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Antibacterial activity of methylated chitosan and chitooligomer derivatives: Synthesis and structure activity relationships

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Abstract

The purpose of this study was to synthesize series of methylated chitosaccharide derivatives, possessing various degree of methylation, and to determine their structure activity relationship (SAR) with regard to their antibacterial effect against *Staphylococcus aureus*. Chitosan polymer and chitooligomers were used as starting materials and were methylated by reaction with methyl iodide. Depending on the reaction conditions the degree of *N*-quaternization ranged from 0% to 74%, with varying degree of *N*,*N*-dimethylation, *N*-monomethylation and *O*-methylation. More selective *N*-quaternization could be obtained with protection group strategy. At pH 5.5 the chitosaccharide polymers and their methylated derivatives were active against *S. aureus* with minimal inhibitory concentration (MIC) ranging from 16 to 512 μg/mL. At pH 7.2 the non-quaternized derivatives were inactive but their highly *N*-quaternized derivatives showed MIC as low as 8 μg/mL. The chitooligomers, as well as their derivatives, were inactive at both pH's. The SAR studies revealed that *N*-quaternization was mainly responsible for the antibacterial effects at pH 7.2, whereas it did not contribute to the antibacterial activity under acidic conditions.

Keywords: Chitosan; Trimethylated chitosan; Quaternary chitosan; Antibacterial activity; Structure activity

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1. Introduction

Chitosaccharides are classified as either chitin or chitosan. These are oligo- and polysaccharides composed of β-1–4 linked *N*-acetyl-glucosamine (GluNAc) and glucosamine (GluN) units. The monomer is mainly *N*-acetyl-glucosamine in chitin, whereas glucosamine predominates in chitosan.

Chitosaccharides have been used as food additives [1], for water treatment [2] and in cosmetics [3], as well as biologically active compounds in pharmaceutical products [4]. Their biological activity includes mucoadhesion [5,6], absorption enhancement [7,8], activation of tissue regeneration [9,10], and antimicrobial activity [11–13]. Chitosan is active against many gram-negative and gram-positive bacteria [14–16] and has the advantage of low toxicity towards mammalian cells as compared to various other disinfective materials [17]. In the food industry, chitosan is used for coating of vegetable and fruits to enhance their shelf-life [17,18]. Their antibacterial activity is also important when chitosan is used as wound-healing agents [19–21].

The antimicrobial activity of chitosaccharides is a function of several structural factors such as their degree of *N*-acetylation and their molecular weight, but the environmental conditions can also have significant effect [13,15,20–22]. The antimicrobial activity has been linked to the ionisation of the glucosamine amino group and, consequently, found to increase with decreasing pH [12,23–26]. The reported antibacterial activities of chitosaccharides show a broad range of activity depending on the properties of the material used and the type of assay. The assay conditions have not been standardized and it is therefore difficult to compare results from one study to another.

There is considerable interest in biologically active derivatives of chitosaccharides. *N*,*N*,*N*-Trimethylchitosan (TMC) is one of the most promising chitoderivatives that currently are being investigated. Trimethylated chitosan has a fixed charge on the quaternized amino groups and is therefore soluble in the lower sections of the gastrointestinal tract, i.e. under neutral and basic conditions [27,28]. Trimethylated chitosan can act as an absorption enhancer at these conditions, whereas chitosan has no activity due to its limited solubility. The methylated derivatives can be produced in a one-step reaction of chitosan with methyl iodine (MeI) in the presence of sodium hydroxide as base using *N*-methyl-2-pyrrolidone (NMP) as solvent. The methylation step can be repeated to increase the trimethylation [29–32]. The reported degree of trimethylation in TMC varies from 10% to 80%. Significant *N*,*N*-dimethylation, *N*-monomethylation and *O*-methylation is also observed [29,33–35]. The *O*-methylation can decrease the water solubility significantly, but the solubility will also decrease with increasing molecular weight [34]. Researches indicate that it is very difficult to get fully *N*,*N*,*N*-trimethylated chitosan with no *O*-methylation with these synthetic strategies [29,33–35]. Various properties of TMC have been investigated and promising results have been obtained in applications such as in gene delivery [36], absorption enhancement of macromolecular drugs [28], colonic drug delivery [37] and as absorption enhancement in Caco-2 membrane model [38]. Structure activity relationship (SAR) investigations associated with these applications have mainly been focused on the role of *N*-quaternization without much attention to the role of degree of *O*-methylation or *N*,*N*-dimethylation that is normally observed in these heterogeneous TMC materials [39–41].

The antibacterial effect of TMC is a useful property in medical applications. Other *N*-quaternized chitosaccharide derivatives, such as *N*-alkyl [26,42] and *N*-[(2-hydroxy-3-trimethylammonium)propyl] chitosan (HTCC) [43] have been shown to posses significant antibacterial activity. However these and other related compounds have only been tested at non-standardized conditions and thus it is difficult to determine which modifications will give the most active materials.

In the present work we have investigated the antibacterial activity of polymeric and oligomeric TMC. We have investigated how the reaction conditions can be adjusted to affect the relative *N*- and *O*-methylation, and protection strategy has also been used to steer the reaction towards *N* modification. The antibacterial activities against *Staphylococcus aureus* were performed at pH 5.5 and 7.2 according to the Clinical and Laboratory Standards Institute (CLSI) methods for dilution antimicrobial susceptibility test from bacteria that grow aerobically (formerly National Committee for Clinical Laboratory Standards (NCCLS)) [44]. The results were used to determine the effect of *O*-methyl-, *N*-mono-, *N*,*N*-di-, and *N*,*N*,*N*-trimethylation on antibacterial effect at different pH.

2. Experimental section

2.1. Materials

Genis EHF (Reykjavik, Iceland) provided the chitosaccharide starting materials. Three starting materials were used; chitosan I with average MW 8.1 kDa as determined by estimating the number of end reducing sugars with method previously described by Miller [47] and with a degree of *N*-acetylation of 5%, chitooligomer II average MW 775 Da determined with MALDI TOF [45] and with degree of *N*-acetylation of 52%, and chitooligomer III with average MW ~775 Da and with *N*-acetylation of 18% (synthesized from chtiooligomer II, see Section 2.5.1). All other chemicals used were commercially available and used as received except pyridine, which was distilled over KOH. Dialysis membrane (Spectrapore@MW cutoff 500 and 3500 Da) was purchased from Spectrum laboratories Inc. (Rancho Dominguez, USA).

2.2. Characterization

2.2.1. Estimation of degree of substitution with NMR spectroscopy

All ^{1}H-NMR and ^{13}C-NMR samples were measured with Bruker AVANCE 400 (Bruker Biospin GmbH, Karlsruhe, Germany) operating at 400.13 and 100.61 MHz, respectively at 298 °K. The *N*-acetyl peak was used as internal reference with DMSO, D_2O or D_2O/DCl (DCl = deuterium chloride) as solvents. The measurements were done without water suppression.

The degree of substitution for the TMC derivatives was calculated using the combined integrals, in the ^{1}H NMR spectra (D_2O or D_2O/DCl as solvents), for the H-2 (GluNAc), H-3, H-4, H-5, H-6, and H-6′ (6H) peaks at δ 3.6–4.5 and H-2 (GluN) peak at 3.10 ppm (3.37 with D_2O/DCl as solvent). This integral ([H-2, H-3, H-4, H-5, H-6, H-6′]) represented six protons. Following equations were used to estimate the substitution degree:

(1) % *N*,*N*,*N*-Trimethylation = $[[N(CH_3)_3]/[\text{H-2, H-3, H-4, H-5, H-6, H-6}'] \times 6/9] \times 100$
(2) % *N*,*N*-Dimethylation = $[[N(CH_3)_2]/[\text{H-2, H-3, H-4, H-5, H-6, H-6}'] \times 6/6] \times 100$
(3) % *N*-Monomethylation = $[[N(CH_3)]/[\text{H-2, H-3, H-4, H-5, H-6, H-6}'] \times 6/3] \times 100$
(4) % *O*-Methylation = $[[O(CH_3)]/[\text{H-2, H-3, H-4, H-5, H-6, H-6}'] \times 6/6] \times 100$
(5) % *N*-Acetylation = $[[C{=}O(CH_3)]/[\text{H-2, H-3, H-4, H-5, H-6, H-6}'] \times 6/3] \times 100$ [46]

where $[N(CH_3)_3]$, $[N(CH_3)_2]$, and $[N(CH_3)]$ are the integrals of the *N*,*N*,*N*-trimethyl- (δ 3.30 ppm), *N*,*N*-dimethyl- (δ 2.87 ppm or 3.00 with D_2O/DCl as solvent), and *N*-monomethylamino (δ 2.77 ppm or 2.80 with D_2O/DCl as solvent) singlet peaks, respectively. $[O(CH_3)]$ and $[C{=}O(CH_3)]$ are the integrals of the *O*-methyl (δ 3.35 ppm, for O^3–CH_3 and 3.43 for O^6–CH_3) and acetyl (δ 2.0 ppm) singlet peaks, respectively. The substitution degree is given in percentage.

2.2.2. FTIR analysis of the derivatives

All IR-measurements were done in AVATAR 370 FT-IR (Thermo Nicolet Corporation, Madison, USA). Samples (2–10 mg) were mixed thoroughly with KBr. For the TMC I derivatives the material was dissolved in water and mixed with NaCl and freeze-dried to give crystalline material which could be grounded and mixed with KBr. The sample was pressed into pills with a Specac compressor (Specac Inc., Smyrna, USA). FTIR was used to identify the attachment and removal of protection groups in the phthaloyl and trityl chitosan derivatives that were poorly soluble in common NMR solvents.

2.2.3. Characterization with elemental analysis

Elemental analysis was performed with a Thermo Quest CE EA1110-CHNS-O elemental analyzer (Thermo Nicolet Corporation, Waltham, USA). The C/N ratio found with elemental analysis was used to evaluate the accuracy of the ^{1}H NMR results. Elemental analysis was also used to evaluate the degree of substitution for the compounds that were not soluble in the NMR solvents.

2.3. Antibacterial activity measurements

The antibacterial tests were performed according to the CLSI standard. The media used for MIC measurements was Mueller Hinton broth (Oxoid) (Hamshire, UK) at pH 7.2 and blood agar (heart infusion agar (Oxoid) with 5% (v/v) defibrinated horse blood) for measurements of minimum lethal concentration (MLC). The Mueller–Hinton broth was also adjusted with HCl (5 N) to pH 5.5 for measurements at the lower pH. The samples were dissolved in the broth to obtain a concentration of 8192 μg/mL. The measurements were expressed in μg/mL. The broth dilutions and the agar plates were incubated

for 24 h at 37 °C, in ambient air. *S. aureus* (ATCC 25923) was selected from the American Type Culture Collection (ATCC), representing clinically important species and a strain recommended by the CLSI for quality control in susceptibility testing.

2.4. Surface activity measurements and calculations

Surface tension was measured at room temperature (RT) using the ring method (ring radius 4.900 mm) with K9Mk1 tensiometer (from Krüss, Hamburg, Germany). Measurements were done with the compounds dissolved in deionized water (Milli-Q®, Millipore Corporation, Billerica, USA) or 0.01 M acetate buffer, pH 5.5. Sodium dodecyl sulfate (SDS) was used as reference compound. Sample concentration for the measurements ranged from 0.00156% to 1% (w/v) using five to eight different concentrations for the investigation of each compound. Three replicates for each concentration were measured. The critical micelle concentration (CMC) can be determined as the intercept of the two linear segments of the surface tension vs concentration curve.

2.5. The synthesis

2.5.1. Chitooligomer III

Two grams of chitooligomer II was deacetylated by dissolving it in 600 mL of a freshly made 40% (w/v) sodium hydroxide solution. The mixture was stirred under N_2 for 48 h at RT. The mixture was then neutralized with 1 M HCl, precipitated with acetone and washed with ether. The product was desalted by nanofiltration (filter type AFC 30 NF, PCI membranes, Hampshire, UK) for 12 h resulting in a 4% (w/w) NaCl chitosan dry material after freeze-drying (Snijders Scientific LY-3-TT, Tilburg, Netherlands). Salt concentration was determined with titration.

^{1}H NMR (400 MHz, D_2O) δ ppm 2.1 (s, $CH_3C{=}O$), 3.2 (m, H-2 GluN), 3.6–4.1 (m, H-2 (GluNAc), H-3, H-4, H-5, H-6, H-6′), 4.9 (partly overlapped by water peak, H-1). ^{13}C NMR δ ppm 19.5 (CH_3), 51.5 (C-2), 57.4 (C-6), 65.6 (C-4), 71.6 (C-3), 73.6 (C-5), 95.1 (C-1 GluN), 98.6 (C-1 GluNAc), and 171.9 (C=O). FTIR (KBr):*v* 3376 (br, OH), 2880 (m, C–H), 1651 (vs, C=O amide I), 1556 (vs, C=O amide II) cm^{-1}.

2.5.2. Methylation procedure A

The starting chitosan material (Table 1) was dissolved in DMF:H_2O [50:50] (v/v). Methyl iodide (MeI) and sodium hydroxide (NaOH) were then added to the solution. Equivalent quantities (Table 1) were calculated on the basis of the number of free amino groups in the starting material. The reaction mixture was vigorously stirred for a set time at the given temperature. Water was then removed by evaporation under reduced pressure. The methylated material was precipitated and washed with cold ethanol (96%) (TMC I) or acetone (TMC II and III). In some cases half of the precipitated material (Table 1) was taken through a second step with the same reaction conditions as in the first step. The filtered compounds were dissolved in water and dialyzed against two changes of freshly distilled water using 3500 molecular weight cutoff (MWCO) dialysis membrane (Spectrum Laboratories Inc., Rancho Dominguez, USA) for the high molecular weight material (chitosan I) and 500 MWCO dialysis membranes for the chitooligomer (chitooligomer II and chitooligomer III). The solution was then freeze-dried. The sponge like material was ion exchanged by dissolving it in a 5% (w/v) NaCl solution and precipitated with cold ethanol (96%) (TMC I) or acetone (TMC II and TMC III). The material was dialyzed and freeze-dried again as described above.

^{1}H NMR (400 MHz, D_2O) δ ppm 2.00 (s, $CH_3C{=}O$), 2.77 (2.80 when D_2O/DCl as solvent (m, N–CH_3)), 2.87 (3.00 when D_2O/DCl as solvent (s, N–$(CH_3)_2$)), 3.10 (3.37 when D_2O/DCl as solvent (m, H-2 GluN and GluN substituted)), 3.30 (s, N–$(CH_3)_3$), 3.6–4.5 (m, H-2 (GluNAc), H-3, H-4, H-5, H-6, H-6′), 4.9–5.3 (m, partly overlapped by water peak, H-1). ^{13}C NMR δ ppm 19 ($CH_3C{=}O$), 34 (CH_3), 44 (($CH_3)_2$), 56 (($CH_3)_3$), 59 (C-2), 63 (C-6), 70 (C-4), 77 (C-3), 79 (C-5), 99 (C-1), and 173 (C=O). FTIR (KBr):*v* 3423 (br, OH/NH), 2930 (m, C–H), 1625 (vs, C=O amide I) and 1476 (tertiary N-CH_3) cm^{-1}.

2.5.3. Methylation procedure B

The starting material (Table 2) was dissolved in NMP along with 4.8 g NaI forming a clear solution. 1.5 eq. of freshly prepared 20% (w/v) sodium hydroxide solution was added to the mixture followed by addition of 5.4 eq. of MeI, which was added carefully. The equivalent was calculated from the free hydroxyl and amino content from the starting material. The reaction was carried out at 60 °C for 1 h. After cooling the solution down to room temperature the compound was precipitated with cold ethanol (96%) (chitosan I and 6-*O*-Tritylchitosan I

Table 1
Reaction conditions for the synthesis of the methylated compounds, using 50:50 DMF:H_2O as solvent, and degree of substitution determined by ^1H NMR spectroscopy

Product	Starting material	Eq. MeI	Eq. NaOH	Temperature (°C)	Time (h)	Steps	% $N(CH_3)_3$	% $N(CH_3)_2$	% $N(CH_3)$	% O–(CH_3)	% *N*-Acetyl
TMC I 75 A	Chitosan I	6	3	75	2	1	3	42	21	0	2
TMC I 50 A	Chitosan I	6	3	50	8	1	3	37	24	0	2
TMC I 21 A	Chitosan I	6	3	21	48	1	4	40	17	0	2
TMC I 75 B	Chitosan I	6	3	75	2	2	12	66	7	0	2
TMC I 50 B	Chitosan I	6	3	50	8	2	20	70	9	0	1
TMC I 21 B	Chitosan I	6	3	21	48	2	29	59	2	0	2
TMC I 30	Chitosan I	12	3	75	0.5	1	2	18	28	0	4
TMC I 60	Chitosan I	12	3	75	1	1	3	21	33	0	4
TMC I 120	Chitosan I	12	3	75	2	1	4	28	30	0	4
TMC I 1 1/2	Chitosan I	6	1.5	75	2	1	1	18	26	0	2
TMC I 6	Chitosan I	6	6	75	2	1	1	30	27	0	3
TMC I 9	Chitosan I	6	9	75	2	1	0	12	23	0	3
TMC II 75 A	Chitooligomer II	6	3	75	2	1	0	8	13	0	34
TMC II 50 A	Chitooligomer II	6	3	50	8	1	2	17	12	0	35
TMC II 21 A	Chitooligomer II	6	3	21	48	1	1	17	6	0	46
TMC II 75 B	Chitooligomer II	6	3	75	2	2	4	30	13	0	35
TMC II 50 B	Chitooligomer II	6	3	50	8	2	10	42	16	0	34
TMC II 21 B	Chitooligomer II	6	3	21	48	2	15	40	9	0	35
TMC III 75 A	Chitooligomer III	6	3	75	2	1	3	25	21	0	17
TMC III 50 A	Chitooligomer III	6	3	50	8	1	7	36	17	0	19
TMC III 21 A	Chitooligomer III	6	3	21	48	1	5	31	18	0	17
TMC III 75 B	Chitooligomer III	6	3	75	2	2	3	25	21	0	17
TMC III 50 B	Chitooligomer III	6	3	50	8	2	26	48	2	0	16
TMC III 21 B	Chitooligomer III	6	3	21	48	2	24	42	4	0	15

Table 2
Reaction conditions for the synthesis of the methylated compounds in Section 2.5.3 and 2.5.7 and degree of substitution

Product	Starting material	Steps	% $N(CH_3)_3$	% $N(CH_3)_2$	% $N(CH_3)$	% O^3–(CH_3)	% O^6–(CH_3)	% *N*-Acetyl	% O–Tr.	% *N*-pht	Analysis method
TMC I 60 A[a]	Chitosan I	1[c]	18	34	14	13	32	2	–	–	NMR
ONPM-Chitosan I[a]	Chitosan I	3[c]	74	15	1	29	65	2	–	–	NMR
ONPM-Chitosan II[a]	Chitooligomer II	3[c]	71	0	0	37	45	22	–	–	NMR
ONPM-Chitosan III[a]	Chitooligomer III	3[c]	63	20	4	21	48	18	–	–	NMR
6-*O*-Trityl-*N*-methyl chitosan I A[b]	6-*O*-Tritylchitosan I	1[c]	28	29	12	10	15	2	76	0	Elemental analysis
6-*O*-Trityl-*N*-methyl chitosan I B[b]	6-*O*-Tritylchitosan I	3[c]	69	11	0	31	39	2	76	0	Elemental analysis
N-methyl chitosan I A[a]	6-*O*-Trityl-*N*-methyl chitosan I A	1[d]	28	29	12	10	15	2	0	0	NMR
N-methyl chitosan I B[a]	6-*O*-Trityl-*N*-methyl chitosan I B	1[d]	69	11	0	31	39	2	0	0	NMR

[a] Measured with D_2O/DCl as solvent.
[b] Not soluble in DMSO, D_2O or D_2O/DCl.
[c] Methylation procedure B, Section 2.5.3.
[d] Trityl deprotection, Section 2.5.7.

as starting materials) or acetone (chitooligomer II and chitooligomer III as starting materials), filtered and then washed three times with diethyl ether. In some cases the precipitated material was taken through two additional steps (Table 2). The material was dissolved in NMP along with NaI. 1.5 eq. of NaOH followed by addition of 5.4 eq. of MeI and stirred for 30 min at 60 °C. Then 2.2 eq. of methyl iodine was added again along with 1.5 eq. of NaOH tablets and the reaction continued for additional hour. The isolation was conducted by the same procedure as for compounds in Section 2.5.2.

(TMC III 60 A, *O*,*N*-pentamethyl chitosan (ONPM-Chitosan) I, II, III) ^{1}H NMR (400 MHz, D_2O) δ ppm 2.00 (s, $CH_3C{=}O$), 2.77 (2.80 when D_2O/DCl as solvent (m, N–CH_3)), 2.87 (3.00 when D_2O/DCl as solvent (s, N–$(CH_3)_2$)), 3.10 (3.37 when D_2O/DCl as solvent (m, H-2 GluN and GluN substituted)), 3.30 (s, N–$(CH_3)_3$), 3.35 (s, O^6–CH_3), 3.43 (s, O^3–CH_3) 3.6–4.5 (m, H-2 (GluNAc), H-3, H-4, H-5, H-6, H-6′), 4.9–5.3 (m, partly overlapped by water peak, H-1). ^{13}C NMR δ ppm 19 ($CH_3C{=}O$), 34 (CH_3), 44 ($(CH_3)_2$), 56 ($(CH_3)_3$), 61 (O^6–CH_3), 63 (O^3–CH_3), 58 (C-2), 63 (C-6), 71 (C-4), 77 (C-3), 79 (C-5), 99 (C-1), and 173 (C=O). FTIR (KBr):*v* 3423 (br, OH/NH), 2888 (m, C–H), 1625 (vs, C=O amide I) and 1476 (tertiary N–CH_3) cm^{-1}. (**6-*O*-Trityl-*N*-methyl chitosan I A and B**) FTIR (KBr):*v* 3440 (br, OH), 3055 (vs, C–H trityl), 2929 (m, C–H), 1665 (vs, C=O amide I) and 1488 (tertiary N–CH_3) cm^{-1}, 1448 (C=C trityl), and 764, 747 (arom, trityl) cm^{-1}.

2.5.4. *N*-Phthaloylchitosan I

15.00 g Chitosan I was dissolved in DMF:H_2O mixture [95:5] (v/v) and stirred overnight. 41.35 g of phthalic anhydride (three equimolar calculated in the same manner as in Section 2.5.2) was added and continued stirring at 120 °C for 8 h. The phthaloylated material was precipitated by pouring the reaction mixture into ice water. The precipitated material was filtered and washed with methanol and diethyl ether. The product was then washed with warm acetone using soxhlet apparatus and finally dried under vacuum. The substitution degree was calculated from elemental analysis.

(*N*-phthaloylchitosan I) FTIR (KBr):*v* 3446 (br, OH/NH), 2929 (vs, C–H), 1772 (C=O imide) and 1716 (C=O imide), 1387 (C=C pht), and 724 (arom, pht) cm^{-1}. Anal. Calculated $(C_8H_{13}NO_4)_{0.03}$

$(C_6H_{11}NO_4)_{0.44}$ $(C_{14}H_{13}NO_6)_{0.53}$: C, 51.24; H, 4.85; N, 5.56. Found: C, 51.32; H, 5.26; N, 5.60.

2.5.5. *N*-Phthaloyl–6-*O*-tritylchitosan I

9.99 g of *N*-Phthaloylchitosan I was mixed in 125 mL of dry pyridine. 96.25 g of triphenylmethane chloride was added and the solution was stirred at 100 °C under nitrogen for 15 h. The solvent was evaporated to dryness and the product washed thoroughly with acetone and diethyl ether. The substitution degree was calculated from elemental analysis.

FTIR (KBr):*v* 3468 (br, OH), 3055 (vs, C–H trityl), 1772 (C=O imide) and 1716 (C=O imide), 1490, 1448 (C=C trityl), 1381 (C=C pht), 764, 747 (arom, trityl) and 724 (arom, pht) cm^{-1}. Anal. calculated: $(C_6H_{11}NO_4)_{0.24}$ $(C_{25}H_{25}NO_4)_{0.20}$ $(C_{27}H_{27}NO_5)_{0.03}$ $(C_{33}H_{27}NO_6)_{0.53}$: C, 69.13; H, 5.29; N, 3.23. Found: C, 68.54; H, 5.46; N, 3.20.

2.5.6. 6-*O*-Tritylchitosan I

N-phthaloyl-6-*O*-tritylchitosan I derivative was dissolved in H_2O. Hydrazine monohydrate was added (66 eq.) and the solution was stirred for 13.5 h at 100 °C and then 17.5 h at RT. The material was precipitated with H_2O. The product washed with diethyl ether.

FTIR (KBr):*v* 3446 (br, OH), 3055 (vs, C–H trityl), 1658 (C=O amide I), 1596 (C=O amide II), 1490, 1448 (C=C trityl), and 764, 747 (arom, trityl) cm^{-1}. Anal. calculated: $(C_6H_{11}NO_4)_{0.21}$ $(C_{25}H_{25}NO_4)_{0.76}$ $(C_{27}H_{27}NO_5)_{0.03}$: C, 65.41; H, 5.71; N, 3.58. Found: C, 66.45; H, 6.20; N, 4.73.

2.5.7. *N*-methylated chitosan I A and *N*-methylated chitosan I B

One gram of 6-*O*-Trityl-*N*-methyl chitosan I A and 6-*O*-Trityl-*N*-methyl chitosan I B (Table 2) was stirred in 124 mL of 1 M HCl for 24 h. The solvent was evaporated and the solid material washed with ethanol (96%) and diethyl ether.

^{1}H NMR (400 MHz, D_2O) δ ppm 2.00 (s, CH_3C=O), 2.77 (2.80 when D_2O/DCl as solvent (m, N–CH_3)), 2.87 (3.00 when D_2O/DCl as solvent (s, N–$(CH_3)_2$)), 3.10 (3.37 when D_2O/DCl as solvent (m, H-2 GluN and GluN substituted)), 3.30 (s, N–$(CH_3)_3$), 3.35 (s, O^6–CH_3), 3.43 (s, O^3–CH_3) 3.6–4.5 (m, H-2(GluNAc), H-3, H-4, H-5, H-6, H-6′), 4.9–5.3 (m, partly overlapped by water peak, H-1). ^{13}C NMR δ ppm 19 (CH_3C=O), 34 (CH_3), 44 (($CH_3)_2$), 56 (($CH_3)_3$), 61 (O^6–CH_3), 63 (O^3–CH_3), 58 (C-2), 63 (C-6),71 (C-4), 77 (C-3), 79 (C-5), 99 (C-1), and 173 (C=O). FTIR (KBr):*v* 3414 (br, OH/NH), 2937 (m, C–H), 1655 (vs, C=O amide I) and 1483 (tertiary N–CH_3) cm^{-1}.

3. Results and discussions

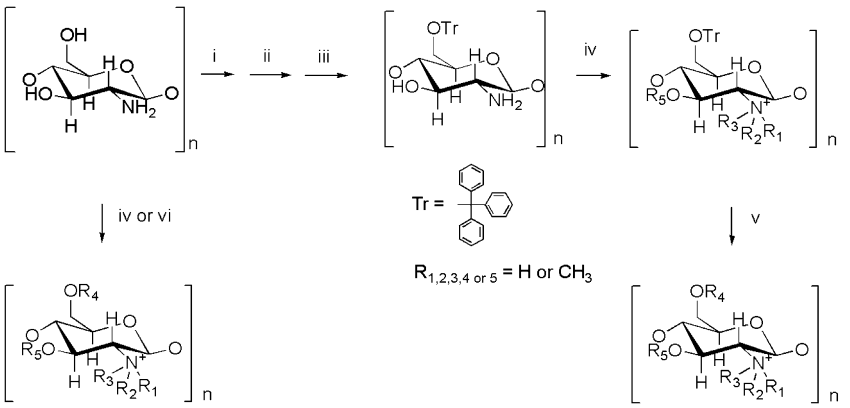
3.1. The chitosaccharide starting materials

Three starting materials with different molecular weight and degree of *N*-acetylation were used in this study. The starting oligomeric material (chitooligomer II) had a average molecular weight of 775 Da, as determined with MALDI TOF [45] and with 48% degree of deacetylation determined with ^{1}H NMR with a method adopted from Lavertu [46]. The deacetylation of the low MW chitooligosaccharide (chitooligomer II) resulted in an 82% deacetylated product (chitooligomer III). The starting polymeric material (chitosan I) was 95% deacetylated and an average molecular weight of 8.1 kDa as determined by dinitrosalicylic acid (DNS) end reducing sugar assay [47]. The oligomers had good solubility in water, whereas the polymeric starting material (chitosan I) was less soluble. The oligomers were also readily soluble in organic solvents such as DMF, NMP and pyridine and to some extend in ethanol and methanol. The polymeric material had good solubility in NMP and could also be dissolved in 50:50 mixture of DMF and water.

3.2. The synthesis and characterization

Series of synthesis were carried out where reaction parameters such as reaction temperature, molar ratios of reagents, solvent system, time and number of reaction steps were changed (Table 1). Syntheses were done in a mixture of DMF and water [50:50] (v/v) (Scheme 1). Reaction at high temperature (75 °C) resulted in significant darkening of oligomeric material. The reaction time at this temperature was therefore kept at 2 h or less. The reaction times at 50 °C and 21 °C were 8 and 48 h, respectively. The new solvent mixture dissolved the polymer just as well as NMP, which is commonly used for methylation reactions of chitosan [34]. The new solvent system has the advantage of lower boiling point than NMP. The reaction mixture can therefore be concentrated by evaporation of water and to some degree DMF before precipitation with ethanol.

In the one-step synthesis the reaction time affected *N*-quaternization, which increased from 2% to 4% and *N*,*N*-dimethylation increased from



Scheme 1. Synthetic route of the methylated chitosan derivatives. *Note*: Reagents and conditions: (i) phthalic anhydride, DMF/water, 120 °C; (ii) triphenylchloromethane, pyridine, 100 °C; (iii) hydrazine monohydrate, water, 100 °C; (iv) methyl iodide, NaOH, NMP, 60 °C (Section 2.5.3); (v) aqueous HCl, room temperature; (vi) methyl iodide (6 or 12 eq.), NaOH (1.5, 3, 6 or 9 eq.), DMF/water, various temperature (room temperature, 50 °C or 75 °C) (Section 2.5.2).

18% to 28% for 0.5 vs 2 h reaction at 75 °C. The equimolar ratio of NaOH was varied to find the optimal conditions. Polnok et al. [33] studied the effect of alkaline environment on degree of methylation. Low concentration of NaOH resulted in a less *O*-methylated product when done in NMP. The methylation degree was highest with 3 eq. of NaOH or 42% *N*,*N*-dimethylation. Less *N*,*N*-dimethylation was obtained with other molar ratios or 18%, 30% and 12% degree of substitution with 1.5, 6 and 9 eq. NaOH, respectively. The one step reaction series made in the DMF:water solvent system (Table 1) resulted in significant *N*-mono- and *N*,*N*-dimethylation, which was 20–40% in the case of the deacetylated chitosan I and chitooligomer III. The desired *N*,*N*,*N*-trimethylation (*N*-quaternization) of the amino groups was less than 7%. Due to *N*-acetylation on the amino group the products were less methylated with chitooligomer II as starting material. Significant *N*,*N*,*N*-trimethylation, up to 29%, could be obtained by a two-step procedure and the *N*-monomethylation was less than 21%. In general, better results (i.e. increase in *N*,*N*,*N*-trimethylation) were obtained with extended reaction at low temperature than with reaction at high temperature. No *O*-methylation was observed in any of these products. The series was compared to products from a slightly modified procedure previously reported by Siveal [34] (TMC I 60 A and ONPM-Chitosan I, II, III). In this case NMP was used as solvent and NaI as a catalyst in the reaction (Scheme 1). These reactions gave highly methylated products, with methylation on both the amino and hydroxyl groups (Table 2). One-step reaction resulted in 18% *N*,*N*,*N*-trimethylation and three-step reaction resulted in 63–74% *N*,*N*,*N*-trimethylation. The total *O*-methylation after one step was 22% and 34–46% after three-step reaction.

When looking at the oligomers (chitooligomer II and III), it is clear that the high degree of *N*-acetylation reduces the reactivity of the chitosaccharides. The more *N*-acetylated starting material (chitooligomer II) reached only half of the maximum *N*-quaternization of the less *N*-acetylated product (chitooligomer III) as seen in Table 1. When the material is highly *N*-acetylated like starting material chitooligomer II the *N*-acetylation decreases from 52% to approximately 35%. This is not the case for the less *N*-acetylated products (chitosan I and III).

Protection group strategy [48–50] was used to obtain more site specific methylation of the amino moiety (Scheme 1). The *N*-phthaloylchitosan I intermediate with 53% *N*-substitution was *O*-protected with a trityl protection group resulting in a 76% 6-*O*-protected material (Table 3). The methylation (Scheme 1) resulted in a highly quaternized product with 50% less 6-*O*-methylation compared to the unprotected chitosan methylated in the same manner (TMC I 60 A vs *N*-methyl chitosan I A, Table 2). Quaternary amino group can be introduced trough *N*-selective reaction with a quaternized alkylamino aldehyde and reduction. The quaternized aldehyde is produced by reaction of the dialkylamino group with alkyl halide [35]. Jia et al. [42] used reaction with alkyl aldehyde to obtain

Table 3
Antibacterial activity of the methylated products at two different acidic conditions

Compounds	Antibacterial activity against *S. aureus* ATCC 29213 at pH 5.5		Antibacterial activity against *S. aureus* ATCC 29213 at pH 7.2	
	MIC (μg/ml)	MLC (μg/ml)	MIC (μg/ml)	MLC (μg/ml)
Chitosan I	64	64	⩾8192	⩾8192
Chitosan II	⩾8192	⩾8192	⩾8192	⩾8192
Chitosan III	⩾8192	⩾8192	⩾8192	⩾8192
TMC I 75 A	64	64	4096	⩾8192
TMC I 75 B	32	32	⩾8192	⩾8192
TMC I 50 A	16	16	2048	4096
TMC I 50 B	16	32	4096	⩾8192
TMC I 21 A	16	16	⩾8192	⩾8192
TMC I 21 B	32	32	128	256
TMC I 60 A	32	32	64	64
TMC I 1 1/2	16	16	1024	1024
TMC I 6	16	32	1024	1024
TMC I 9	16	16	1024	512
TMC I 120	16	16	1024	1024
ONPM-Chitosan I	512	⩾8192	16	256
N-methyl chitosan I A	32	32	32	32
N-methyl chitosan I B	256	1024	8	8

N-alkyl chitosan derivatives which can then be quaternized by reaction with alkyl halide.

The substitution degree of the methylated derivatives was calculated by using the integration of the protons on the chitosaccharide (H-2 (GluNAc), H-3, H-4, H-5, H-6 and H-6′ at δ 3.6–4.4 ppm) and H-2 (GluN and GluN substituted) 3.10 ppm (δ 3.37 with D_2O/DCl as solvent) in the ^1H NMR spectrum and compared to the integration of either; *N*-mono- (δ 2.8 ppm), *N*,*N*-di- (δ 2.9 ppm), *N*,*N*,*N*-trimethyl (δ 3.3 ppm) or *O*-methyl (δ 3.4–3.5 ppm) peaks (Fig. 1). Fig. 2 shows comparison of TMC I 50 B measured in D_2O and D_2O/DCl. The assignment of these peaks has previously been described by Sieval et al. [34]. The *N*-monomethyl and *N*,*N*-dimethyl peaks shifted downfield when DCl was added. The relative error in substitution degrees obtained from the ^1H integrals was estimated by comparison with the C/N ratio calculated from the elemental analysis result. The error ranged between 3% and 10%.

3.3. The antibacterial effects of the TMC derivatives

The series of compounds made where used for SAR investigations against *S. aureus*. The activity of the derivatives was measured at two pH values, pH 5.5 where all the free non-quaternized amino groups are protonated giving the material a positive charge, and at pH 7.2, which represent the physiological acidity and the non-quaternized groups are not cationic.

The oligomers (TMC II and III derivatives) showed no activity against the microorganism. Similar molecular weight effect has been observed in earlier studies of the antibacterial effect of chitosan against *S. aureus* [12,16] where chitosan with molecular weight ranging between 1 and 1671 kDa were studied.

The polymer derivatives showed a considerable activity at pH 5.5 but were less active at pH 7.2 (Table 3). Researches have shown this activity relationship for chitosan [12] and other quaternized *N*-alkyl derivatives of chitosan [42,51]. The structure activity relationship for the degree of *N*-mono- and *N*,*N*-dimetylation was investigated for the current series of compounds but no direct correlation was found. According to the results the *N*-mono- and *N*,*N*-dimethyl amino groups have the same function as a free amino group since there is a negative correlation between the degree of *N*-quaternization and activity (Fig. 3) at pH 5.5. The structure activity relationship at pH 5.5 indicates that the protonated amino groups, which are not *N*,*N*,*N*-trimethylated (*N*-quaternized), contribute to the antibacterial activity. When the activity at pH 7.2 is analyzed the *N*-quaternized group has a positive effect on activity (Fig. 3). No correlation was found with degree of *N*-mono- and *N*,*N*-dimethylation, and the contribution seems not to be

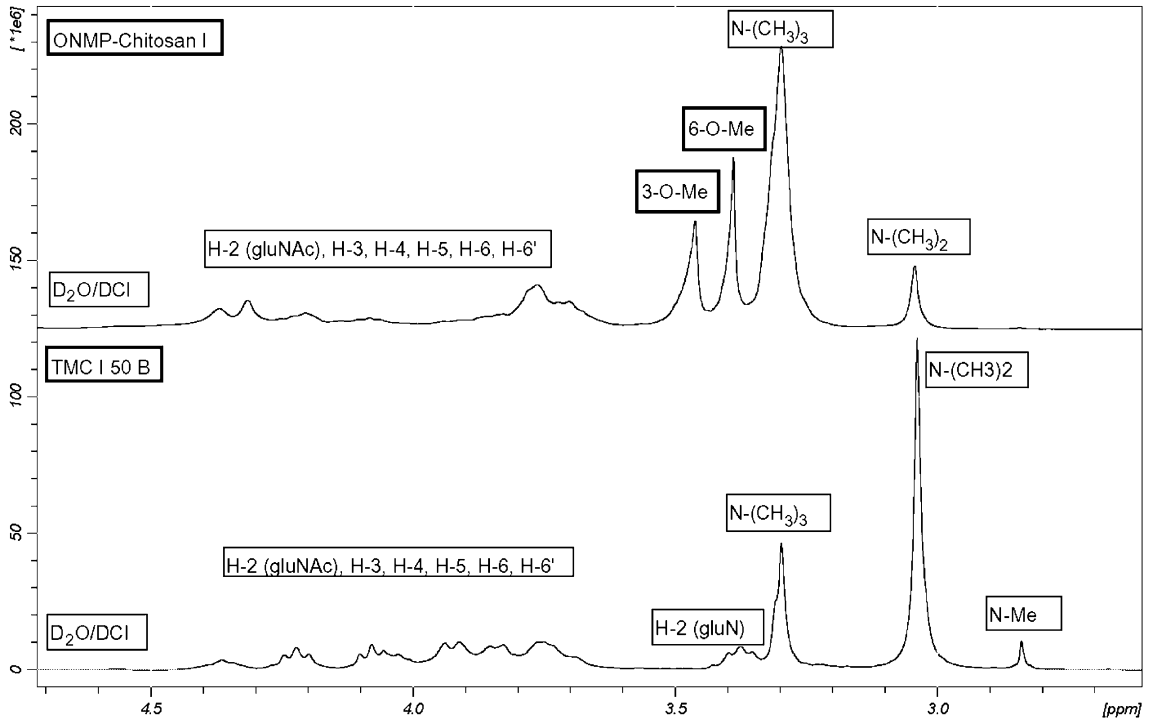


Fig. 1. ^{1}H-NMR spectra of ONPM-Chitosan I with high degree of *O*-methylation and *N*,*N*,*N*-trimethylation **(A)**, and TMC I 75 B which is 7% *N*-monomethylated, 66% *N*,*N*-dimethylated and 12% *N*,*N*,*N*-trimethylated, with no *O*-methylation **(B)**.

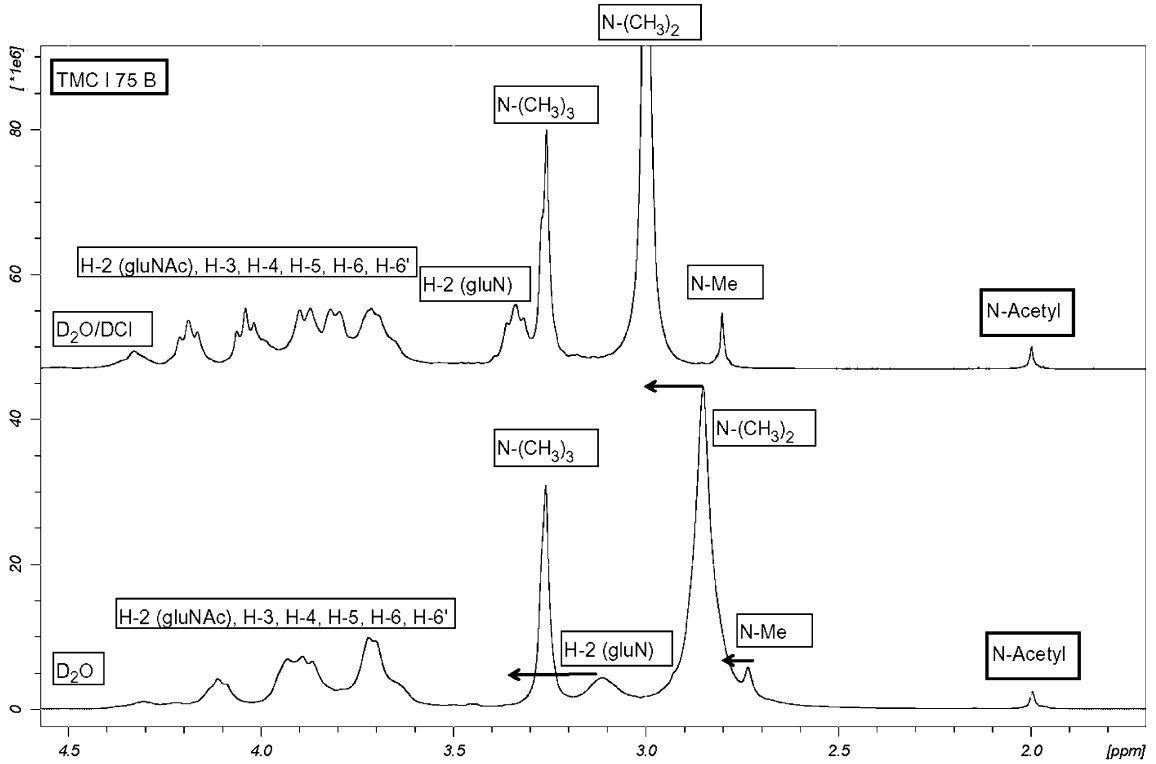


Fig. 2. ^{1}H-NMR spectra of TMC I 50 B measured in D_2O compared to spectra measured in D_2O/DCl where the H-2 (GluN), *N*-monomethyl- and *N*,*N*-dimethyl peaks shifts downfield upon acidification.

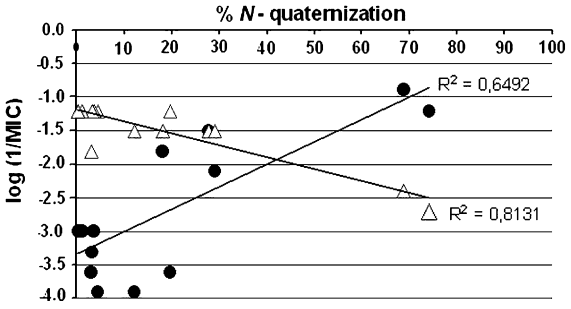


Fig. 3. The relationship between degree of *N*-quaternization and antibacterial activity at pH 7.2 (●) and pH 5.5 (△) of the TMC I derivatives.

different from the free amino group. *O*-methylation does not seem to contribute to the activity but it decreases the solubility of the polymeric TMC I derivatives.

The antibacterial activity of TMC derivatives has not been studied in detail and therefore it is difficult to compare with other studies on TMC antibacterial activity. For example, the effect of degree of substitution on antibacterial activity has not been reported. Jia et al. [42] reported the effect of equally trimethylated TMC derivatives with different molecular weights on *E. coli*, where the low MW derivatives were most active. It is difficult to compare current results for other *N*-quaternized chitosan derivatives since many factors such as the assay conditions and stain of bacteria are different. In general, when antibacterial activity of other *N*-quaternized chitosan materials (e.g. *N*-[(2-hydroxy-3-trimethylammonium)propyl] chitosan (HTCC)) are analyzed, the *N*-quaterization leads to a more soluble material and therefore antibacterial activity is also increased [22,43,52].

In the current study the antibacterial activity was determined according to the standard CLSI method. Thus we can better compare the activity in the current study to activity found in other studies performed according to this standardized method. Our previous study on antibacterial effect of chitosan *N*-betainate was done under the same standardized conditions as the present study [53]. In this case the activity decreased with increasing substitution with the cationic *N*-betainoyl moiety. It was concluded that the attachment of the quaternary ammonium moiety on the amino group of chitosan is not necessarily sufficient to obtain antimicrobial action. The key issue is the optimal positioning of the positive charge in relation to the polymer backbone. The current study verifies this conclusion. This can be observed since TMC I derivatives are more active than the *N*-betainate and the activity increases with higher degree of *N*-quaterization under physiological pH.

Surface active cationic compounds show antibacterial activity [54]. It is therefore possible that increase in surface activity contributes to the activity of methylated chitosan derivatives. The chitosan derivatives had no effect on surface tension, of pure water or pH 5.5 acetate buffer, up to 1% (w/v) concentration. The initial surface tension of the pure water or acetate buffer ranged between 31.4 and 33.2 mN/m. However, chitosan I lowered the surface tension from 32.3 to 25.5 mN/m in acetate buffer in the concentration range 0–1%, the chitooligomers had no effect on surface tension. It was not possible to evaluate the CMC since the chitosaccharides were not surface active. This indicates that the surface tension does not play a role in the antibacterial activity of the chitosan derivatives.

4. Conclusion

Series of methylated derivatives of chitosan and chitooligomers were prepared with different degree of methylation. By increasing the reaction time and reaction steps a higher degree of *N*-quaternization was achieved. Using a solvent system with 50% water in DMF minimized *O*-methylation. We have introduced an approach where the antibacterial activity is measured by a standardized method and thus we can better compare the results from the current study with results in other studies. Chitooligomers and their methylated derivatives were inactive against *S. aureus*, whereas the chitosan polymer and its derivatives were active. The antibacterial activity measurements show that quaternization is vital for the derivatives to be active at pH 7.2 but has a negative effect on activity at pH 5.5. At acidic conditions the protonation of the free-, *N*-mono- and *N*,*N*-dimethylated amino groups is important for antibacterial activity. Surface activity does not contribute to antibacterial activity of these compounds.

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