Chitotriazolan (poly(β(1-4)-2-(1*H*-1,2,3-triazol-1-yl)-2-deoxy-D-glucose)) Derivatives: Synthesis, Characterization, and Evaluation of Antibacterial Activity

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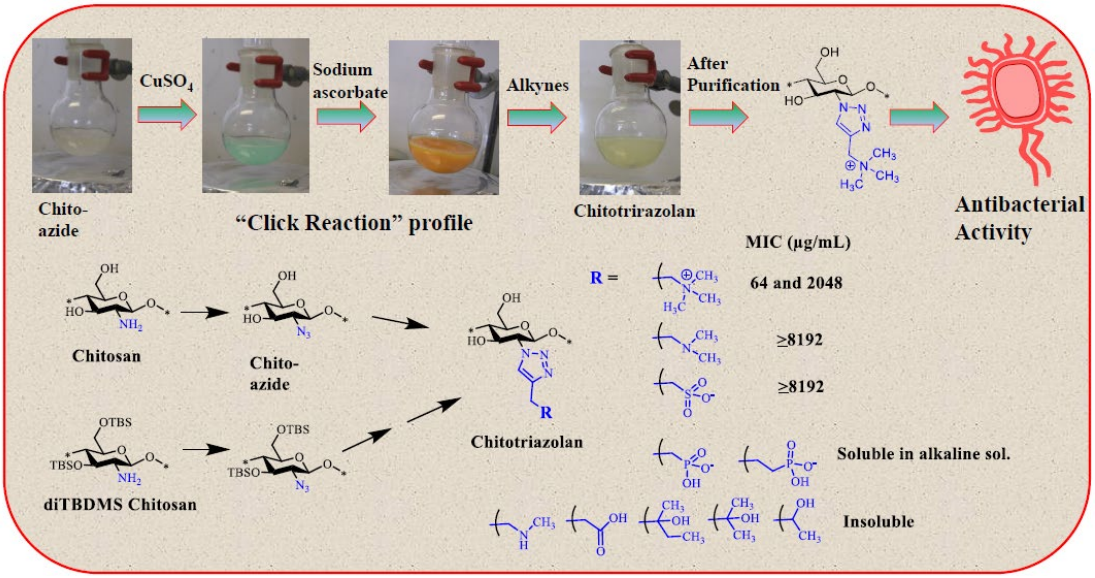
Highlights

- The first synthesis of water-soluble chitotriazolan derivatives
- Poly(β(1-4)-2-deoxy-D-glucose with aromatic triazole side group in the second position
- Two cationic and two anionic derivatives analyzed by 1H, ^{13}C APT, COSY, and HSQC NMR
- The quaternary *N,N,N*-trimethylammoniummethyl derivative was highly active against *S. aureus* and *E. coli*

Abstract

Here we describe the first synthesis of a new type of polysaccharides derived from chitosan. In these structures, the 2-amino group on the pyranose ring was quantitively replaced by an aromatic 1,2,3-triazole moiety. The 2-amino group of chitosan and di-TBDMS chitosan was converted into an azide by diazo transfer reaction. The chitosan azide and TBDMS-chitosan azide were poorly soluble but could be fully converted to triazoles by "copper-catalysed Huisgen cycloaddition" in DMF or DMSO. The reaction could be done with different alkynes but derivatives lacking cationic or anionic groups were poorly soluble or insoluble in tested aqueous and organic solvents. Derivatives with *N,N*-dimethylaminomethyl, *N,N,N*-trimethylammoniummethyl, sulfonmethyl, and phosphomethyl groups linked to the 4-position of the triazole moiety were soluble in water at neutral or basic conditions and could be analyzed by ^{1}H, ^{13}C APT, COSY, and HSQC NMR. The quaternized cationic chitotriazolan's had high activity against *S. aureus* and *E. coli*, whereas the anionic chitotriazolan's lacked activity.

Graphical abstract



Keywords

Chitosan; Click Chemistry; CuAAC; 1,2,3-Triazole; Antimicrobial activity.

1. Introduction

Chitosan is an abundant, renewable polysaccharide derived from chitin that exhibits attractive biopolymer properties for many biomedical applications such as non-toxicity, biocompatibility, and biodegradability(Elsabee & Abdou, 2013; Jayakumar, Prabaharan, Nair, & Tamura, 2010). It has antimicrobial activity(Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003; Zheng & Zhu, 2003), and regenerative properties(Dash, Chiellini, Ottenbrite, & Chiellini, 2011). Chitosan is also used in drug delivery applications as an absorption enhancer(Kotzé, Lueßen, de Boer, Verhoef, & Junginger, 1999), mucoadhesive polymer(He, Davis, & Illum, 1998), to form nanoparticles(Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010; Qi, Xu, Jiang, Hu, & Zou, 2004), and for gene delivery applications(Park, Saravanakumar, Kim, & Kwon, 2010). Chemical modification of chitosan to improve the properties for the intended application or biological activity is also a very active research field(Harish Prashanth & Tharanathan, 2007). The glucosamine monomer in chitin has three nucleophilic functional groups, the C-2 amino group, the C-3 hydroxyl group, and the C-6 hydroxyl groups, which have been targeted for modification. Most commonly, this is done through either *N*- or *O*- alkylation or acylation(Ifuku, 2014; Sahariah & Másson, 2017). The primary C-6 has also been replaced with other functional groups such as Br, $N(CH_3)_3^+$ or N_3(Gao, Zhang, Chen, Gu, & Li, 2009; Satoh et al., 2006; Zampano, Bertoldo, & Ciardelli, 2010). Chitosan is poorly soluble in most organic solvents, which are often required as the medium for the reactions, and the reported conversion or substitution is only partial with generally less than 50% conversion of targeted groups on the polymer chain. Lack of selectivity is also an issue with many reactions, and a mixed *N*, *O* modification is common.

One way to address this issue is to use protecting groups in the synthesis of chitosan derivatives. The purpose of the protecting groups is to prevent the reaction of the groups that are not targeted for modification and also to improve the solubility in organic solvents. The *tert*-butyl dimethyl silyl (TBDMS or TBS) protection of the hydroxyl groups is especially useful in this regard. Di-3,6-*O*-TBDMS chitosan is well soluble in moderately polar organic solvents, such as dichloromethane and chloroform and has been used for *N*-selective synthesis of *N,N,N*-trialkyl and *N*-acyl derivatives and conjugates with 100% degree of substitution(Rathinam, Ólafsdóttir, Jónsdóttir, Hjálmarsdóttir, & Másson, 2020a; Sahariah, Óskarsson, Hjálmarsdóttir, & Másson, 2015). These derivatives have been investigated as antimicrobial agents(Rathinam, Ólafsdóttir, et al., 2020a; Rathinam, Solodova, Kristjánsdóttir, Hjálmarsdóttir, & Másson, 2020; Sahariah, Óskarsson, et al., 2015), absorption enhancers(Benediktsdóttir, Gudjónsson, Baldursson, & Másson, 2014), and for photo-activated delivery of genes and cancer drugs(Gaware et al., 2017; Gaware et al., 2013).

"Click chemistry" is a term that was first introduced by K. B. Sharpless to describe selective reactions that afford carbon-heteroatom bonds in high yield(Kolb, Finn, & Sharpless, 2001). The copper (I) catalyzed azide-alkyne cycloaddition (CuAAC) was proposed to fit these criteria. The "click chemistry" approach is now commonly used to synthesize bio-conjugates, especially for functionalizing peptides and proteins with different moieties(El-Sagheer & Brown, 2010; Elchinger et al., 2011; Hein, Liu, & Wang, 2008) or conjugating them and other functional moieties to nanoparticles(Lu, Shi, & Shoichet, 2009), liposomes(Fritz et al., 2014), solid surfaces(Sun, Stabler, Cazalis, & Chaikof, 2006), and carbohydrates(Nielsen, Wintgens, Amiel, Wimmer, & Larsen, 2010). Thus, a substituent containing a terminal alkyne or azide group is first introduced by acylation or alkylation, and then the functional moieties are introduced by reaction with a corresponding azide or alkyne.

CuAAC modifications of chitosan have been mainly focused on reactions with the azide introduced at the C-6 position and with the C-2 amine protected with phthaloyl groups(Gao et al., 2009; Luan et al., 2018). The 2-amino group has also been modified with acyl moieties carrying terminal alkyne or azide groups that can subsequently be converted to triazole by the CuAAC reaction. This approach has been used for grafting peptides(Barbosa, Vale, Costa, Martins, & Gomes, 2017; Sahariah, Sørensen, et al., 2015), poly(ethylene glycols)(Kulbokaite, Ciuta, Netopilik, & Makuska, 2009), drug conjugates, and nanoparticles(Q. Li, Sun, Gu, & Guo, 2018; Qing Li, Tan, Zhang, Gu, & Guo, 2015; Sarwar, Katas, Samsudin, & Zin, 2015).

Primary amines, like the 2-amino group of chitosan, can be converted to azide by Cu(II) catalyzed diazo transfer reaction with imidazole-1-sulfonyl azide hydrochloride(Goddard-Borger & Stick, 2007). This approach has been used to convert chitosan prior to CuAAC to introduce PEG moieties(Kulbokaite et al., 2009), or to modify chitosan antimicrobial coatings(Barbosa et al., 2019). This procedure has also been used for the synthesis of insoluble chitosan derivatives(Zhang et al., 2008). The reported grafting ratio for water-soluble derivatives has not been high. For example, a peptide was grafted at a 2 mg/g ratio corresponding to 0.2% degree of substitution (DS)(Barbosa et al., 2017). A previous study found that chitosan could not be converted in more than 40% from amines to triazole via *N*-azidated chitosan(Kulbokaite et al., 2009). In the present work, we aimed to use the CuAAC reaction to synthesize new types of water-soluble carbohydrate polymers starting from chitosan. In these structures, all C-2 primary amino groups of chitosan are to be converted to aromatic 1,2,3-triazole, and thus chitotriazolan is the suggested name for these new structures. Herein, the chitotriazolans were synthesized by two different pathways, starting from di-TBDMS protected chitosan or unmodified chitosan. Six of the derivatives could be solubilized in water and were characterized by FT-IR, NMR, and SEC-MALS. Five

derivatives were insoluble and therefore only analyzed by FT-IR. Antibacterial activity of soluble derivatives was evaluated against *S. aureus* and *E. coli* at pH 7.2.

2. Materials and Methods

2.1. Material

Chitosan (S160302-1-2-3-4, DA of 17%, and MW 108 kDa) was obtained from Primex ehf Siglufjördur, Iceland. All reagent grade chemicals were purchased from Sigma Aldrich (Germany): Methanesulfonic acid, acetic acid, *tert*-butyldimethylsilyl chloride (TBDMS-Cl), imidazole, sodium azide, sulfuryl chloride, trimethylamine, copper sulfate, sodium ascorbate, acetyl chloride, hydrochloric acid, propargyl bromide, *N*-methylpropargylamine, *N,N*-dimethylpropargylamine, 3-butynoic acid, 3-methyl-1-pentyn-3-ol, 2-methyl-3-butyn-2-ol, 3-butyn-2-ol, sodium sulfite, *N,O*-bis(trimethylsilyl)acetamide, tris(trimethylsilyl) phosphite, and 4-bromo-1-butyne. All solvents, including dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF), dichloromethane (DCM), acetone, methanol, ethanol, and acetonitrile, were also obtained from Sigma Aldrich. De-ionized water was treated using a Milli-Q™ filtration system. Dialysis membranes (RC, Spectra/Por, MW cutoff 3500 Da 45 mm) were purchased from Spectrum® Laboratories Inc. (Rancho Dominguez, USA).

2.2. Methods and preparations

2.2.1. Preparation of imidazole sulfonyl azide hydrochloride salt

The imidazole sulfonyl azide hydrochloride salt was prepared following a previously published procedure(Goddard-Borger & Stick, 2007). Briefly, sulfuryl chloride (2.48 mL, 30.77 mmol) was added dropwise at 0 °C to the suspension of sodium azide (2.0 g, 30.77 mmol) in anhydrous acetonitrile (40 mL) under nitrogen, and the reaction mixture was stirred at room temperature overnight. Then imidazole (4.19 g, 61.54 mmol) was added portion-wise

to the reaction mixture at an ice-cooled condition, and the reaction mixture was stirred at room temperature for 3 hours. After that, the reaction mixture was diluted with ethyl acetate (100 mL) and washed with water (3 × 100 mL), and saturated aqueous $NaHCO_3$ solution (2 × 100 mL) in a separatory funnel. The organic phase was dried over Na_2SO_4 and filtered. A solution of HCl in ethanol [50 mL, dropwise addition of acetyl chloride (12 mL) to ice-cooled ethanol (40 mL)] was added to the filtrate, and the mixture stirred at 0 °C to get a white precipitate. The solids were filtered and washed with ethyl acetate to obtained small white needle crystals as a product. The mother liquors were discarded - HAZARD statement: Concentration of mother liquors at this step may result in an explosion(Goddard-Borger & Stick, 2007).

2.2.2. Synthesis of *N*-propargyl *N,N,N*-trimethylammonium bromide salt

The title compound was synthesized according to a reported procedure(Nguyen, Fournier, Asseline, Thuong, & Dupret, 1999). Briefly, trimethylamine (1.48 mL, 16.81 mmol) was dissolved in acetonitrile (100 mL) at -20 °C. Then propargyl bromide (1.27 mL, 16.81 mmol) was added slowly at -20 °C. The reaction mixture was warmed to room temperature and stirred for 24 h, and then the solvent was removed using rotary evaporation and dried under reduced pressure to provide a white solid as a product. Procedures for the synthesis of propargyl sulfonate and butynyl phosphonate are reported in the supplementary information.

2.2.3. OTBDMS-Chitosan amine to azide conversion (A2)

Chitosan OTBDMS(Rathinam, Ólafsdóttir, Jónsdóttir, Hjálmarsdóttir, & Másson, 2020b) (500 mg, 1.26 mmol) was dissolved in 15 mL of DCM and 15 mL of MeOH. After that, imidazole sulfonyl azide hydrochloride (0.395 g, 1.89 mmol) and Et_3N (0.26 mL, 1.89 mmol) were added to the solution. A solution of $CuSO_4$ $5H_2O$ (31 mg, 0.125 mmol dissolved in 1 mL water) was added to the reaction mixture. The color of the reaction mixture changed to a

blue tinge, and the product started to precipitate. The reaction was further stirred at room temperature for 60 h under an N_2 atmosphere. The material was concentrated under reduced pressure. A precipitate was formed, and this was filtered and washed with ethanol and dried for more than one hour by suction. The resulting material had a light bluish color, and the product could be confirmed by IR spectroscopy.

2.2.4. OTBDMS-Chitosan azide to triazole conversion (A3)

OTBDMS-Chitosan azide (700 mg, (1.75 mmol) was dissolved in DMF (20 mL). Then $CuSO_4$ (56 mg, 0.23 mmol in 2.5 mL water) and sodium ascorbate (174 mg, 0.87 mmol in 2.5 mL water) were added to the reaction mixture, followed by *N,N*-dimethylamino-1-propyne (0.94 mL, 8.76 mmol) under nitrogen atmosphere. The reaction mixture was stirred at 50 °C for 48 h. Then, the resulting material was dialyzed against water for three days and freeze-dried. Full conversion of starting material to the product was confirmed by the absence of the azide peak in the FT-IR.

2.2.5. OTBDMS-Chitosan deprotection (1)

O-TBDMS -Chitosan triazole (**A3**) (600 mg) was dissolved in methanol (30 mL) and conc. HCl (5 mL was diluted with 10 mL of methanol) was added slowly. The reaction mixture was then stirred at room temperature for 24 h. After that, the reaction mixture was dialyzed against water for three days (first day 5% NaCl, next two days water) and then freeze-dried. Yield: 325 mg, ^{1}H NMR (400 MHz, D_2O): δ 2.08 (*N*-$COCH_3$), 2.81 (H6'), 2.95 [*N*-$(CH_3)_2$], 3.14 (H6), 3.52 (H5), 3.77 (H4), 3.94 (H3) 4.40 (H2), 4.56 (triazole CH_2), 5.17 (H1), 8.46 (triazole CH).

2.2.6. Chitosan amine to azide conversion (A5)

Chitosan (500 mg, 2.958 mmol) was dissolved in 40 mL of 0.1 M HCl solution, then $NaHCO_3$ (0.248 g, 1.0 equiv) was added to the solution, and the mixture was stirred vigorously for 30 mins. After that, imidazole sulfonyl azide hydrochloride (0.93 g, 4.437 mmol) and $NaHCO_3$ (2.48 g 10.0 equiv) were added slowly in small portions. Then a solution of $CuSO_4$ $5H_2O$ (95 mg, 0.384 mmol) in 1 mL of water and 10 mL of methanol solution was added to the reaction mixture. The reaction mixture was turned to bluish color and was stirred at room temperature for 24 h. Finally, the material was precipitated out using acetone. The precipitate was filtered and washed with water five times and acetone. The product was dried, and the presence of the azide group was confirmed by IR spectroscopy.

2.2.7. General procedure for chitosan azide to triazole conversion (derivatives 3-11)

Chitosan azide (1 equiv.) was dissolved in DMSO (15 mL) at 50 °C. Then CuSO4 (0.13 equiv. in 2.5 mL water) and sodium ascorbate (0.5 equiv. in 2.5 mL water) were added to the reaction mixture followed by alkyne (5.0 equiv.) under nitrogen atmosphere. The reaction mixture was stirred at 50 °C for 48 h. Then, the resulting material was dialyzed against water for three days (first day 5% NaCl, next two days water) and freeze-dried. The products were confirmed by FT-IR to show that the azide peak (at 2109 cm^{-1}) had completely disappeared and by ^1H NMR when solutions in D_2O could be prepared.

2.2.8. Synthesis of derivative 3

Chitosan azide (200 mg, 1.07 mmol) was dissolved in DMSO (15 mL) at 50 °C. Then $CuSO_4$ (34 mg, 0.139 mmol in 2.5 mL water) and sodium ascorbate (106 mg, 0.534 mmol in 2.5 mL water) were added to the reaction mixture, followed by *N*-propargyl-*N,N,N*-trimethylammonium bromide (523 mg, 5.34 mmol). ^1H NMR. Yield: 270 mg for **3**, ^1H NMR (400 MHz, D_2O): δ 2.08 (N-$COCH_3$), 2.90 (H6'), 3.20 [H6, *N*$(CH_3)_3$], 3.52 (H5), 3.78 (H4),

4.44 (H3), 4.58 (H2), 4.77 (triazole CH_2 was merging with D_2O peak), 5.18 (H1) 8.59 (triazole CH). The procedure for derivatives (**4-11**) is reported in supporting information.

2.3. Characterization

2.3.1. NMR and FTIR spectroscopy

The chitotriazolan derivatives were characterized by using ^{1}H NMR and ^{13}C NMR spectroscopy. ^{1}H and COSY NMR spectra were recorded on a Bruker Avance 400 spectrophotometer operating at 400 MHz. The ^{13}C NMR and HSQC spectra were recorded on a Bruker 500 MHz spectrometer equipped with a cryoprobe. NMR samples were prepared in $CDCl_3$, D_2O, or D_2O/DCl in concentrations of 7 – 15 mg/mL. The N-acetyl peak at 2.08 ppm was used as an internal reference in all proton NMR spectra. The FT-IR spectra of the chitosan (CS) and chitotriazolan derivatives were measured using a Thermo Scientific™ Nicolet™ iS10 FTIR spectrometer in the 500 – 4000 cm^{-1} wavelength region. The set number of scans was 64, and the resolution was 4.0 cm^{-1}. Few milligrams of the material were used for each IR spectra and all compounds were measured against a blank background.

2.3.2. Gel permeation chromatography

Average Molecular weight (MW) determination was carried out using gel permeation chromatography (GPC). GPC measurements were done using the Polymer Standards Service (PSS) (GmbH, Mainz, Germany), Dionex Ultimate 3000 HPLC system (Thermo Scientific-Dionex Softron GmbH, Germering, Germany), Dionex Ultimate 3000 HPLC pump, and Dionex Ultimate 3000 autosampler (Thermo Scientific-Dionex Softron GmbH, Germering, Germany), Shodex RI-101 refractive index detector (Shodex/Showa Denko Europe GmbH, Munich, Germany), PSS's ETA-2010 viscometer and MALS detector (PPC SLD 7100). WINGPC Unity 7.4 software (PSS GmbH, Mainz, Germany) was used for data collection and

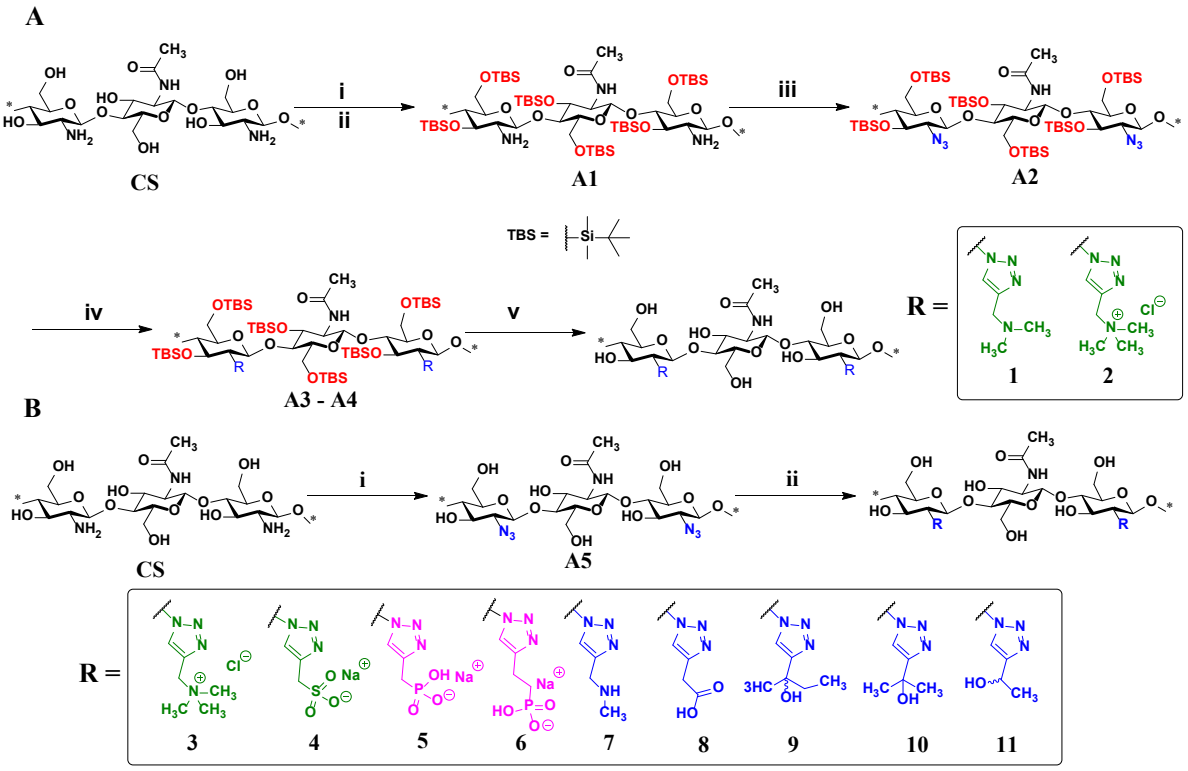
processing. A series of three columns [PSS Novema 10 µ guard (50 x 8 mm), PSS Novema 10 µ 30 Å (150 x 8 mm) and PSS Novema 10 µ 1000 Å (300 x 8 mm)] (PSS GmbH, Mainz, Germany) were used in the HPLC system. Ready Cal-Kit Pullulan standards with M_p (180 – 708000 Da) from PSS (GmbH, Mainz, Germany) were used for calibration. The eluent used was 0.1 M NaCl/0.1% TFA solution. Each sample was dissolved in the same eluent as mentioned above at a concentration of 1 mg/mL at 25 °C using a flow rate of 1 mL/min. Each sample had an injection volume of 100 µL, and the time between injections was 30 min.

2.4. Antibacterial Assay of the Chitosan derivatives

Minimal inhibition concentration (MIC) was measured according to the CLSI standard(CLSI, 2009). The antibacterial activity was tested against two different bacterial species, Gram-positive bacteria *Staphylococcus aureus* (*S. aureus*, ATCC 29213) and Gram-negative bacteria *Escherichia coli* (*E. coli*, ATCC 25922). Before MIC testing, the bacterial strains were cultured on blood agar at 37 °C for 12-18 hours. The bacterial colonies were suspended in saline water and adjusted to 0.5 McFarland and further diluted in Mueller-Hinton broth (MHB) to reach the final concentration of 5×10^5 colony forming units (CFU)/mL in the test wells. The MHB was used for MIC measurement at pH 7.2. Gentamicin, a well-known antibiotic was used as a performance control, MHB without chitosan derivatives or the bacterial solution as a sterility control, and MHB with only the bacterial solution as growth control. The stock solution of compounds was prepared in sterile water at a concentration of 8192 µg/mL, 50 µg/ml of the compounds were added to a microtiter 96-well plate, and two-fold dilutions were done in MHB for concentrations 16 – 8192 µg/ml. Later 50 µL of bacterial 5×10^5 (CFU)/mL suspension was added to each well. The microtiter plates were incubated at 37 °C for 20 to 24 h. The MIC values were observed by the naked eye and determined as the lowest concentrations of the antibacterial agent to completely inhibit the visible growth of microorganisms in the microtiter 96-well plate.

3. Results and Discussion

The main aim of the research work was to develop a procedure to quantitatively convert the primary amino groups of chitosan first to azide groups and then to 1,2,3-triazole moieties to enhance solubility in water. Previous investigations have shown that chitosan azides are insoluble in aqueous solutions and organic solvents(Kulbokaite et al., 2009), limiting the conversion of the amino groups(Zhang et al., 2008). We have used di-OTBDMS protected chitosan to address potential issue with the solubility of the product derivatives(Rathinam, Ólafsdóttir, et al., 2020b). It has been shown that O-TBDMS-chitosan and its derivative is soluble, in most cases, in solvents such as dichloromethane and chloroform(Rúnarsson, Malainer, Holappa, Sigurdsson, & Másson, 2008) (Sahariah, Másson, & Meyer, 2018). Thus, the synthesis was initially attempted starting from O-TBDMS chitosan (**Scheme. 1A**). The conversion to the corresponding azide **(A2)** could be confirmed by FT-IR (**Fig.1),** but to our surprise, it turned out that the O-TBDMS chitosan azide had low solubility in organic solvents and thus could not be fully characterized by NMR. The O-TBDMS chitosan azide did not dissolve in aqueous and instead of organic solvents such as water, aqueous 0.1 M HCl, 0.1 M NaOH, MeOH, acetonitrile, chloroform, dichloromethane, and NMP. Mixed solvents like 1:1 ratio of MeOH:0.1 M HCl solution and acetonitrile:0.1 M HCl solution could neither be used to solubilize this polymer. The material was partially soluble in DMF, and DMSO (this required the material to be stirred for 1−2 h at room temperature or 50 °C). Thus the subsequent CuAAC was carried out in DMF to obtain 4-(*N,N*-dimethylaminomethyl)chitotriazolan **1** and 4-(*N,N,N*-trimethylammoniumethyl)chitotriazolan **2** following the deprotection step.



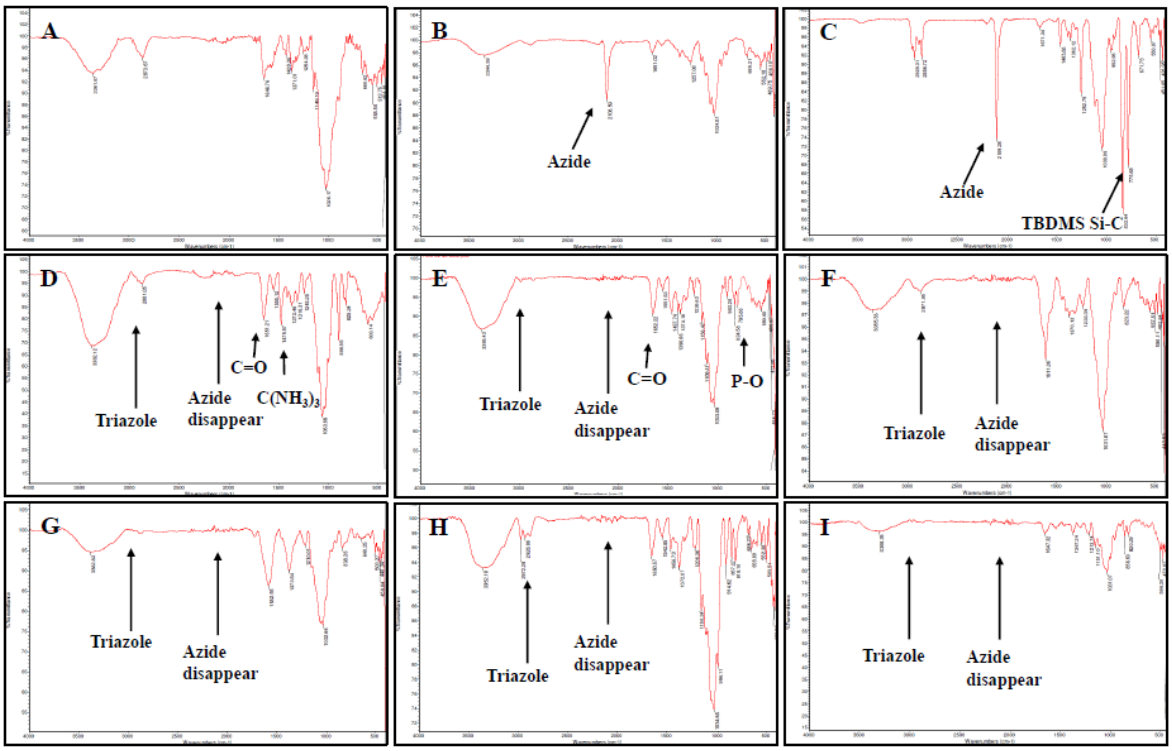
Scheme 1A. Synthesis of chitotriazolan via TBDMS (TBS) protection routes and conditions: (i) methane sulfonic acid, deionized water, 10 °C; (ii) imidazole, TBDMS-Cl, DMSO, RT; (iii) imidazole sulfonyl azide HCl salt, triethylamine, $CuSO_4$ $5H_2O$, DCM, methanol, RT; (iv) $CuSO_4$ $5H_2O$, sodium ascorbate, terminal alkyne, DMF 50 °C; (v) Conc. HCl, methanol RT. **B.** Synthesis of chitotriazolan via without TBDMS protection synthetic routes and conditions: (i) 0.1 M HCl solution, sodium bicarbonate, imidazole sulfonyl azide HCl salt, $CuSO_4$ $5H_2O$, water, methanol, RT; (ii) $CuSO_4$ $5H_2O$, sodium ascorbate, terminal alkyne, DMSO, 50 °C.

In parallel, an alternative route where chitosan was directly converted to azide without the use of protecting groups, was investigated. The conversation to azide could be confirmed with FT-IR, and the aromatic triazole conversion was achieved in near quantitative, which was similar to previous work(Kulbokaite et al., 2009). We found that the material was insoluble in an aqueous solution and organic solvents. However, CuAAC reaction with *N*-propargyl-*N,N,N*-trimethylammonium bromide in DMSO proved to be successful, and the resulting product was soluble in H_2O and could be purified by dialysis, and the product was freeze-dried. Full conversion to the chitotriazolan product was confirmed by the disappearance of the azide peak in the IR spectra and the appearance of a triazole peak at 8.5 ppm in ^1H NMR, corresponding to a 90% degree of substitution for the triazole group.

This procedure was also used to synthesize 4-substituted chitotriazolan derivatives with *N*-methylaminomethyl, carboxymethyl, 2-hydroxybut-2-yl, 2-hydroxyprop-2-yl, and 1-hydroxyethyl side groups. Propargyl sulfonate and propargyl phosphonates were synthesized (see in the supporting information) according to reported procedures(Ouadahi, Allard, Oberleitner, & Larpent, 2012; Wanat et al., 2015) and used to synthesize 4-substituted sulfomethyl, phosphomethyl, and phosphoethyl chitotriazolan derivatives (**Scheme 1B).**

3.1. Characterization by FT-IR spectroscopy

The FT-IR spectra of chitosan, chitosan O-TBDMS azide (**A2**), chitosan azide (**A5**), and chitotriazolans **3**, **5**, and **7**-**10** are shown in **Fig. 1**. The characteristic C=O stretching vibration band at 1652 cm^{-1} for the *N*-acetyl group (DA of 17% present in chitosan starting material) was observed in all spectra. New N_3 bands appeared at 2109 cm^{-1} when the amino group was converted to azide (**Fig. 1 B** and **C**). The azide band disappeared after the CuAAC reaction to form the 1,2,3-triazole on the chitosan backbone at the C-2 position. In **Fig.1 C** strong bands at 775 cm^{-1} and 831 cm^{-1} correspond to Si-C stretching vibrations. A new band at 1475 cm^{-1} can be observed in **Fig. 1 D,** which could be assigned to the weak N-CH_3 absorbance, and a new band appeared at 795 cm^{-1}, confirming the P-O bond for the phosphonate group (**Fig 1. E**). The conversion for insoluble chitotriazolan derivatives were confirmed by the disappearance of the sharp azide peaks (**Fig 1. F, G, H, I)**.



316 **Fig. 1**. FT-IR spectra for chitosan and chitotriazolan derivatives: **CS** (A), derivative **A5** (B),
317 derivative **A2** (C), derivative **3** (D), derivative **5** (E). FT-IR spectra for insoluble
318 chitotriazolan derivative **7** (F), derivative **8** (G), derivative **9** (H), derivative **10** (I).

319 **3.2. Characterization by NMR spectroscopy**

320 The ^{1}H NMR spectra of the water-soluble 4-(*N,N,N*-trimethylammoniummethyl)-
321 chitotriazolan and 4-sulfomethylchitotriazolan are shown in **Fig. 2**. For derivative **3**, the
322 1,2,3-triazole structure could be confirmed by the aromatic proton peak at 8.59 ppm. The
323 quarternary trimethylammonium group for derivative **3** appeared at 3.2 ppm, and the
324 methylene (CH_2) group at 4.8 ppm merged with the HDO peak; however, it was clearly
325 visible in the HSQC spectrum **(Fig. 3C)**. The conversion of the free amino group in the C-2
326 position on chitosan to the 1,2,3 triazole leads to a dramatic shift in the C-2 proton peak from
327 around 2.8 ppm to 4.58 ppm. Other protons of the chitosan backbone are also shifted
328 significantly. The C-6 protons could be observed at 2.90 ppm and 3.2 ppm (merged with the
329 $N(CH_3)_3$ peak) and the C-5, C-4, and C-3 protons at 3.52, 3.78, and 4.44 ppm, respectively.
330 The aromatic triazole proton of derivative **4** was broadened and appeared in a slightly up field
331 position (8.13 – 8.43 ppm) relative to that of derivative **3.** The C-6, C-5, C-4, C-3, and C-2

protons were observed at similar shift values in the two derivatives. The peak for the CH_2 adjacent to the sulfonate groups was observed at 4.27 – 4.42 ppm, merged with the C-3 and C-2 proton peaks.

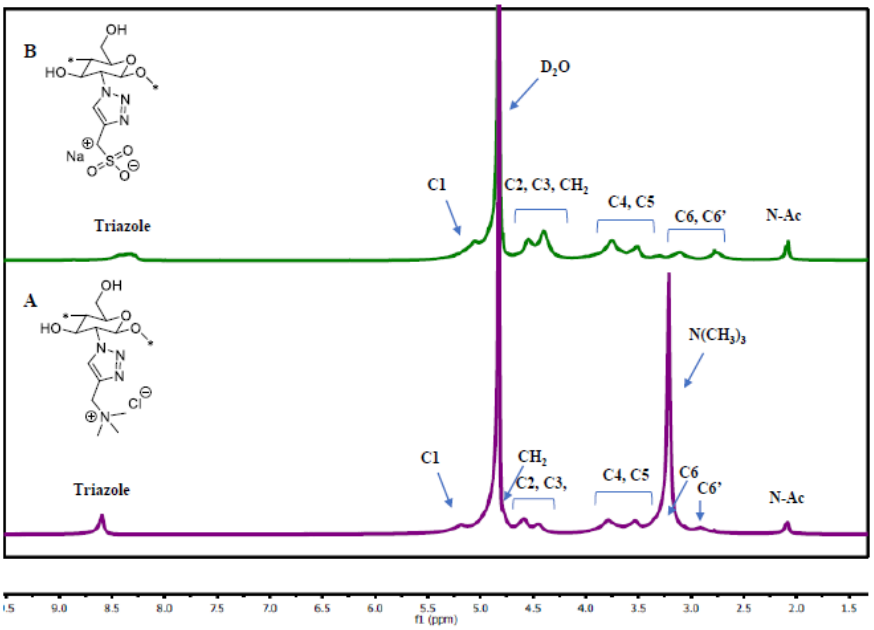


Fig. 2. ^{1}H NMR spectra for derivative **3** (A) and derivative **4** (B).

The aromatic signal for C-4 in the 1,2,3-triazole ring was observed at 137 ppm in the ^{13}C APT NMR spectrum of derivative **1 (Fig. 3A**). The chitosan carbon signals for C-2 to C-6 appeared between 60 – 80 ppm and C-1 at 100 ppm. The correlation between ^{1}H NMR and the COSY spectra further confirmed the assignment of the 1,2,3-triazole peak at 8.59 ppm, and the *N*-acetyl peak at 2.08 ppm (**Fig. 3B)**. The HSQC spectra for derivatives **3** and **4** could be used to confirm the assignment of the proton peaks (**Fig. 3C** and **Fig. 3D).** The complete assignment of all peaks also confirmed that the azide had been fully converted to the new structure**.** The HSQC spectrum clearly shows the trimethylammonium protons at 3.2 ppm for cationic 4-(*N,N,N*-trimethylammonium methyl) chitotriazolan, whereas this peak was not present in the anionic 4-sulfomethyl chitotriazolan spectrum.

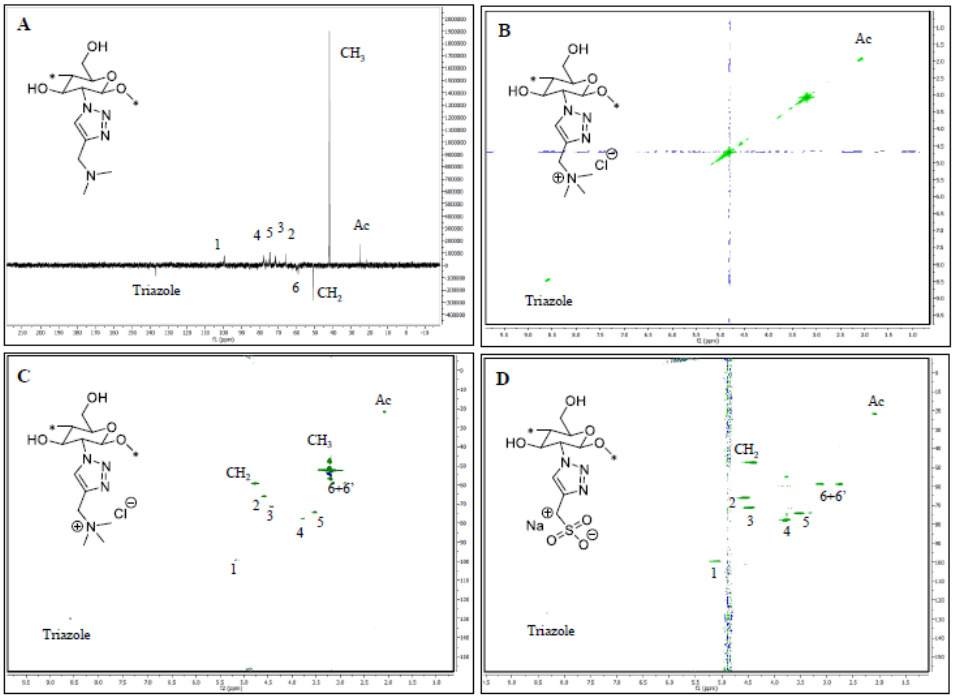


Fig. 3. ^{13}C NMR for derivative **1** (A), COSY NMR for derivative **3** (B), HSQC NMR for derivative **3** (C), and derivative **4** (D).

The degree of substitution (DS), degree of acetylation (DA), and molecular weight (MW) of derivatives **1-6**, are shown in **table 1.** The integration of the NMR peaks in the cationic chitotriazolan derivatives indicated more than 90% conversion from the free amino group in chitosan to the 1,2,3-triazole. However, the peaks were broad, and this could influence the accuracy. Only one peak could be observed for each monomer proton of the chitotriazolan backbone, and this was consistent with 100% conversion. The average molecular weights of derivatives **1** and **2** were more than four times less than the MW of the starting material. This reduction in MW was caused by acid hydrolysis of the polymer chain, which occurs when the chitosan mesylate salt is prepared and in the deprotection reaction to remove TBDMS(Sahariah et al., 2014). The average MW of materials **3** and **5,** synthesized without the use of protection groups had about twice the MW of the starting material, which was consistent with the increase in the MW of the monomer units when chitosan was converted to chitotriazolan derivatives. The MW of 4-sulfomethyl chitotriazolan **4** and 4-phosphoethyl chitotriazolan **6** were found to be around 6 KD which was much less than expected (see SI.

MW. chromatogram profile and **S.Table 1**). This was probably due to low solubility in the mobile phase and that the higher MW material was removed in the filtration of the samples.

Table 1. The degree of substitution (DS), degree of acetylation (DA), and molecular weight analysis for chitotriazolan derivatives.

Derivatives	DS-TM [a b]	DA [a]	DS-Triazole [a]	MW (kDa)	Polydispersity Index (D)
1	0.98	0.08	0.86	28.94	1.76
2	0.73	0.09	0.68	17.05	1.72
3	0.98	0.18	0.9	214.59	1.97
4	NA [c]	0.17	0.8	(6.26) [d]	(1.69) [d]
5	NA	ND [e]	ND	220.02	2.69
6	NA	ND	ND	(5.58) [d]	(1.04) [d]

[a] Degree of substitution (DS), degree of acetylation (DA), and DS-triazole were calculated based on ^{1}H NMR spectroscopy. [b] TM-Trimethylation, [c] Not available, [d] These samples were poorly soluble in the mobile phase but could be analyzed after the insoluble material (about 80%) had been removed by filtration. [e] Not determined.

3.3. Solubility Analysis

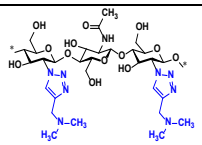
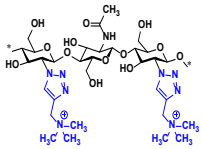
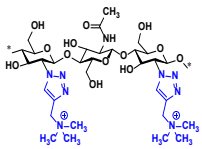
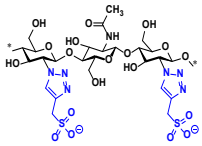
Cationic derivatives **1**, **2**, **3**, and sulfonated anionic derivative **4** was completely soluble in water at neutral pH. 4-Phosphoethyl-chitotriazolan **6** was soluble in 0.1 M sodium hydroxide solution, and 4-phosphomethyl-chitotriazolan **5** was partially soluble. The 4-(*N*-methylaminomethyl)-chitotriazolan **7**, 4-carboxymethyl-chitotriazolan **8,** and the 4-(hydroxyalkyl)-chitotriazolan derivatives **9**-**11** (marked in blue color in the **scheme. 1B**) were insoluble in all solvents and solvent mixtures tested. Derivatives **2**-**6** had fully ionized side groups, and this may contribute to better solubility. The low MW of derivative **1** may explain why it had better solubility than derivative **7,** which has a similar structure with one less *N*-methyl group.

3.4. Antibacterial properties for chitotriazolan derivatives

The antibacterial activity of chitosan and chitosan derivatives is influenced by several factors, including the degree of substitution (DS), molecular weight, ionic interactions, and the structure of the substitutents(Kong, Chen, Xing, & Park, 2010; Sahariah & Másson, 2017).

The water-soluble chitotriazolan derivatives were studied for antibacterial activity against *S. aureus* and *E. coli* bacteria at pH 7.2 (**Table 2**). The cationic chitotriazolan derivative **3** was most active against the bacteria with MIC equal to 64 ug/mL, whereas the anionic derivative **4** was inactive. The monomer structure of derivative **2** was identical to derivative **3** but the former was more than 30 times less active against the bacteria. Derivative **2** had a markedly lower molecular weight than **3,** and there were some residual TBDMS groups (< 3% for **1** and < 0.4% for **2**) left from the deprotection step, which could explain this difference. This is also a consideration for derivative **1,** which was inactive and had a similar structure with one less *N*-methyl group than derivatives **2** and **3**. The most active derivative **3** was also tested against *E. faecalis* (ATCC 29212) and *P. aeruginosa* (ATCC 27853) and the MIC values found to be 1024 µg/mL and 128 µg/mL, respectively.

Table 2. Antibacterial activity of water-soluble chitotriazolan derivatives

Derivatives	Structure	MIC (µg/mL)	
		S. aureus	*E. coli*
1		≥8192	≥8192
2		2048	2048
3		64	64
4		≥8192	≥8192
5	Gentamicin	0.25	1

4. Conclusion

In the current work, we were successful in obtaining a near-complete conversion of the 2-amino group of chitosan to 1,2,3-triazole and obtain the first water-soluble chitotriazolan derivatives. Eleven chitotriazolan derivatives were synthesized through two routes, and four of the structures had good water solubility. The derivatives were characterized by FT-IR, ^{1}H, and 2D NMR techniques as well as SEC-MALS to determine the structure and molecular weight. The antibacterial activity was evaluated against *S. aureus* and *E. coli* at pH 7.2. The cationic chitotriazolan derivatives had significant antibacterial activity, whereas the anionic chitotriazolans were inactive.

Chitoriazolans represent a new class of biopolymers with an aromatic 1,2,3-triazole side group on the 2-deoxyglucopyranose monomer unit. Ionic chitotriazolan derivatives can be water-soluble and the *N,N,N*-trimethylammoniummethyl derivatives **2** and **3** were active against bacteria. The ease of synthesis and structural modification of this new class of biopolymers should stimulate further research into the biological and other properties and utility for diverse applications.

Supplementary data

There is supporting information for this article.

Authors Information

Már Másson (MM) designed the research plan in collaboration and was the principal supervisor for Sankar Rathinam. The synthesis work and some of the characterization was done by Sankar Rathinam (SR). The advanced NMR studies were done by Mikkel B. Thygesen (MBT). The antimicrobial assay was done by SR, supervised by Martha Á. Hjálmarsdóttir (MAH). SR and MM prepared the manuscript, and all co-authors participated

in interpreting the results and approved the final version. MM and MBT were responsible for funding. This work presents no conflict of interest for any of the authors.

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