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Chemical functionalization of polysaccharides with aromatic molecules: A ^{1}H NMR spectrum is not always enough for confirming the derivatization☆,☆☆

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ABSTRACT

Carbohydrate-aromatics interactions are well known in Nature and are also exploited in food and pharmaceutical industry for encapsulating low molecular weight drugs or flavors into polysaccharide carriers. Despite that, the non-covalent entrapment of small molecules containing aromatic moieties into the 3D-structure of a polysaccharide is typically overlooked in the context of the structural characterization of derivatized polysaccharides, that is often limited to the measurement of only ^{1}H NMR spectra. This study demonstrates that it can be misleading in some cases. We report some examples of products – obtained by grafting polysaccharides with small molecules containing aromatic moieties and displaying fully satisfying ^{1}H NMR spectra – that were demonstrated to be instead poorly or even not derivatized at all by 2D-NMR analysis. Although these results do not question any polysaccharide grafting with aromatic functionalities reported so far in literature, they strongly support the necessity of a structural characterization based not merely on ^{1}H NMR spectra for a robust demonstration of a successful polysaccharide derivatization.

1. Introduction

Polysaccharides are among the most abundant and widespread organic molecules on Earth. Their diversity in terms of chemical structures allows them to play multiple roles in Nature, from energy reserves to structural materials to key agents in intercellular communication processes. Natural polysaccharides have been exploited for several purposes by centuries. Since 150 years polysaccharides have been started to be derivatized by chemical reactions, in order to expand their structural diversity further and broaden their fields of application. Among the huge number of structural manipulations that have been reported (Cumpstey, 2013), some of them focus on the conjugation of polysaccharides with naturally occurring or synthetic organic, bioactive molecules in order to improve the physicochemical and biological properties of the former and/or the latter. In this frame, several studies concern the chemical or chemoenzymatic grafting of aromatic molecules onto the polymeric structure of polysaccharides (Boundaoui et al., 2024; Liu et al., 2018). Applications of the obtained conjugates span from chromatography (Bui et al., 2021) to drilling industry (Gao et al., 2022), but most of them are related to food and pharmaceutical industries. In particular, the conjugation of polyphenols (flavonoids, stilbenes, lignans, phenolic acid etc.) onto the polymeric structure of polysaccharides is an intense field of research, that is directed not only to improve and broaden their applications but also to support the detailed understanding of the covalent and, above all, non-covalent interactions occurring between such molecules in the vegetal kingdom (Guo et al., 2022; Liu et al., 2020). Indeed, very often polysaccharides and polyphenols form complexes that are stabilized by a set of non-covalent interactions including hydrogen bonding, electrostatic attractions, hydrophobic forces and π–π and CH-π stacking (Cortés-Ferré et al., 2025; Shahidi & Athiyappan, 2023). Similarly, a very extensive number of such non-covalent interactions can strongly pack polysaccharides with aromatic polymer lignin in lignocelluloses biomass, strongly influencing their nanoscale assembly in plant cell walls (Kirui et al., 2022). Carbohydrate-aromatics interactions mainly based on CH-π stacking are also pivotal factors for oligo- and polysaccharides binding to proteins

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(Asensio et al., 2013), and can contribute to the encapsulation of flavors and drugs containing aromatic moieties into hydrophobic pockets of polysaccharide carriers (Barclay et al., 2019; Buljeta et al., 2021).

In spite of this great body of evidence about the ability of polysaccharides to interact with the aromatic moieties of organic molecules and even to encapsulate them in the tridimensional polymeric structure, this feature is not taken into consideration when the product of a conjugation reaction between a polysaccharide and an aromatic functionality is analyzed for structural characterization. Indeed, in most cases the confirmation of the success in the conjugation and the determination of the degree of derivatization is ascribed to a comparison of the ^{1}H NMR spectra of the starting materials (i.e. a polysaccharide and the organic molecule to be grafted to) and of the suitably purified reaction product. The presence in the latter of characteristic signals shown in the former spectra is typically retained as a sufficient proof to claim that a covalent linkage between the polysaccharide and the reaction partner has been formed. Instead, the ability of polysaccharides to bind small organic molecules through a network of non-covalent interactions can make the purification of the reaction product not effective and the occurrence of a physical mixture of the underivatized reagents in the sample rather than a covalent conjugate between them is overlooked. This can afford misleading results in structure-activity relationship screenings, taking also into consideration that a polysaccharide grafted with an aromatic molecule can display different properties with respect to a mixture of the two components not linked together through a covalent bond (Yongvongsoontorn et al., 2025).

In order to discriminate between a covalent conjugation and a physical mixture, a diffusion-ordered (DOSY) NMR spectrum is sometimes measured. Indeed, in the case of a covalent conjugation the signals related to polysaccharide and the grafted molecule are characterized by the same diffusion coefficient. Conversely, if the sample is constituted by a physical mixture of the two components, a DOSY spectrum should allow to distinguish between signals associated with low-molecular weight species, typically faster self-diffusing in solution, and peaks related to slowly diffusing macromolecules such as polysaccharides are (Bedini et al., 2023). Unfortunately, encapsulation complexes formed by entrapment of small organic molecules into the tridimensional structure of the polysaccharide cannot be evidenced by a DOSY spectrum, because the polysaccharide vehicle and the loaded small molecule move in solution with a unique diffusion coefficient.

In this work we hypothesize that some reactions attempting the grafting of polysaccharides (at least the two treated in this study: alginic acid and unsulfated chondroitin) with small organic molecules containing aromatic functionalities and giving apparently satisfying ^{1}H NMR and 1D-DOSY spectra, can be shown to be instead scarcely efficient or completely unsuccessful, if a detailed 2D-NMR analysis and/or a more extensive purification are conducted. The goal is obviously not to question any polysaccharide grafting with aromatic functionalities achieved so far in literature, but just to warn about the robustness of claimed conjugation reactions based exclusively on measurement of ^{1}H NMR spectra.

2. Experimental

2.1. General methods

Commercial grade reagents and solvents were used without further purification, except where differently indicated. Unsulfated chondroitin (42 ± 3 kDa, 1.34 dispersity) deriving from the fed-batch fermentation of *Escherichia coli* O5:K4:H4 (Cimini et al., 2018) was a kind gift of Prof. Chiara Schiraldi (Department of Experimental Medicine, University of Campania "L.Vanvitelli", Naples, Italy). M-rich alginic acid (poly-M, 9 ± 2 kDa, 1.33 dispersity) was purchased from Biosynth. Its D-mannuronic acid (M) to L-guluronic acid (G) composition was evaluated by ^{1}H NMR anomeric peak integration, in agreement with the literature (Grasdalen, 1983): M = 83%, G = 17%; MM = 57%, MG = GM = 17%, GG = 1%. The term "pure water" refers to water purified by a Millipore Milli-Q Gradient system. Dialyses were conducted on Spectra/Por 3.5 kDa cut-off membranes at room temperature. Centrifugations were performed with an Eppendorf Centrifuge 5804R instrument at room temperature (4600 g, 5 min). Freeze-dryings were performed with a 5Pascal Lio 5P 4 K freeze dryer. NMR spectra were recorded at 298 K in D_2O (acetone as internal standard, ^{1}H: $(CH_3)_2CO$ at δ 2.22; ^{13}C: $(CH_3)_2CO$ at δ 31.5; H_3PO_4 as external standard, ^{31}P: δ 0.0) on a Bruker Avance-III HD (^{1}H: 400 MHz, ^{13}C: 100 MHz, ^{31}P: 162 MHz) instrument. Data were processed using the data analysis packages integrated with Bruker TopSpin 4.0.5 software. 1D-DOSY experiments were measured using a stimulated echo sequence with bipolar gradient pulses and one spoil gradient (stebpgp1s1d) with a diffusion time Δ of 300 ms and a gradient duration of 1.25 ms. ^{1}H,^{13}C-DEPT-HSQC experiments were measured in the ^{1}H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain, using data sets of 2048 × 400 points and typically from 64 to 96 increments, and setting a pulse sequence delay corresponding to a 145 Hz value for J_{CH} coupling constant. ^{1}H,^{31}P-HSQC experiment was measured in the ^{1}H-detected mode via a phase sensitive gradient selection with decoupling during acquisition, using data set of 2048 × 300 points and 64 increments, and setting a pulse sequence delay corresponding to a 20 Hz value for J_{PH} coupling constant. ^{1}H,^{13}C-HMBC experiment was measured in the ^{1}H-detected mode via zero and double quantum coherence, using data sets of 2048 × 300 points and 164 increments, and setting a pulse sequence delay corresponding to a 8 Hz value for long range J_{CH} coupling constant.

2.2. Esterification with benzyl alcohol under neat conditions

A sample of poly-M (17.9 mg, 30.4 μmol repeating unit) was dried in an oven at 60 °C overnight, then mixed with dipyridyldithiocarbonate (DPDTC, 48.8 mg, 195 μmol) and slowly stirred at 60 °C. After 6 h the mixture appeared as a greenish slime. It was cooled to room temperature and then treated with benzyl alcohol (255 mL, 2.26 mmol) and 4-dimethylaminopyridine (DMAP, 11.6 mg, 94.9 μmol). After overnight stirring at 60 °C, the mixture was cooled to room temperature, diluted with pure water (10 mL) and neutralized with few drops of a 0.01 M HCl aqueous solution. The solution was dialyzed and freeze-dried to afford a slightly yellow solid (23.4 mg). The sample was further purified by dissolution in dimethyl sulfoxide (DMSO, 750 μL) followed by treatment with acetone (5.0 mL), that caused the formation of a white precipitate. It was collected by centrifugation and then dried under vacuum overnight to yield **1** as a white solid (17.2 mg).

2.3. Phosphorylation with dibenzyl N,N-diisopropylphosphoramidite

Chondroitin derivative **2** (19.2 mg, 33.0 μmol repeating unit) was treated with dry *N*,*N*-dimethylformamide (DMF, 1.45 mL) under Ar atmosphere and stirred for some minutes. Thereafter, a 0.45 M solution of 1H-tetrazole in acetonitrile (CH_3CN, 1.45 mL, 660 μmol) was added to give a clear brownish solution that was then treated with dibenzyl *N*,*N*-diisopropylphosphoramidite (73.3 μL, 218 μmol). The solution was stirred overnight at room temperature. After cooling to 0 °C, a 30% w/w aqueous solution of hydrogen peroxide (26.4 μL, 462 μmol) was added and stirring was continued at room temperature for 2 h. Diisopropyl ether (11 mL) and acetone (11 mL) were then added to give a precipitate that was collected by centrifugation. It was then dissolved in DMSO (1.0 mL) and treated again with diisopropyl ether (10 mL) and acetone (10 mL) to afford a brownish precipitate, that was collected by centrifugation and dried under vacuum to yield **3** (23.8 mg). The product was suspended in pure water (7 mL) and treated with a few drops of 33% w/v aqueous solution of sodium hydroxide to reach a strongly alkaline pH (~13). The reaction mixture was stirred at room temperature overnight to give a clear solution that was then neutralized with a 1 M HCl aqueous solution. The solution was dialyzed and freeze-dried to afford a

yellowish solid (6.4 mg). The sample was further purified by dissolution in DMSO (400 μL) followed by treatment with acetone (3.0 mL), that caused the formation of a precipitate. It was collected by centrifugation and then dried under vacuum to yield **4** as a slightly yellow solid (5.6 mg). The product was then further purified by dissolving it in pure water (1.0 mL) and eluting the resulting solution through a Waters Sep-Pak C18 cartridge (adsorbent: reverse phase C18-silica gel, 360 mg adsorbent per cartridge; particle size: 55–105 μm, pore size: 125 Å), that was preliminarily conditioned by washing with ethanol (20 mL), then acetonitrile (4 mL) and finally pure water (20 mL). The product was obtained as a white solid (2.3 mg) after freeze-drying.

3. Results and discussion

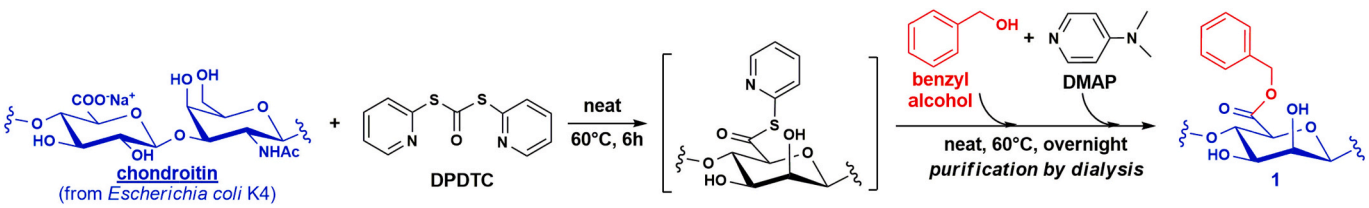
3.1. Esterification of a polysaccharide under neat conditions

Esterification is one of the most versatile and widespread employed derivatization reaction on polysaccharide structures (Heinze et al., 2006). In order to find green protocols to accomplish such transformation, we recently tried to apply a novel solvent-free procedure, that was developed on small organic molecules to some carboxylic acid containing polysaccharides. The original protocol relies upon the in situ activation of carboxylic acids as 2-pyridyl-thioesters by reaction with DPDTC without the use of any reaction medium, followed by the addition of the desired alcohol and a base in aqueous solution or still under neat conditions (Freiberg et al., 2023). Microbial-sourced, unsulfated chondroitin (Cimini et al., 2023) was firstly selected as acidic polysaccharide to be subjected to an esterification test. This polysaccharide shares almost the same structure with an important animal-sourced glycosaminoglycan (GAG), i.e. chondroitin sulfate (CS). It is constituted by a disaccharide repeating unit composed of 2-acetamido-2-deoxy-D-galactose (*N*-acetyl-D-galactosamine, GalNAc) and D-glucuronic acid (GlcA) residues with alternating β-1 → 3 and β-1 → 4 glycosidic bonds, but lacks the sulfate groups showed by CS (Vessella et al., 2019). Due to the enormous biological relevance of GAGs and to the need to access GAG-type bioactive agents from non-animal sources to avoid ethical, safety and sustainability concerns, the development of tailored reactions on microbial-sourced GAG-type polysaccharides is currently pursued more and more (Bedini et al., 2016; Hintze et al., 2022; Sockett et al., 2026). In this frame, an esterification reaction on unsulfated chondroitin was attempted, using DPDTC as carboxylic acid activator, DMAP as base and benzyl alcohol as partner for the esterification reaction performed under neat conditions at 60 °C (Scheme 1).

After dilution of the crude reaction mixture with water, followed by dialysis purification to remove low molecular weight reagents and byproducts and then freeze-drying, product **1** was firstly analyzed by ^{1}H NMR spectroscopy. A comparison with the spectrum of the starting chondroitin polysaccharide clearly revealed the presence of new signals at 6.74, 6.65 and 4.53 ppm (Fig. 1a, b), that could be assigned to the aromatic and benzylic hydrogen atoms of grafted benzyl moieties, respectively. The large line width of these peaks, comparable with the ones related to the signals attributed to the polysaccharide backbone, was in agreement with a covalent grafting of the benzyl moieties onto the chondroitin chain, because the broadening of the benzyl peaks could be attributed to a shortening of transverse relaxation times, that is typical for atoms behaving to polymeric species. The 1D-DOSY spectrum was superimposable with the ^{1}H NMR spectrum (Fig. 1b, c), thus suggesting the covalent linkage between benzyl functionalities and the polysaccharide backbone, because signals related to H atoms belonging to unbound low molecular weight species should have been cut off in a diffusion edited spectrum such as 1D-DOSY. In order to demonstrate unequivocally the conversion of GlcA carboxylic acid moiety into a benzyl ester group, ^{1}H,^{13}C-heteronuclear 2D-NMR spectra were also measured on product **1** (Fig. 1d). The ^{1}H,^{13}C-DEPT-HSQC spectrum showed that the ^{1}H NMR signals at δ_H 6.74 and 6.65 ppm correlated with a ^{13}C signal at 127.7 ppm, thus confirming their assignment to a benzene ring, while the CH_2-tagged signal at $\delta_{H/C}$ 4.53/69.3 ppm was undoubtedly assigned to the benzylic methylene atoms. The ^{1}H,^{13}C-HMBC spectrum showed, at a ^{1}H chemical shift value of 4.53 ppm, three distinct signals at 127.7, 135.1 and 154.8 ppm. The first and the second chemical shift values were expected, as they could be assigned to benzene ring carbon atoms at *ortho* and *ipso* position, that were two and one bonds far from the benzylic carbon atom, respectively. Conversely, the HMBC cross-peak at $\delta_{H/C}$ 4.53/154.8 ppm could not be assigned to a correlation between the benzylic H atoms and an adjacent carboxyl ester C atom, due to a too low ^{13}C-chemical shift value.

A screening of ^{13}C NMR literature data revealed that the chemical shift values for dibenzyl carbonate (Scheme 2) were fully consistent with the detected ones (69.9, 128.5, 128.7, 135.3 and 155.2 ppm for a ^{13}C NMR spectrum measured in $CDCl_3$; Fiorani & Selva, 2014). Indeed, its formation could be ascribed to the nucleophilic attack of two molar equivalents of benzyl alkoxide on DPDTC, the former being generated in turn by deprotonation of benzyl alcohol by DMAP.

In order to demonstrate even more robustly the formation of an encapsulation complex between unmodified chondroitin and dibenzyl carbonate in water or D_2O rather than a covalent grafting of benzyl alcohol on the polysaccharide, the dissolution of the solid sample obtained after freeze-drying in a different solvent – hopefully disfavoring the formation of the encapsulation complex – followed by a selective precipitation of the polysaccharide was attempted. DMSO was found to dissolve very well the product, nonetheless the selective precipitation of unsulfated chondroitin failed in spite several solvents were tested. Conversely, this was possible on another carboxylic acid containing polysaccharide, such as poly-M, i.e. a commercially available alginic acid characterized by a very high D-mannuronic acid to L-guluronic acid ratio (M/G = 83:17; Esposito et al., 2023). In particular, the esterification of poly-M with benzyl alcohol in the presence of DPDTC and DMAP was attempted under the same conditions employed on unsulfated chondroitin (Scheme 3). With respect to starting poly-M, the polysaccharide product recovered after dialysis purification showed a ^{1}H NMR spectrum with additional, broad signals at 6.74, 6.65 and 4.53 ppm, that could be assigned to aromatic and benzylic hydrogen atoms of benzyl moieties, respectively (Fig. 2a, b), as already discussed above for unsulfated chondroitin (Fig. 1a, b). The 1D-DOSY spectrum was superimposable with the ^{1}H NMR spectrum (Fig. 2b, c), thus suggesting the covalent grafting of benzyl alcohol on poly-M. Nonetheless, the dissolution of the product in DMSO, followed by a selective precipitation of the polysaccharide with acetone, afforded a sample showing a ^{1}H NMR spectrum with no signals assignable to aromatic and benzylic hydrogen atoms anymore (Fig. 2d). This result confirmed the formation of an



Scheme 1. Attempted esterification of unsulfated chondroitin with benzyl alcohol under neat conditions.

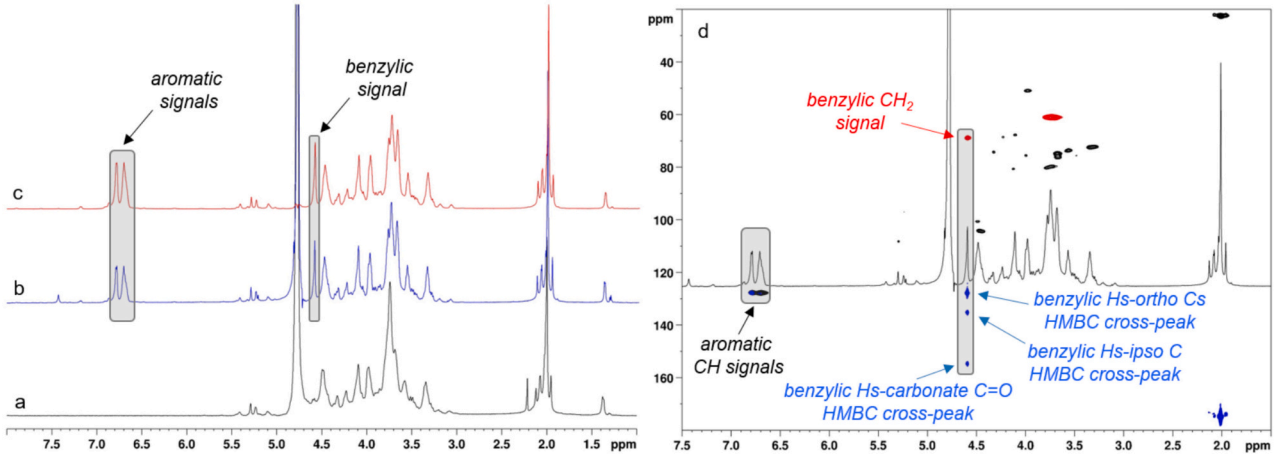
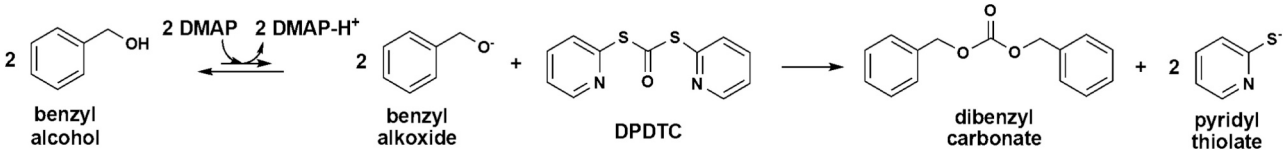
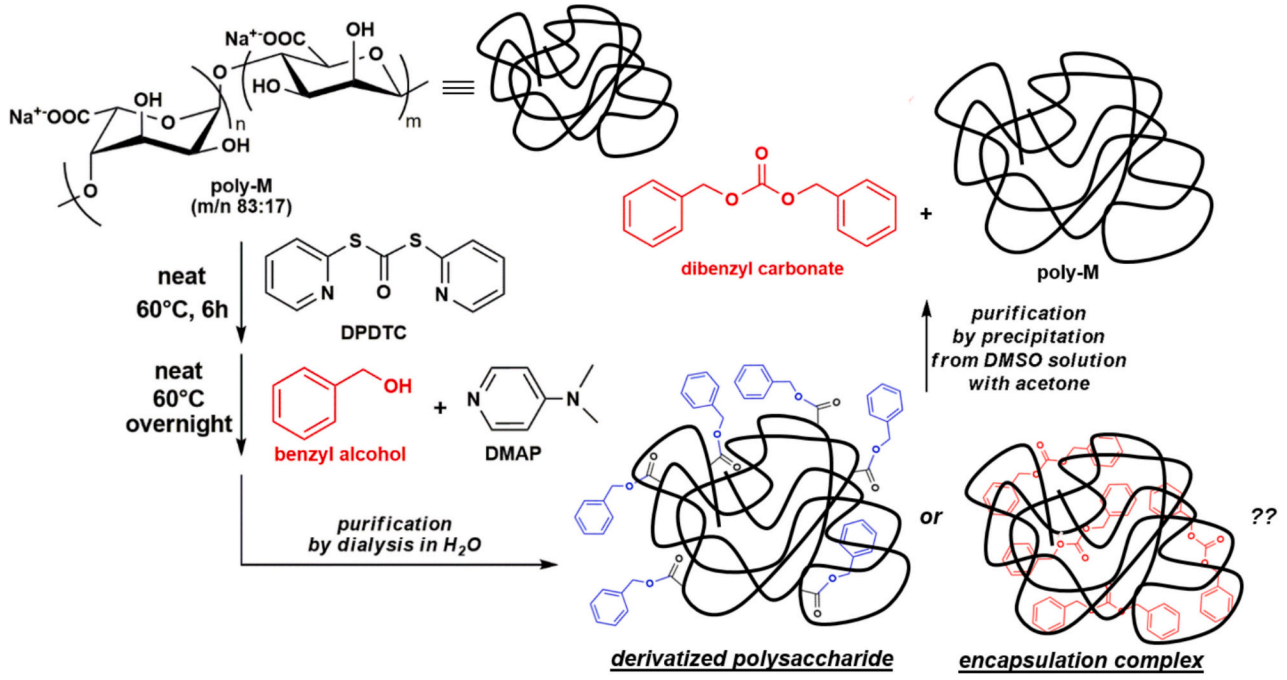


Fig. 1. (a) ^{1}H NMR spectrum of starting chondroitin polysaccharide; (b) ^{1}H NMR and (c) 1D-DOSY spectra of derivative **1**; (d) superimposition of ^{1}H NMR, ^{1}H,^{13}C-DEPT-HSQC (CH_3 and CH: black, CH_2: red) and ^{1}H,^{13}C-HMBC 2D-NMR of derivative **1**.



Scheme 2. Formation of dibenzyl carbonate from benzyl alcohol and DMAP.



Scheme 3. Formation of an encapsulation complex rather than a derivatized polysaccharide by esterification reaction of poly-M with benzyl alcohol under neat conditions.

encapsulation complex rather than a covalent conjugation between unmodified poly-M and dibenzyl carbonate in aqueous solution (Scheme 3), and proved the inadequacy of ^{1}H NMR and 1D-DOSY spectra in this case to demonstrate the grafting of a polysaccharide with an aromatic moiety containing molecule.

3.2. Phosphorylation of a polysaccharide with a dibenzyl phosphoramidite

Phosphorylation is a quite common derivatization reaction of polysaccharides, as the obtained derivatives have been proposed for numerous applications in diverse fields (from textile industry as flame-retardant agents to bioremediation as adsorbents for several transition metals and lanthanide ions), with the biomedical field standing as the

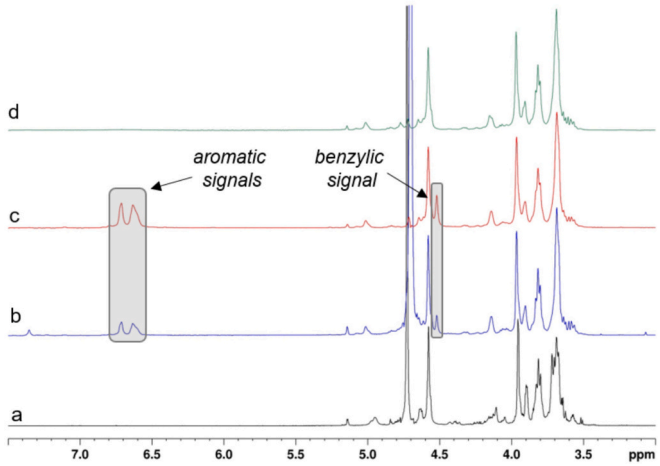
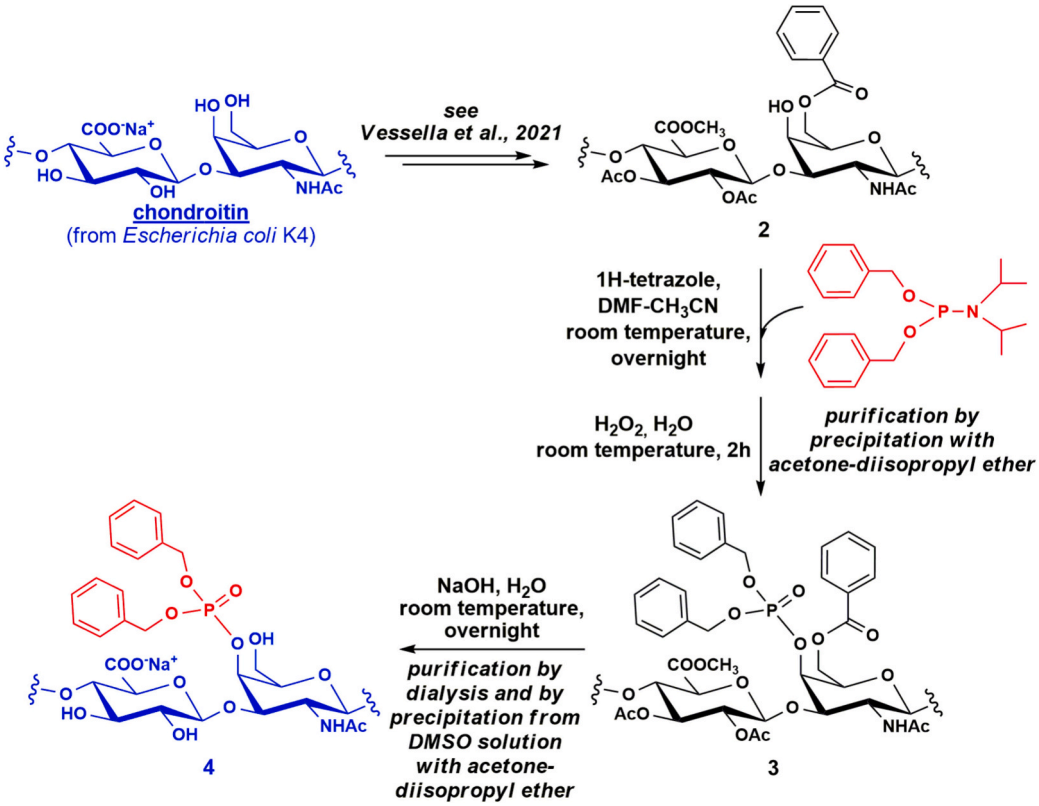


Fig. 2. (a) ^{1}H NMR spectrum of starting poly-M polysaccharide; (b) ^{1}H NMR and (c) 1D-DOSY spectra of the product of esterification after dialysis purification; (d) ^{1}H NMR spectrum of the same product after further purification by precipitation with acetone from a DMSO solution.

most promising one (Laffargue et al., 2023). Unfortunately, phosphorylation reactions on common polysaccharides are typically characterized by harsh conditions (with phosphoric acid employed as reagent at very high temperatures), poor yields and degrees of phosphorylation as well as no control of regiochemistry (Illy et al., 2015; Wang et al., 2022; Zhou & Huang, 2021). In order to overcome these limitations, the use of phosphorylation agents milder than phosphoric acid or the employment of more efficient heating techniques were recently tested for the phosphorylation of the microbial-sourced chondroitin polysaccharide cited above (Esposito et al., 2024, 2026), with the aim to open an access to regioselectively phosphorylated chondroitin derivatives as promising glycosaminoglycan mimics (Bojarski et al., 2019). In this frame known chondroitin derivative **2** – having its disaccharide repeating unit with the carboxylic acid and all the alcohol functionalities protected except for the axially oriented, secondary hydroxyl at *C*-4 of GalNAc units (Vessella et al., 2021) – was subjected to a phosphorylation attempt at the latter position by reaction with dibenzyl *N*,*N*-diisopropylphosphoramidite in the presence of 1H-tetrazole in a DMF-acetonitrile solvent mixture, followed by one-pot P(III) to P(V) oxidation with aqueous hydrogen peroxide (Scheme 4). These are typical conditions for the introduction of a protected phosphate monoester on alcohol functionalities in oligonucleotide chemistry. Recently they were successfully employed for the phosphorylation of synthetic polysaccharide mimetics (i.e. poly-amidosaccharides; Varghese et al., 2022), while to the best of our knowledge their use is completely unprecedented on natural polysaccharides and derivatives thereof. Phosphorylated product **3** was collected by precipitation from the reaction mixture with 1:1 v/v acetone-diisopropyl ether, and treated in turn with an aqueous alkaline solution to cleave all the carboxylic ester protecting groups and afford the water-soluble, phosphorylated chondroitin polysaccharide **4**.

Derivative **4** was subjected to dialysis, then freeze-dried and purified further by precipitation from a DMSO solution with 1:1 v/v acetone-diisopropyl ether, in order to remove low molecular weight reagents and byproducts as most extensively as possible. The obtained product was analyzed firstly by measuring ^{1}H- and 1D-DOSY NMR spectra. They suggested the presence of benzyl moieties covalently linked to chondroitin polymer chain, because they both showed two broad signals at 7.16–7.45 and 4.75–5.00 ppm – assigned to benzene ring and benzylic methylene H atoms, respectively – that could not be detected in the starting polysaccharide chain (Fig. 3a–c). The cross-peaks at $\delta_{H/C}$ 7.16–7.45/130.0 and 4.75–5.00/69.2 ppm displayed in the ^{1}H,^{13}C-DEPT-HSQC spectrum confirmed such assignments (see Supporting Information). Furthermore, the ^{31}P NMR spectrum of product **4** showed signals in the region around 0 ppm (Fig. 3e), that could be assigned to the presence of phosphate groups decorating the polysaccharide chain.



Scheme 4. Phosphorylation of a partially protected chondroitin derivative through phosphoramidite method.

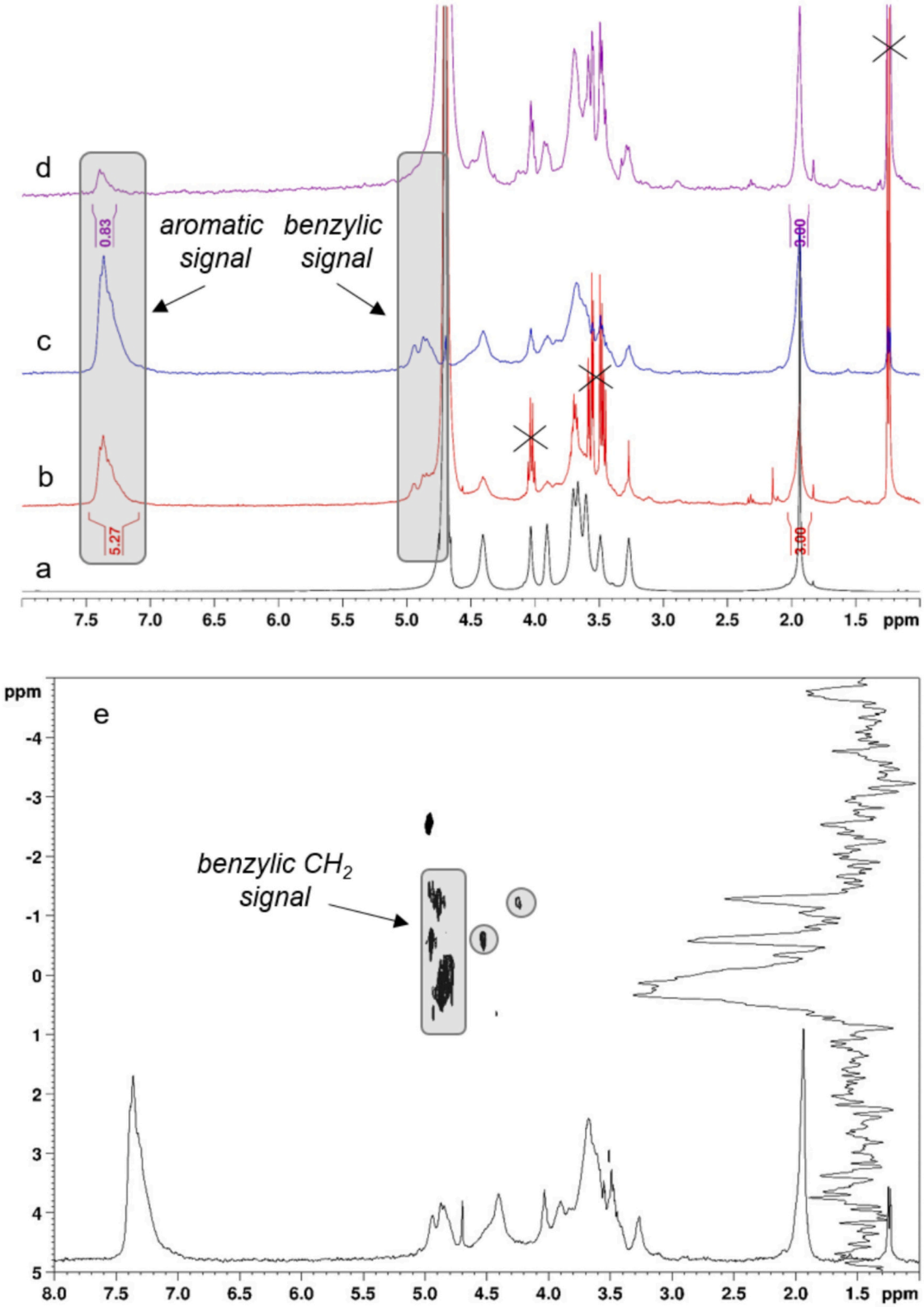


Fig. 3. (a) ^{1}H NMR spectrum of starting chondroitin polysaccharide; (b) ^{1}H NMR and (c) 1D-DOSY spectra of derivative **4** after purification by dialysis and precipitation (peaks marked with × symbol could be assigned to solvent impurities); (d) ^{1}H NMR of derivative **4** after further purification on a reverse phase C18-silica gel cartridge; (e) superimposition of 1D-DOSY, ^{31}P NMR and ^{1}H,^{31}P-HSQC of derivative **4** after purification by dialysis and precipitation.

All these NMR data could allow to conclude that dibenzyl phosphate moieties were effectively grafted onto the chondroitin backbone, as postulated for the structure of derivative **4**. Moreover, the relative integration of the benzene ring peak at 7.16–7.45 ppm and the *N*-acetyl signal at 1.90–2.00 ppm in ^{1}H NMR could give an estimation of the degree of derivatization (DD). A value equal to 0.53 could be calculated by applying Eq. (1).

$$DD = \left(I_{\text{benzene signal}}/10\right)/\left(I_{N-\text{acetyl signal}}/3\right) \quad (1)$$

Nonetheless, in spite of such non-negligible derivatization, a comparison between ^{1}H,^{13}C-DEPT-HSQC spectra of derivative **4** and starting chondroitin polysaccharide (see Supporting Information) did not reveal in the former any clearly visible ^{1}H and ^{13}C-downfield shifted signal that could be assigned to a phosphorylated CH-O site (Speciale et al., 2022). To check it out more precisely, a ^{1}H,^{31}P-HSQC 2D-NMR spectrum was measured for product **4**. As expected, cross-peaks were found in the ^{1}H,^{31}P-HSQC NMR at the ^{31}P chemical shifts (0.3, −0.6 and − 1.3 ppm) detected for the three signals displayed in the ^{31}P NMR spectrum (Fig. 3e). Their corresponding ^{1}H chemical shifts values at 4.80–5.02 ppm confirmed the phosphoester linkage with benzyl groups. Nonetheless, only the signals at δ_P -0.6 and − 1.3 ppm displayed additional, low intense cross-peaks – at δ_H 4.53 and 4.25 ppm, respectively – suggesting the presence of phosphoester bonds with CH atoms of the polysaccharide backbone. Conversely, the major ^{31}P signal at 0.3 ppm did not show any additional cross-peak and could be therefore assigned to a dibenzyl phosphoric species encapsulated into rather than covalently linked on the polysaccharide chain. In order to provide a definitive demonstration of this hypothesis, a further purification of product **4** was attempted by dissolving it in water and eluting the resulting solution through a reverse phase C18-silica gel cartridge. The ^{1}H NMR spectrum (Fig. 3d) of the obtained sample displayed much less intense signals at 7.16–7.45 and 4.75–5.00 ppm, with a DD equal to 0.08, as calculated from the former spectrum by applying Eq. (1). This demonstrated that a marked over-estimation of the DD occurred before the further purification, most likely due also in this case to having overlooked the encapsulation of an aromatic low molecular weight species into the tridimensional network of the polysaccharide.

4. Conclusions

The successful grafting of polysaccharides with small organic molecules is very often demonstrated in literature by ^{1}H NMR spectra displaying both signals assigned to the polysaccharide backbone and peaks related to the grafted functionality. In this work we have proved that this can be misleading in some cases. Indeed, we reported some examples of chemical derivatization reactions of polysaccharides with aromatic containing moieties, affording products that were demonstrated by 2D-NMR analysis and extensive purification to be very poorly or even not derivatized at all, in spite they clearly showed both polysaccharide chain and aromatic signals not only in their ^{1}H- but also in 1D-DOSY NMR spectra. The blunder can be caused by the non-covalent entrapment of aromatic molecules into the tridimensional architecture of the polysaccharide, a complexation phenomenon that is typically overlooked in the context of the structural characterization of derivatized polysaccharides, even if it is very well known and even exploited in food and pharmaceutical applications. We believe that this work gives the due emphasis to the necessity of supporting the claim of a successful derivatization reaction on a polysaccharide with as much experimental evidence as possible. On one side, the structural characterization of the sample should be based not only on a ^{1}H NMR spectrum but on a proper set of 2D-NMR spectra. On the other side, an adequate purification of the derivatized polysaccharide should be pursued. Unfortunately, no general protocol ensuring it can be suggested, because purification success strongly depends on the type and tridimensional architecture of the polysaccharide as well as on the structure of the small molecule to be grafted onto, as clearly displayed in the examples reported in this work. Therefore, we suggest to measure and compare ^{1}H NMR spectra on the sample just after a first purification step (i.e. dialysis, in the present work) and after a further purification step, or even more than one. The additional purification can rely upon the dissolution and ri-precipitation of the sample, or elution on an inverse phase chromatographic adsorbent, as in the present work, but different techniques (e.g. size-exclusion chromatography) could be useful as well.

CRediT authorship contribution statement

Fabiana Esposito: Writing – review & editing, Visualization, Investigation, Data curation. **Simona Bruno:** Writing – review & editing, Data curation. **Alfonso Iadonisi:** Writing – review & editing, Validation. **Serena Traboni:** Writing – review & editing, Supervision. **Emiliano Bedini:** Writing – original draft, Visualization, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2026.125559.

Data availability

Data will be made available on request.

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