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Oxidation and metal-ion affinities of a novel cyclic tetrasaccharide☆

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Abstract

The cyclic tetrasaccharide, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}, was oxidized in high yield to a dicarboxylic acid, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→}. The parent and oxidized compound were then screened for the ability to form stable complexes with 20 metal cations. Ion-exchange thin-layer chromatography was utilized to survey binding in aqueous and 50% methanolic solutions. The screening identified Pb^{2+}, Fe^{2+} and Fe^{3+} as forming strong metal chelates with the oxidized cyclic tetrasaccharide. The stoichiometry of the oxidized cyclic tetrasaccharide and Pb^{2+} complex was determined to be 1:1 using aqueous gel-permeation chromatography. Perturbations between the free and complexed structure were examined using NMR spectroscopy. Molecular simulations were used to identify a probable structure of oxidized cyclic tetrasaccharide complexed with Pb^{2+}.
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Keywords: Cyclic tetrasaccharide; TEMPO oxidation; Pb chelator; Pb binding; Oligosaccharide oxidation

1. Introduction

The novel cyclotetrasaccharide, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (CTS), is produced by hydrolyzing alternan by the enzyme alternanase.[1–3] Alternan is a dextran-like polysaccharide composed predominantly of an alternating sequence of α-1,3-linked and α-1,6-linked D-glucose units[4] and is produced from sucrose by alternansucrase from *Leuconostic mesenteroides*.[5] CTS has also recently been synthesized by two novel glycosyltransferases from *Bacillus globisporus* C11 from maltooligosaccharides[6] and starch.[7]

The unique structure and properties of *cyclo*-oligosaccharides has led to their use in many applications, such as drug delivery systems, foods, cosmetics, packaging and textiles.[8–12] The properties and potential applications of CTS have yet to be studied in any detail. The crystal structure of CTS revealed a plate-shaped molecule with a shallow depression on one side.[13]

Carbohydrates have long been known to form stable complexes with metal ions.[14] Cyclo-oligosaccharides are also capable of forming complexes with metal ions using a variety of mechanisms. Cyclofructan has been shown to bind metal ions in a crown-ether like manner.[15,16] β-Cyclodextrin was recently shown to coordinate Pb^{2+} in alkaline solutions.[17] The structure of CTS suggests it may offer potential metal-ion binding sites. It has two free primary alcohols that could be oxidized to acids, which can form strong interactions with metal ions.[14] Carbohydrate chelation can increase the bioavailability and solubility of metal ions. Carbohydrate-based chelators have found clinical use in the treatment of iron

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overload, iron deficiency, promoting the removal of toxic metals, and in the treatment of ulcers.[18–22]

2. Experimental

2.1. General

All chemicals, metal salts and solvents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) in ACS grade or better. Sodium hypochlorite (5.6%) was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). All metals were provided as the appropriate chloride salts, with the exception of Ag^+ and Pb^{2+}, which were provided as the nitrate salts. Polygram Ionex-25 SA Na sheets were purchased from Macherey-Nagel, Inc. (Easton, PA, USA). CTS was prepared as previously described.[2,3]

2.2. Preparation of *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→}

A 50 mM aqueous solution of CTS, (*cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}), (typically on 1–2 g scale) was prepared, 0.15 equiv of NaBr and 0.01 equiv of TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy, free radical) were added to the solution and allowed to cool to in an ice bath (4 °C). While monitoring the pH, chilled 5.6% NaClO was added dropwise until the pH reached 11.0. NaClO was added as needed to maintain the pH between 10.5 and 11.0, until 6 equiv of NaClO were consumed, after which 1.0 M NaOH was used to maintain the pH. The reaction was considered complete when the pH stabilized, typically during 15–30 min. HCl (3.0 M) was added to adjust the pH to 3.0–4.0. The sample was purified using aq gel-permeation chromatography over Bio-Gel P-2, which resulted in removing the salts and producing a product 90–95% pure. Final purification was carried out by HPLC using a Synergi 10-μm RP 80 Å reversed-phase preparative column (21.2 × 250 mm, Phenomenex Corp., Torrance, CA). Elution was accomplished in water at a flow rate of 3–4 mL/min with refractive index detection. Byproducts isolated from both chromatography steps were analyzed by ESI mass spectroscopy. When necessary, complete conversion to the free-acid form was accomplished by eluting over a column of Dowex-50 resin in the H^+ form. Average yield of the recovered free acid was 86% for three trials. The compound was recovered as a white solid, mp 120 °C (dec), $[\alpha]_D^{25}$ +206° (H_2O). ^1H, ^{13}C, COSY, HMQC, and HMBC NMR spectra were recorded on a Bruker 400 MHz instrument in D_2O at 27 °C, pD 6.0. The chemical shifts are relative to external TMS. ^{13}C NMR (D_2O): δ Glc; 97.28 (C-1), 71.92 (C-2), 73.19 (C-3), 70.97 (C-4), 70.49 (C-5), 68.23 (C-6), GlcA; 99.19 (C-1), 70.13 (C-2), 75.25 (C-3), 73.80 (C-4), 72.47 (C-5), 176.6 (C-6). ^1H NMR data are provided in the text. Mass spectra were recorded on a Micromass Q-tof II electrospray ionization instrument; measured mass ($M+Na^+$) 699.159, calculated mass ($M+Na^+$) 699.159.

2.3. Metal-binding assay

TLC sheets were prepared as described by Briggs et al.[23] Briefly, the sheets were immersed in deionized water until saturated. The sheets were then placed in 100 mM solution of the appropriate metal salt for 1 h with slow shaking. The sheets were then washed with deionized water three times and allowed to air dry. The sheets were stored at 4 °C until used. Sheets were then spotted with 1 μL of 1% (w/v) of the cyclic tetrasaccharide, and the chromatograms were then developed in deionized water or in 1:1 deionized water:MeOH. Compounds were detected using, 0.2% *N*-(1-naphthyl)ethylenediamine in 97:3 MeOH–concd sulfuric acid. The sheets were dried and sprayed until wetted with the reagent and heated at 125 °C for 10 min. Some metal ions (especially Ag^+ and Fe^{2+}) were prone to create streaking, rather than discrete spots. In these cases the area with the highest intensity was assigned the retention value.

2.4. Pb^{2+} binding

Stoichiometry of the complex was determined using aqueous gel-permeation chromatography over Bio-Gel P-2. An aqueous solution (0.5 mL) of 2 mM OCTS with and without 1 mM $Pb(NO_3)_2$ adjusted to pH 6.0 was eluted from a 1-cm × 50-cm column. The fractions were monitored using TLC. ^1H NMR spectra were recorded on a Bruker 400-MHz instrument in D_2O at 27 °C, pD 6.0. Samples of OCTS and OCTS (2 mM) with $Pb(NO_3)_2$ (1:1) were prepared in H_2O, pH adjusted to 5.6 (pD = pH +0.4) and freeze dried. These were then resuspended in D_2O and the pD checked.

2.5. Computational methods

Molecular simulations were carried out using InsightII/Discover (v. 4.0, Molecular Simulations Inc.) software.[24] Energy was calculated using the all-atom force field, AMB99C[25,26] at a dielectric constant value of one. The carboxyl groups in OCTS are given a −1e total charge spread among the C–COO^- atoms. The partial atomic charges on the glucose residues are treated as previously described.[13] The molecule was defined with all atoms and fully flexible during energy minimization. The starting structure was taken as the symmetric conformer[13] found using molecular dynamics simulations in water. The Pb^{2+} atom parameters were derived for this problem using experimental results from struc-

tural studies on Pb^{2+} bearing minerals or glasses.[27] The Pb–O distance was fitted by adjusting the van der Waals radius to give 2.51 Å, a distance found to be in agreement with experimental tetra-coordinated structures.[27]

3. Results

The oxidation of CTS was accomplished using the TEMPO–NaBr–NaClO system, which was originally shown to oxidize partly protected monosaccharides.[28] In more recent applications it has been used to oxidize high molecular weight polysaccharides[29–32] and smaller maltodextrins.[33] The method of Thaburet et al.[33] was utilized in the current report, where CTS was oxidized to OCTS, (*cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→}, as depicted in Fig. 1. The only modification of the method was the addition of more oxidant (NaClO). This resulted in higher yields (~90% based on RP-HPLC) and coproducts that were easier to remove. Mass spectra of the coproducts formed showed them to consist mainly of over-oxidized versions on the product compound, which were easier to remove with size-exclusion chromatography than under-oxidized products. High selectivity and yield was obtained using this method by controlling the amount of oxidant added and monitoring product formation.

The ability of CTS and OCTS to bind metals was screened using ion-exchange thin-layer chromatography

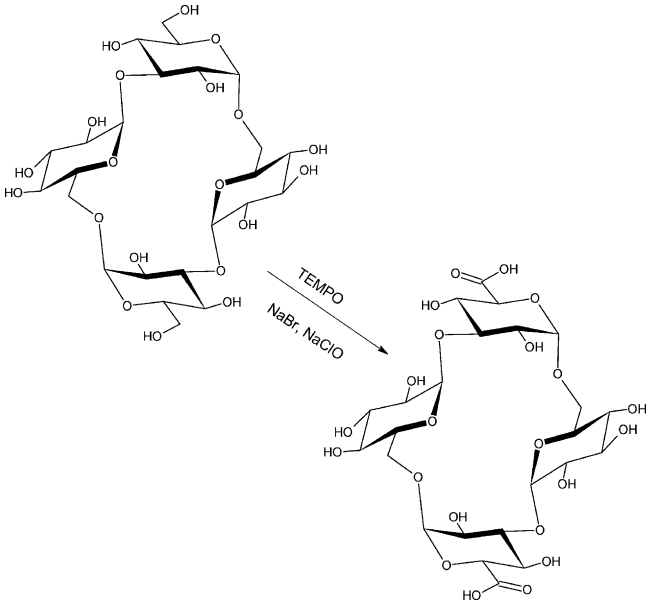

Fig. 1. The TEMPO-catalyzed oxidation of *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (CTS) to the dicarboxylic acid, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→} (OCTS).

(IETLC), as originally described by Briggs et al.[23] IETLC provided a convenient method of qualitatively screening CTS and OCTS for binding to metal ions. This method was previously used to identify complex formation between cycloinulino-oligosaccharides and Ba^{2+}.[16] D-Glucuronic acid (GlcA) and methyl α-D-glucopyranosiduronic acid (GlcAOMe) were also screened for comparative purposes. Twenty metal cations were screened, and the observed R_f values are reported in Fig. 2. Fig. 2(A) shows the behavior of the potential chelators with water as the mobile phase. Fig. 2B shows the behavior with 1:1 water–methanol as the mobile phase. In the aqueous system, CTS has little or no complex formation with all the metals, except Ag^+, which is known to bind nonspecifically to carbohydrates. OCTS, on the other hand, demonstrated strong affinity for Pb^{2+}, Fe^{2+}, Fe^{3+}, and to a lesser degree Al^{3+}. GlcA and GlcAOMe showed a metal preference similar to that of OCTS, although with lower affinity. The carboxylic acids were screened in the free acid form. Tests with the Na^+ salts of the acids showed only minor differences in their behavior, which suggests the pH is controlled by the TLC plate and not the compound screened. The water–methanol system increased the affinities of all the ligands. In this system, the carboxylic acid compounds specifically showed much higher affinity for Ag^+, Ba^{2+}, Cu^{2+}, and Sr^{2+}, when compared to the aqueous system. The lower dielectric constant of the water–methanol system lowers the desolvation energy for the cations and makes it energetically more favorable for the ligands to bind.[34,35]

The IETLC screening identified Pb^{2+} as forming the strongest complex with OCTS and was chosen for additional study. First, we determined the stoichiometry of the complex with aq gel-permeation chromatography. It was observed that free OCTS and OCTS with $Pb(NO_3)_2$ (2:1) elute at the same eluent volume, which suggests $OCTS{\cdot}Pb^{2+}$ exists as a 1:1 complex. During the preparation for these experiments, the low solubility of the $OCTS{\cdot}Pb^{2+}$ complex was also noted, with precipitate forming at approximately 2 mM at pH 6.0.

In order to obtain a better understanding of the structural changes that occur upon binding, ^{1}H NMR spectroscopy was performed on free and Pb^{2+}-bound OCTS. The results are summarized in Table 1. The results show very little perturbation in the structure of the pyranose rings upon binding to Pb^{2+}, with only minor changes observed in both the chemical shift and coupling constants. The lack of significant change in the ^{1}H NMR spectrum also prevented us from using NMR to quantify an equilibrium constant from the complex. Additionally, preliminary experiments with a lead-ion selective electrode lacked the sensitivity and reliability needed to accurately quantify the equilibrium constant. In the future, a polarographic or a detailed potentio-

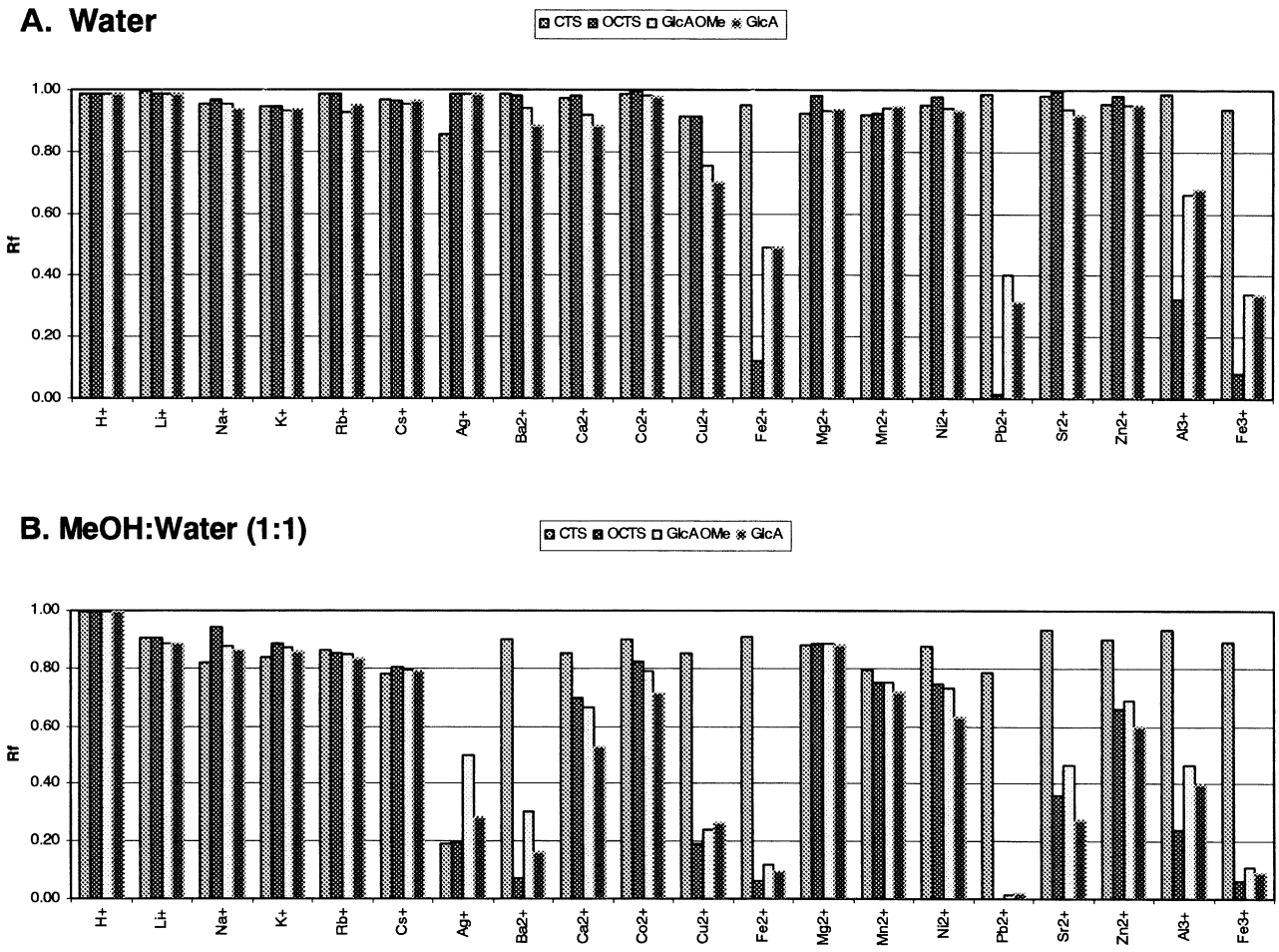

Fig. 2. Retention values (R_f) of carbohydrates on ion-exchange TLC plates impregnated with the appropriate metal. **CTS**; *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}, **OCTS**; *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→}, **GlcAOMe**; methyl α-D-glucopyranosiduronic acid, **GlcA**; D-glucuronic acid. **A**. Water is the mobile phase. **B**. Water–methanol (1:1) is the mobile phase.

metric study should be conducted to accurately determine the equilibrium constant.

In order to obtain a better understanding of how Pb^{2+} may bind to OCTS, a molecular simulation study was performed. Briefly, distance constraints were applied to bring the two carboxyl groups in OCTS to a binding configuration with the Pb^{2+} ion, slowly drawing these groups together to a distance of ~ 5 Å. The Pb^{2+} ion was then placed above the two carboxyl groups and the energy minimized. The resulting energy-minimized structures for the free and complexed OCTS are shown in Fig. 3. The OCTS molecule retained a symmetric conformation, even after being stressed into the complex OCTS·Pb^{2+} conformation. Upon removal of the Pb^{2+} ion, the resulting conformation was stable upon energy minimization with only small changes in the dihedral angles, the energy being somewhat higher than the original OCTS molecule. The molecule did not return to the original Pb-free conformation, which suggests this is a stable local-energy minimum conformation.

The differences between the free and bound OCTS simulated structures are primarily limited to changes in the dihedral angles encompassing the anomeric oxygens. These results are consistent with the information obtained from the NMR experiments. The NMR experiments show a symmetric conformation as found in the simulations, and little change in the individual ring structures as confirmed by the minor changes in coupling constants and chemical shifts. The changes in the dihedral angles between the glucose moieties would also be predicted to have little influence on the ^{1}H NMR spectrum.

Fig. 4 illustrates the Pb–O bond distances obtained for the complex. The oxygens of the C-4 hydroxyls are only 2.87 Å away. The bond distances and distorted

Table 1
^{1}H NMR data for OCTS and OCTS+Pb^{2+}

Chemical shifts (ppm) [a]	H-1	H-2	H-3	H-4	H-5	H-6A	H-6B
Glc							
OCTS	5.49	3.47	3.65	3.16	4.35	3.69	3.64
OCTS+Pb^{2+}	5.50	3.44	3.66	3.17	4.35	3.70	3.65
Difference	−0.01	0.03	−0.01	−0.01	0.00	−0.01	−0.01
GlcA							
OCTS	4.82	3.55	3.95	3.66	3.93		
OCTS+Pb^{2+}	4.83	3.53	3.95	3.62	3.96		
Difference	−0.01	0.02	0.00	0.04	−0.03		
Coupling constants (Hz)	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6}$	
Glc							
OCTS	3.88	9.83	9.63	9.63	10.2	2.23	
OCTS+Pb^{2+}	3.92	9.81	9.16	9.41	9.93	2.65	
Difference	−0.04	0.02	0.47	0.22	0.25	−0.42	
GlcA							
OCTS	3.54	9.74	9.56	10			
OCTS+Pb^{2+}	3.55	9.85	9.39	10.1			
Difference	−0.01	−0.11	0.17	−0.07			

[a] Chemical shifts relative to external TMS.

geometry are similar those reported for the complex of Pb^{2+} with furan-3-carboxylate [36]. A variety of distorted coordination geometries have been reported for Pb^{2+} compounds [36–39], and some have been ascribed to the lone 6*s* electron pair activity [38,39].

4. Discussion

The current study demonstrates a simple and efficient method of modifying the properties of this cyclic tetrasaccharide. The oxidation to the dicarboxylic acid

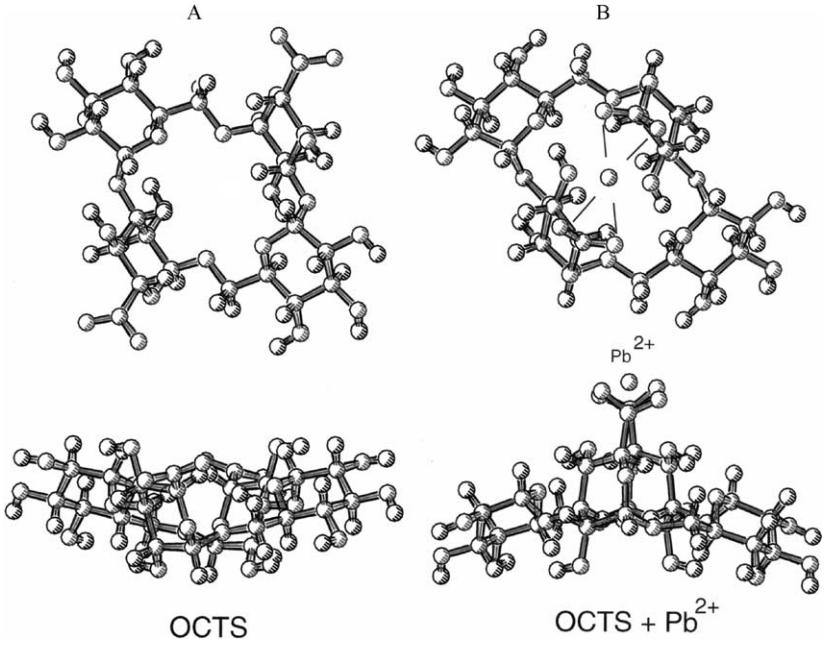

Fig. 3. Structures of OCTS obtained through molecular simulations. **A**. Free OCTS. **B**. The complex of OCTS and Pb^{2+}.

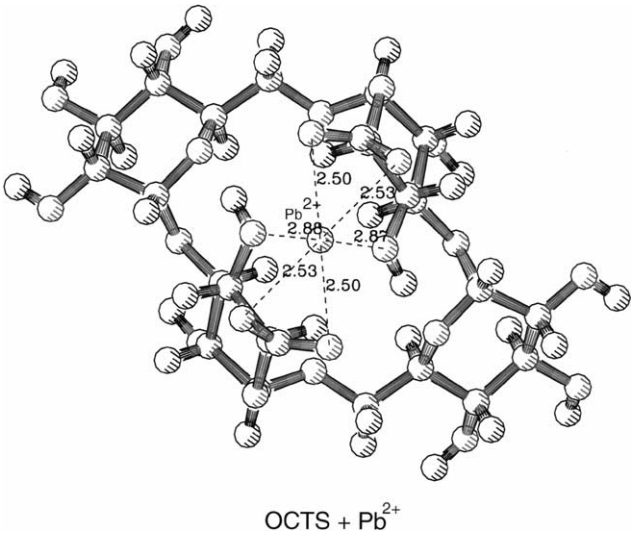

Fig. 4. Pb–O bond distances obtained from molecular simulations.

yielded a selective chelator for Pb^{2+}, Fe^{2+} and Fe^{3+}. Experimental and modeling results suggest the formation of stable 1:1 complexes.

In conclusion, the architecture of the cyclic tetrasaccharide provides an interesting platform for creating molecules with new properties. It also provides a convenient site for chemical modification, with two symmetrical primary alcohols. It is possible to envision a number of modifications (i.e., phosphorylation, alkylation, etc.) which would alter its properties for chelation or other applications.

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