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Rapid Communication

Galactose dialdehyde: the forgotten candidate for a protein cross-linker?

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Abstract

Oxidation of D-galactose mediated by D-galactose oxidase [EC 1.1.3.9] gave a dialdehyde that occurs as two bicyclic structures in aqueous solution as established by ^{13}C labeling and NMR spectroscopy. The dialdehyde is very reactive at basic pH leading to complex reaction mixtures. A comparison was made with glutaraldehyde to assess its potential as protein cross-linker. © 2001 Published by Elsevier Science Ltd.

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The well known and most frequently used protein cross-linker, glutaraldehyde (GA), appears in over 4500 publications, while its sugar analogue, galactose dialdehyde (D-*galacto*-hexodialdose, GALA), is represented in only a few publications. None of these reports mention its use as a protein cross-linker, and essentially no information is presented on its solution structure and properties. GALA can be generated from D-galactose by oxidation with molecular oxygen catalyzed by the enzyme, D-galactose oxidase,[1] which is produced by molds such as *Polyporus circinatus*[2–4] and *Dactylium dendroides*. As GALA has the potential of being a food-grade cross-linking agent that can be prepared in situ through the addition of D-galactose oxidase and D-galactose, we have first studied its solution structure and properties by the use of ^{13}C labeling and NMR spectroscopy. A GA analogue based on a sugar offers interesting possibilities: (1) Sugars are plentiful and cheap. (2) It is enzymatically easy to modify; and (3) biodegradability of such an oxidized sugar derivative is expected to be a desirable property.

The properties of GALA are largely unknown. Synthetic applications are customarily based on reactions in situ with the dialdehyde, without prior isolation. This is in part due to its reactive nature, which makes isolation impracticable. With the aid of ^{13}C NMR spectroscopy, analysis in situ of GALA's structure in aqueous solutions is feasible, making isolation unneeded. Consequently, untimely reactions are avoided, and the door for examination of complex reaction products is opened. For a further increase in intensity of carbon signals, most preparations were mildly

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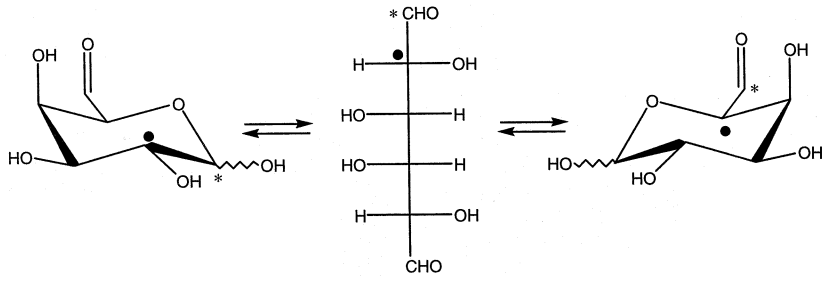
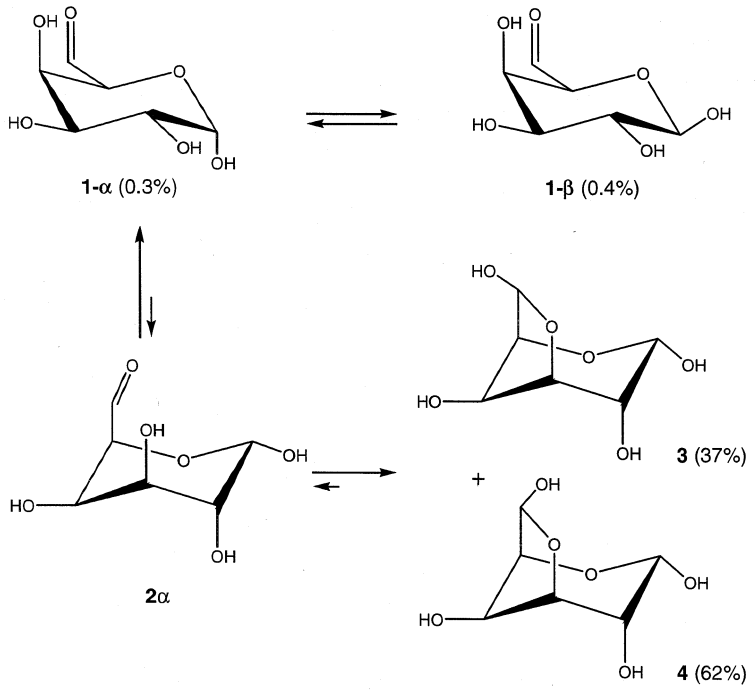


Scheme 1. Formal pyranose and open-chain forms of GALA. Oxidation of D-(1-^{13}C)-galactose gives a mixture of D-(1-^{13}C)- and L-(6-^{13}C)-GALA pyranose forms (indicated with *). The same is indicated for D-(2-^{13}C)-galactose towards D-(2-^{13}C)- and L-(5-^{13}C)-forms (indicated with •).



Scheme 2. Interconversion and composition of GALA structures in water (only one of the enantiomeric forms depicted).

concentrated up to 0.5 M. The sugar dialdehyde has a prominent property: it is symmetrical. So with the use of both D-(1- and 2-^{13}C)-galactose, information about atoms C-5 and C-6 is also obtained (Scheme 1).

Structure.—Preparation of the dialdehyde is a straightforward enzymatic reaction. Formation of galactonic acid as a byproduct has been reported on occasion in literature, but its formation was not observed in our hands, and it must therefore arise from contamination with other oxidases such as glucose oxidase (for which D-galactose is a substrate).

The spatial structure GALA is more puzzling, however (Scheme 2). From measurements of chemical shifts (Table 1) of labeled (1,6- and 2,5-^{13}C)[†] and natural-abundance GALA, signals are found that are typical for α- and β-pyranose forms (major ~63% and a minor ~37%). Most striking is the D-galactose C-6 value of ~62 ppm that has disappeared, with the appearance of a new resonance at 88 ppm, indicating the formation of a hydrated aldehyde or a carbon in a glycosylic bond. Although this seems, at first sight, appealingly straightforward, we have found proof that the C-6 of GALA is not associated

[†] For simplicity, the mixture D-(1-^{13}C)-GALA and L-(6-^{13}C)-GALA is denoted as 1,6-^{13}C GALA; the mixture D-(2-^{13}C)-GALA and L-(5-^{13}C)-GALA as 2,5-^{13}C-GALA.

Table 1
^{13}C chemical shift (δ, ppm) of GALA (0.5 M) in 100 mM P_i buffer (5% D_2O), pH 7.3 [a]

Compound	C-1	C-2	C-3	C-4	C-5	C-6
Structure **1α** (0.3%)						201.24
Structure **1β** (0.4%)						200.39
Structure **3** (37%)	88.61 [b,d]	68.37 [c,d]	73.46 [d]	69.27 [d]	72.28 [c,d]	92.35 [b,d]
Calculated **3** [e]	87.3	66.6	81.1	70.8	73.7	96.0
Structure **4** (62%)	88.35 [b,d]	71.86 [c,d]	72.86 [d]	68.34 [d]	76.92 [c,d]	96.66 [b,d]
Calculated **4**[e]	87.4	66.7	79.1	69.7	75.6	96.7

[a] Internal standard: MeOH = 49 ppm.
[b] 1,6-^{13}C enriched.
[c] 2,5-^{13}C enriched.
[d] Natural abundance.
[e] GALA's highest chemical shift (96.66 ppm) was used for calibration.

with the signal at 88 ppm. To determine this, the relationship with some facts from the literature has to be acknowledged first. In a quantitative study,[5] it was found that less than 0.05% of an aldopentose in water is in the free aldehyde form, and less than 0.1% is in the hydrated form. In the ^{13}C NMR spectrum of 1,6-^{13}C-enriched GALA, some free aldehyde can indeed be found (estimated 0.7%), whereas the hydrated form appears in the region round 95 ppm where many small peaks are found from, for example, the α- and β-furanose forms, making identification impossible. The majority of the carbons are thus not associated with the free aldehyde form, and the amount of hydrated aldehyde is expected to be of the same order of magnitude. Clearly, also acetalization of the second aldehyde moiety by one of the hydroxyl groups has taken place. In this respect, only the hydroxyl group on C-3 is positioned favorably for acetal formation after inversion of the α-pyranose chair form **1-α** into **2-α**. This inversion is followed by fast ring closure to the bicyclic sugars **3** and **4** (Scheme 2). Chair inversion of **1-β** can be ruled out due to 1,3,5-triaxial repulsion in the inverted chair. Apparently, only two of the four possible bicyclic GALA structures are present as the ^{13}C NMR data show only two sets two of peaks for the anomeric carbon atoms (Fig. 1(A) shows a mixture of 1,6-^{13}C and 2,5-^{13}C enriched GALA). This means that only one of the two anomeric carbons actually has a hydroxyl group in both the α- and β-form. Molecular models show that an axial hydroxyl group at C-1 is sterically hindered by the C-3–O–C-6 bridge and gives 1,3-dipolar repulsions with the C-1 and C-3 oxygen atoms.[6] The hydroxyl group on C-6 is relatively free to be in either position, giving rise to both α- and β-forms, although the 1,3-dipolar interaction makes structure **4** more favorable than **3**.

With the aid of labeled and natural-abundance GALA, a pre-assignment in three sets of two peaks could be established. For a total assignment of the NMR signals, chemical shift calculations of structure **3** and **4** were performed[7] using GAUSSIAN98. The calculated values for C-1 and C-6 (Table 1) are 87.4 and 96.7 ppm, respectively. (GALA's highest chemical shift (96.66 ppm) was used for calibration.) The major isomer with 1,6-^{13}C labels has measured values of 88.30 and 96.66 ppm. With the difference between C-1 and C-6 being 8.3 ppm, reversed assignment is highly unlikely. The lower shift can thus be assigned to C-1, and the higher shift to C-6. Although calculation of the C-6 shift did not show much difference between that for the α- and that for the β-glycosyl species, the signal at 92.35 ppm must be associated with it. For C-2 and C-5 the assignment is now set and a distinction can again be made between the two carbon atoms of the peak set without the chance of switching them. The correspondence is fairly good. Left behind are C-3 and C-4. Although the distinction is apparent again, the calcu-

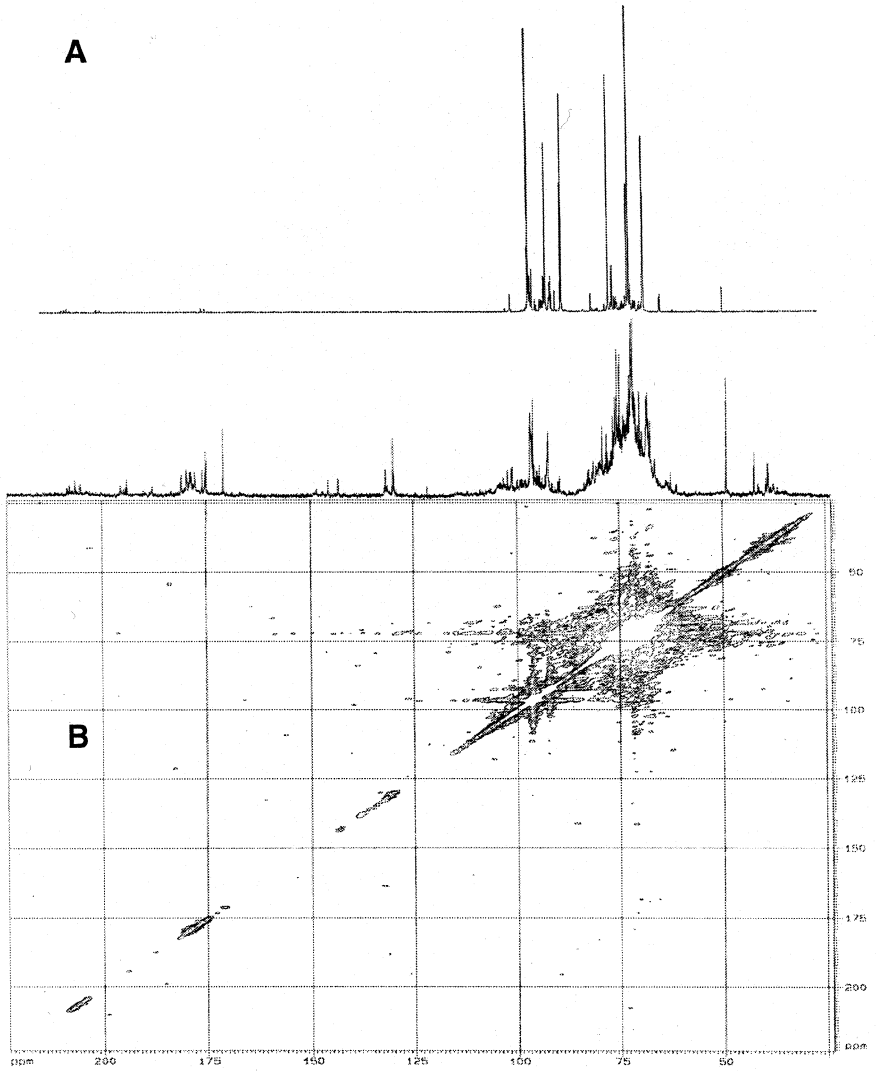


Fig. 1. ^{13}C NMR spectra of a 0.5 M 1,6-^{13}C and 0.5 M 2,5-^{13}C enriched GALA (**A**) and its product mixture after treatment for 1 h at pH 10 at rt (**B**), in the $^{13}C-^{13}C$ COSY no proof of aldol products was found. $CH_3OH = 49$ ppm (internal standard).

lated value for C-3 is quite in error. However, only differentiation between the higher and the lower chemical shift is needed, which makes the fit for C-4 quite good. Altogether, pre-assignment with labeled and natural-abundance GALA makes a total assignment with the aid of chemical shift calculation possible.

An alternative for the bicyclic structure of GALA is the formation of acetals via one hydrated aldehyde function similar to that of GA.[8] In order to undergo this acetalization, GALA must form a seven-membered ring. Formation of a seven-membered ring is possible (albeit less probable than the formation of a pyranose ring) by acetalization of a hydrated aldehyde group with the other aldehyde group. Additionally seven-membered rings are formed when the aldehyde forms a hemiacetal with a hydroxyl group of a second dialdehyde molecule, giving rise to polymers. A model of hydrated GALA shows that all the hydroxyl groups can be in equatorial positions giving rise to a very favorable conformation which is, however, symmetrical and thus in disagreement with the large difference in chemical shift between the peaks in the three peak pairs. It has been reported[9] that GALA can form intermolecular acetal structures upon drying. In aqueous solution there is no proof for the formation of such dimers, which share a substituted dioxane ring. With electrospray mass spectroscopy evidence for such a hydrated dialdehyde was also not found. Another proof of change in conformation after

oxidation is reflected by glucose oxidase, for which D-galactose is a substrate and GALA is not.

Reactivity.—To exclude Amadori rearrangements and Maillard reactions as pathways for newly formed products, D-galactose oxidase and catalase — and thereby reactive amino groups — were removed by membrane filtration. In the 2,5-^{13}C labeled GALA NMR spectrum, four peaks were found at $\sim$209 ppm[‡] ($<1\%$). It is likely that some enolization took place, giving rise to carbonyl carbons at C-2 and C-5. Immediately after oxidation, small amounts of products were detected with chemical shifts of $\sim$70 ppm. The amount increased only slowly on storage of the dialdehyde at pH 7.3 and room temperature. Brief heating (45 min at 80 °C), however, gave complete conversion of the initial oxidation product into a spectrum of products. NMR analysis showed the presence of at least 30 new signals, all of which emerged from a single ^{13}C label. Elevation to pH 10 gave the same results in 20 min at room temperature. No reverse reaction was observed (at room temperature or after readjustment of the pH), and the newly formed products appeared to be stable. At pH 6 heating did not lead to new product formation. To explain these findings it can be expected that GALA has undergone dehydration and aldol reactions, in parallel with the behavior of GA. The latter is known to undergo dehydration forming unsaturated bonds and rapid polymerization in weakly alkaline solutions.

To find proof for aldol reactions, a concentrated mixture was prepared containing both 1,6- and 2,5-^{13}C GALA (Fig. 1(A)). This mixture was subjected to the base treatment (Fig. 1(B)). An aldol reaction would imply an α-C (C-2) attack on the carbonyl (C-1) giving rise to adjacent ^{13}C atoms. This combination would result in cross-peaks, which can be detected with ^{13}C–^{13}C COSY NMR spectroscopy. When the GALA molecules combine, the 'yield' of coupled atoms would be 12.5% maximum; it could be higher when the aldol reactions continue. Examination of the ^{13}C–^{13}C COSY (Fig. 1(B)) spectrum revealed that the contribution of aldol reactions between C-1 and C-2 to the sum of products created by the basic conditions is less than the estimated detection limit of 0.5%, since no cross-peaks were found. Still, there is a possibility that carbon–carbon bond forming reactions occur, but it must then take place between other carbons, e.g., a carbonyl and a double bond (unlabeled C-3 or C-4) from a dehydrated (α,β-unsaturated) GALA molecule. Additionally the occurrence of retroaldol reactions that ultimately led to racemic sugars can also be excluded. Evidence for GALA dehydration is very clear. Multiple NMR signals around 180 and 40 ppm (even proton carbons in APT) proved loss of water, which results (after enol–keto tautomerization) in the formation of a carbonyl and an aliphatic carbon atom with expected chemical shifts in accordance with the values found. These signals appeared in both 1- and 2-^{13}C-labeled GALA reaction products, but 1-C gave much stronger intensities and more peaks for both the carbonyl and aliphatic regions. Prolonged heating did not result in complete dehydration. Elimination of one, two or three molecules of water all yield products for which there were carbon signals corresponding with peaks found in the NMR spectra. Taking into account the distribution of product peaks, up to 30 different structures might be present.

In conclusion, the use of labeled D-galactose allowed assignment of all galactose dialdehyde (GALA) carbons and proved that aqueous GALA exists in two bicyclic structures that must be formed by inversion of the D-galactose pyranose ring. Furthermore, the use of ^{13}C labels made it possible to examine complex reaction products from GALA. By comparing the chemical properties of GALA with glutaraldehyde (GA), it became clear that the two molecules display similarities in properties. Both molecules underwent reactions under basic pH that led to dehydration, and NMR spectroscopy confirmed formation of carbonyl and aliphatic carbon atoms. Proof of aldol reactions was not found, however. In contrast with GA, GALA reaction products are perfectly water soluble due to the remaining hydroxyl groups. Cross-linking capabilities (interconnection of protein amine residues) of GALA are currently under investigation.

[‡] Resonances at 210.45, 209.92, 209.17 and 208.45 ppm.

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