxidized by D-galactose oxidase, further investigations are in progress with lactose as the cross-link moiety, since the stability of this dialdehyde under basic conditions is found to be better than GALA, and the D-galactose unit is unchanged during the Amadori rearrangement of the glucose unit. Now, post-Amadori oxidation of the galactosyl 6-CH_2OH is much more feasible.

Table 1
^{13}C NMR composition and chemical shifts of compounds **2**–**8** [a]

Compound	% [b]	C-1	C-2	C-3	C-4	C-5	C-6
2 α-GALA[17]	37	88.61	68.37	73.46	69.27	72.28	92.35
2 β-GALA[17]	63	88.35	71.86	72.86	68.34	76.92	96.66
3 GALA-$(NHBu)_2$	99	93.43	67.54	83.24	72.19	80.59	94.01
3 GALA-$(NHBu)_2$ imine	1	162.80 162.34					
4 GALU-$(NHBu)_2$	100	51.74	76.15			76.15	51.74
5 GalNHBu β-pyr	100	90.36	70.39	75.15	71.99	77.18	62.48
6 Tag-NHBu α-pyr	32	52.50	95.78	65.51	70.47	69.05	60.20
6 Tag-NHBu β-pyr	55	52.79	95.41	71.99	70.59	65.88	62.70
6 Tag-NHBu β-fur	13	56.60	103.53				
7 β-Gal-gelatin	100	90.29	70.83				
8 α-Tag-gelatin	30	52.74	95.97				
8 β-Tag-gelatin	70	52.94	95.47				

[a] δ in ppm, with methanol as internal standard (49 ppm); **2**, **4**, **6**, and **8** in 10 mM P_i buffer (5% D_2O), pH 7.3; **3** in ethylene glycol; **5** and **7** in water, pH 11.
[b] Proportion of isomers.

4. Experimental

General.—NMR experiments were performed with DPX-300 spectrometers (Bruker, Karlsruhe, Germany) at 75.48 MHz for ^{13}C with MeOH as internal standard (49 ppm) and 5% D_2O or CD_3OD. These additives do not affect enzymatic reactions. 99% enriched D-(1-^{13}C)- or D-(2-^{13}C)-D-galactose were purchased from ARC Technologies (SIL Cambridge). D-Galactose oxidase was a gift from Hercules (Barneveld, The Netherlands), which had the same specificity as that from Sigma. All other enzymes and chemicals were purchased from Sigma.

galacto-*Hexodialdose (GALA*, **2***): typical procedure.*—In an 8-mL vial with a gas-tight Teflon cap, 9 mg of D-(1-^{13}C)- or D-(2-^{13}C)-galactose (0.05 mmol, 50 mM) was added to 1 mL of 10 mM phosphate buffer, pH 7.3, containing 1 mg (26 U) of D-galactose oxidase (26 U/mg) and 1 mg catalase (2390 U/mg). Oxygen was applied for 30 s while the vial was shaken. This was repeated after 1 day. The flask was shaken at rt for 2 days.

Labeled GALA (**2**)*: large scale procedure.*—In a 250-mL flask fitted with a septum, 90 mg of D-(1-^{13}C)- or D-(2-^{13}C)-galactose (0.5 mmol, 50 mM) was dissolved in 10 mL of 10 mM phosphate buffer, pH 7.3. Then 5 mg (130 U) D-galactose oxidase (26 U/mg) and 5 mg catalase (2390 U/mg) was added, and oxygen was applied for 3 min. After 2 days the solution was concentrated by freeze drying. The enzymes were filtered off with a 10 kDa Ultrafree centrifugal filter (Amicon®). The final volume was 1 mL, corresponding to a 0.5 M solution of GALA.

*Bis(*N*-butyl)-β,α-*D-galacto-*hexodialdo-6,3-furanosyl-1,5-pyranosyldiamine (GALA-(NHBu)$_2$,* **3***).*—A 2-mL solution of 50 mM 1,6- or 2,5-^{13}C-GALA was freeze dried, and 1 mL of dry ethylene glycol was added. After complete solvation, 17 μL of butylamine (0.2 mmol) was added, and the sample was immediately analyzed with NMR spectroscopy.

D,L-*1,6-Bis(butylamino)-1,6-dideoxy-*erythro-*hexo-2,5-diulose (GALU-(NHBu)$_2$,* **4***).*—To a solution of **3** in MeOH, 9 mg (1 mmol) of oxalic acid was added. After 2 days at rt, the solution was pale yellow. The yield estimated from NMR spectroscopy was about 5%.

N-*Butyl-*D-*galactosylamine (Gal-NHBu*, **5***).*—In a 50-mL Erlenmeyer flask, 1.8 g (10 mmol) of D-galactose was added to 1.24 mL (12.5 mmol) of butylamine, and the mixture was heated to 50 °C until a yellow melt was formed. Then 15 mL of 2-propanol was added, and after warming 15 mL of petroleum ether (bp 40–60 °C) was gradually added, and crystals were allowed to form slowly. After washing with more petroleum ether, the crystals were collected and dried. The yield was quantitative. ESIMS: m/z 236 (100%, $[M + H]^+$). Anal. Calcd for $C_{10}H_{21}NO_5$: C, 51.05; H, 9.00; N, 5.95. Found: C, 51.39; H, 8.94; N, 5.89.

N-*Butylamino-1-deoxy-*D-*tagatose (Tag-NHBu*, **6***).*—To 1 mmol of glycosylamine, 3 mL of MeOH was added, as well as 1.1 mmol of dry oxalic acid. The solution was stirred for 2 days at 50 °C. Evaporation of the MeOH gave a quantitative yield of **6** as its oxalic acid salt. ESIMS: m/z 235.97 (100%, $[M + H]^+$). Anal. Calcd for $C_{12}H_{23}NO_9 \cdot 16\ H_2O$: C, 40.70; H, 7.46; N, 3.96. Found: C, 40.62; H, 7.54; N, 3.86.

Small-scale procedure for labeled Tag-NHBu (**6**).—In an 8 mL vial with a gastight Teflon cap, 18.0 mg (0.1 mmol) of D-(1-^{13}C)- or D-(2-^{13}C)-galactose was added to 1 mL of dry MeOH and 10 μL (0.1 mmol) of butylamine. The mixture was stirred for 1 h at 60 °C, after which time all the D-galactose had dissolved. During this treatment no coloring was observed. Then 9 mg of oxalic acid was added, and stirring was continued for 2 days at 50 °C. NMR spectroscopy (in MeOH) showed no residual galactose, indicating quantitative conversion.

N-*Gelatinyl-β-*D-*galactopyranosylamine (β-Gal-gelatin*, **7***) and* N-*gelatinyl-α,β-*D-*tagatopyranosylamine (α,β-Tag-gelatin*, **8***).*—To 9 mg (50 mM, 0.05 mmol) of D-(1-^{13}C)- or D-(2-^{13}C)-galactose, 100 mg of gelatin was added in 1 mL of water at pH 11. After complete solvation of the gelatin, EtOH was added (MeOH gave a very sticky precipitate) until no more precipitate was formed. The pellet was centrifuged and washed with EtOH and centrifuged again. Then 1 mL of EtOH and 4.5 mg (0.05 mmol) of oxalic acid were added. The suspension was stirred at 50 °C for 2 days. After evaporation of the EtOH 50 mM phosphate buffer, pH 7.3, and D_2O were added for NMR analysis.

Oxidation of N-*butylamino-1-deoxy-*D-*tagatose* (**6**).—Compound **6** (23.5 mg, 0.1 mmol) was resolved in 1 mL of pH 7.3 10 mM phosphate buffer, and 1 mg (26 U) of D-galactose oxidase (26 U/mg) and 2 mg of catalase (2390 U/mg) were added. Oxygen was applied, and the vessel was shaken gently for 3 days. NMR spectroscopy did not show any change in the spectrum, and addition of butylamine (10 μL, 0.1 mmol) also did not indicate any aldose by formation of an imine.

NMR ^{13}C shift calculations of GALA.—The ^{13}C chemical shift calculations aiding assignment of GALA peaks have been performed using the GIAO method as implemented in GAUSSIAN98, within the density functional theory framework, with the B3LYP exchange-correlation functional and a 6-31G (d, p) basis set. TMS absolute shielding was 187.5 ppm.

Acknowledgements

The authors express their gratitude to Fons Lefeber who assisted with the acquisition of the NMR spectra

and Sylvia Touw who performed the chemical shift calculations. DSM is acknowledged for financial support and Hercules for their generous gift of D-galactose oxidase.

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