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Synthesis and Characterization of Chitosan *N*-Betainates Having Various Degrees of Substitution

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ABSTRACT: An efficient five-step synthetic route was developed for full N-substitution of chitosan with a quaternary betaine moiety. The developed synthetic procedure can also be controlled to produce chitosan *N*-betainates having lesser degrees of substitution. 6-*O*-Triphenylmethylchitosan, which is highly soluble in organic solvents, was used as an intermediate for *N*-acylation reactions. Intermediate products were characterized by ^{13}C CP/MAS NMR, FT-IR, and elemental analysis. The water-soluble quaternary chitosan *N*-betainates were thoroughly characterized by ^{1}H NMR and ^{13}C NMR and by 2D ^{1}H−^{1}H COSY NMR and ^{13}H−^{1}H HSQC NMR. Degrees of substitution were determined from the ^{1}H NMR spectra. A significant degradation of the polysaccharide backbone during the synthetic procedure was determined by GPC with a light scattering detector.

Introduction

Chitosan (poly-1,4-β-D-glucosamine) is a biocompatible,[1] biodegradable,[2] and mucoadhesive[3] polysaccharide that has attracted considerable attention in the pharmaceutical and biomedical fields.[4−6] Chitosan has many conventional formulation applications, mostly for its ability to form films and gels. Novel applications of chitosan are delivery of peptides,[7] vaccines,[8] and genes.[9] Biomedically, the most explored feature of chitosan is its antimicrobial property.[10]

The polycationic properties of chitosan are probably responsible for most of its observed activity.[4] The amino groups on chitosan are only partially protonized at physiological pH 7.4, and the major drawback of chitosan is its poor aqueous solubility, when considered as a pharmaceutical excipient. Chitosan is soluble in dilute aqueous acids due to protonation of the amino groups.

Quaternary ammonium derivatives of chitosan are therefore interesting in view of pharmaceutical applications. These derivatives have two major advantages over the parent chitosan: (1) they are water-soluble at physiological pH, and (2) they have a permanent positive charge on the polysaccharide backbone. Quaternary chitosan derivatives have various potential pharmaceutical applications, e.g., as antimicrobials,[11] as permeation enhancers,[12] and as gene delivery systems.[13]

The aim of the present study was to develop a synthetic route that would enable the preparation of *N*-betainate derivatives of chitosan having various degrees of substitution. The structurally simple quaternary betaine moiety was selected because it is nontoxic natural product, which could yield to more uniform quaternary chitosan derivative having physicochemical, biological, and pharmaceutical properties comparable to *N*-trimethylchitosan. It is important to study the impact of differences in substitution degrees when chitosan derivatives are tested for different applications. Efforts were focused on the exact characterization of chitosan *N*-betainate end products with NMR spectroscopy, which is the most reliable method for the characterization of chitosan derivatives. Aiedeh and coworkers[14] published a synthetic route for chitosan *N*-betaine earlier. However, they published only the preparation and characterization of a product with one degree of substitution. In addition, no NMR data or data on the synthetic procedure's effect on molecular weight were reported.

Chitosan *N*-betainates were characterized by 1D ^{1}H and ^{13}C NMR and also by 2D ^{1}H−^{1}H COSY and ^{13}C−^{1}H HSQC NMR. Degrees of substitution (ds) of the end products were determined from the ^{1}H NMR spectra. GPC-LS was used to analyze effects of the synthetic procedure on the molecular weight and molecular weight distribution of the polymer. Structures of the intermediate products were characterized by solid-state ^{13}C CP/MAS NMR, FT-IR, and elemental analysis. Degrees of substitution of the intermediate products were calculated from the C/N ratios, which were obtained from the elemental analysis.

Experimental Section

Materials. Chitosan, used as a starting material, was donated by Primex ehf (Reykjavik, Iceland). Weight-average molar mass (M_w) 148 200 Da and number-average molar mass (M_n) 94 200 were determined by GPC-LS. The degree of deacetylation (85%) was confirmed by ^{1}H NMR. All other chemicals used were commercially available and used as received. Pyridine was distilled over KOH. Dialysis membrane (M_w cutoff 12 kDa) was purchased from Sigma (St. Louis, MO).

Characterization. ^{1}H and ^{13}C spectra were recorded on a Bruker AVANCE DRX 500, operating at 500.13 and 125.76 MHz, respectively. Samples of chitosan *N*-betainates (40 mg) were dissolved in 600 μL of D_2O. Chemical shifts (δ) are

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reported in parts per million (ppm) using sodium 3-(trimethylsilyl)propionate-d_4 as an internal standard. All measurements were carried out at 343 K. In addition, ^{1}H spectra were also measured at 298 K. For proton spectra, 128 transients were recorded in a routine way using a 90° flip angle. A repetition time of 8.5 s was used. ^{1}H decoupled ^{13}C spectra without NOE were recorded with decoupling only during acquisition to obtain quantitative spectra. A repetition time of 9 s was used. To ensure that magnetization recovery was sufficient for quantitative work, manganese(II) chloride ($MnCl_2$) was added to the samples as a relaxation agent to a final concentration of 8.3 μM before carbon spectra measurements. ^{1}H−^{1}H homonuclear correlation (gradient-enhanced COSY) experiments were carried out in the magnitude mode. The data matrix was 128 × 2K and the spectral width 5900 Hz. For each FID, two transients were accumulated. ^{13}C−^{1}H gradient-enhanced heteronuclear single quantum correlation (ge-HSQC) experiments were carried out in the phase-sensitive mode using the Echo/Antiecho-TPPI gradient selection. The data matrix was 256 × 1K, and the spectral width was 5900 Hz for proton and 20 800 Hz for carbon. An evolution time of $1/(4J_{CH}) = 1.72$ ms was used. For each FID, two transients were accumulated. A pure cosine squared sine window function was applied in both dimensions prior to Fourier transform.

Solid-state ^{13}C CP/MAS NMR spectra were recorded on a Bruker AMX 400 standard bore, high-resolution NMR spectrometer operating at 100.6 MHz. The powder samples were placed in zirconia rotors (diameter of 7 mm). The Hartmann−Hahn matching condition for cross-polarization and the magic angle were set using adamantane and glycine, respectively. The ^{13}C CP/MAS spectra were obtained at a spinning speed of 4.5 kHz using a 30 kHz spectral window with 7.4 Hz data points. The single contact pulse sequence used a 1 ms contact time. Scans of 10 000 were acquired with a 4 s recycle delay. The chemical shifts are reported relative to TMS with the use of glycine as an external reference sample.

FT-IR spectra were recorded with a Nicolet 510 P spectrometer from KBr pellets.

Elemental analyses were performed with a Thermo Quest CE EA1110-CHNS-O elemental analyzer.

Molecular weights and molecular weight distributions were determined by a GPC system consisting of a Gilson 321 pump, a Gilson 243 automatic injector, two Polymer Labs PL aquagel-OH MIXED (300 × 7.5 mm, 8 μm) columns at 40 °C, and a Waters 2410 refractive index detector containing a PD2010 dual-angle laser light scattering detector. A mobile phase of acetic acid (0.3 M)/sodium acetate (0.2 M) (pH 4.5) at a flow rate of 1 mL/min was used. Chromatograms were collected by PrecisionAcquire32 software, provided by Precision Detectors Inc., Bellingham, MA, and analyzed with Matlab 6.1 (MathWorks Inc., Natick, MA) using self-made programs. Dextran samples of known molecular weights were used as standards in molecular weight calculations. Weight-average molar mass (M_w) and number-average molar mass (M_n) were determined by comparing the signals from the refractive index detector to that of the light scattering detector 90° signal time slice by time slice. The refractive index increment (dn/dc) was determined for each sample using a series of five concentrations. Molecular weights were corrected using the refractive index increments of the sample and the standards.

***N*-Phthaloylchitosan.**[15] Chitosan (10 g, free amino group content 50.78 mmol) was dispersed in 200 mL of DMF containing 5% (v/v) water and stirred overnight. Phthalic anhydride (27.6 g, 186 mmol) was added, and the mixture was stirred at 120 °C for 8 h. The reaction mixture was poured into ice water, filtered, and washed with copious amounts of methanol. The yield of the product was 15.04 g (92%). The degree of substitution (ds) was 0.81 according to the C/N ratio of the elemental analysis; i.e., 95% of the free amino groups of the starting material were substituted. IR (KBR): ν 3600−3100 (O−H), 2980−2830 (C−H, pyranose), 1779 (C═O, imide), 1713 (C═O, imide), 1390 (C═C, phth), 1150−950 (C−O, pyranose), 720 cm^{-1} (arom, phthaloyl). ^{13}C CP/MAS NMR: δ 22.40 (CH_3, *N*-acetyl), 57.72 (C-2), 61.49 (C-6), 71.34 (C-3), 75.90 (C-5), 83.04 (C-4), 100.99 (C-1), 131.37 (arom, phth), 168.08 ppm (C═O, phth). Elemental analysis: Calculated for $(C_{14}H_{13}NO_6)_{0.81}$ $(C_8H_{13}NO_5)_{0.15}$ $(C_6H_{11}NO_4)_{0.04}$·$0.2H_2O$: C, 55.53; H, 4.86; N, 5.07. Found: C, 55.52; H, 4.83; N, 5.06.

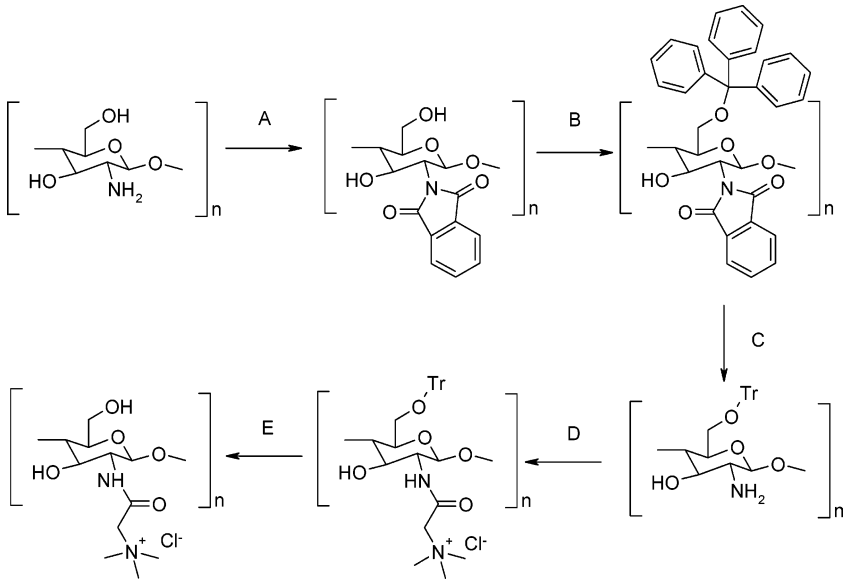
***N*-Phthaloyl-6-*O*-triphenylmethylchitosan.**[16] Triphenylchloromethane (43.8 g, 157.2 mmol) was added to a solution of 14.31 g of *N*-phthaloylchitosan (6-OH group content 52.45 mmol) in 215 mL of pyridine. The reaction mixture was stirred at 90 °C for 24 h. Solvents were evaporated, and the product was washed with ethanol and diethyl ether. The yield of the product was 27.35 g (100%). The ds of 6-*O*-triphenylmethylation was 0.83 according to the C/N ratio of the elemental analysis. IR (KBR): ν 3600−3100 (O−H), 3100−3000 (C−H, trityl), 2980−2830 (C−H, pyranose), 1779 (C═O, imide), 1714 (C═O, imide), 1491 (C═C, trityl), 1449 (C═C, trityl), 1380 (C═C, phth) 1150−950 (C−O, pyranose), 765 (arom, trityl), 747 (arom, trityl) 720 (arom, phth), 703 cm^{-1} (arom, trityl). ^{13}C CP/MAS NMR: δ 22.84 (CH_3, *N*-acetyl), 57.31 (C-2), 63.70 (C-6), 75.02 (C-3), 82.93 (C-5), 86.70 (C-4), 97.45 (C-1), 120−135 (arom, phth and trityl), 168.04 ppm (C═O, phth). Elemental analysis: Calculated for $(C_{33}H_{27}NO_6)_{0.6723}(C_{27}H_{27}NO_5)_{0.1245}$-$(C_{25}H_{25}NO_4)_{0.0332}(C_{14}H_{13}NO_6)_{0.1377}(C_8H_{13}NO_5)_{0.0255}(C_6H_{11}$-$NO_4)_{0.0068}$: C, 72.35; H, 5.22; N, 2.96. Found: C, 72.43; H, 5.29; N, 2.96.

6-*O*-Triphenylmethylchitosan.[16] *N*-Phthaloyl-6-*O*-triphenylmethylchitosan (26.37 g, phthalimido group content 45.06 mmol) and 148 mL (3.04 mol) of hydrazine monohydrate were stirred in 295 mL of water at 100 °C for 15 h. Solvents were evaporated, and the product was washed with water, ethanol, and diethyl ether. The yield of the product was 19.19 g (98%). FT-IR and ^{13}C CP/MAS NMR spectra and elemental analysis confirmed the complete removal of the phthalimido moiety. IR (KBR): ν 3600−3100 (O−H), 3100−3000 (C−H, trityl), 2980−2830 (C−H, pyranose), 1672 (amide I, *N*-acetyl), 1597 (amide II, *N*-acetyl), 1491 (C═C, trityl), 1449 (C═C, trityl), 1150−950 (C−O, pyranose), 765 (arom, trityl), 747 (arom, trityl), 703 cm^{-1} (arom, trityl). ^{13}C CP/MAS NMR: δ 22.77 (CH_3, *N*-acetyl), 57.11 (C-2), 65−87 (C-6, C-3, C-5, C-4), 104.40 (C-1), 127.68 ppm (arom, trityl). Elemental analysis: Calculated for $(C_{25}H_{25}NO_4)_{0.7055}(C_{27}H_{27}NO_5)_{0.1245}(C_6H_{11}NO_4)_{0.1445}$-$(C_8H_{13}NO_5)_{0.0255}$: C, 71.92; H, 6.27; N, 3.80 Found: C, 72.30; H, 6.37; N, 3.85.

***N*-Betainate-6-*O*-triphenylmethylchitosan.** Three different conditions were tested for the reaction between 6-*O*-triphenylmethylchitosan and *N*-chlorobetainyl chloride. The amounts of *N*-chlorobetainyl chloride used were 1 equiv (1.15 g, 6.67 mmol), 2 equiv (2.23 g, 12.94 mmol), and 4 equiv (4.95 g, 28.75 mmol), compared to the free amino groups in 6-*O*-triphenylmethylchitosan (2.7 g, 6.23 mmol; 2.7 g, 6.23 mmol; 3.0 g, 6.92 mmol, respectively). 6-*O*-Triphenylmethylchitosan was dissolved in pyridine (20 mg/mL). *N*-Chlorobetainyl chloride was added as a solid, and reaction mixtures were stirred at room temperature for 72 h. Reaction mixtures were evaporated to give dark, viscous syrups. Products were precipitated with a methanol/diethyl ether mixture (1:1, v/v), filtered, and washed with copious amounts of this solvent mixture. The yields of the products were 2.94 g (1 equiv), 3.35 g (2 equiv), and 4.55 g (4 equiv). Relative yields cannot be determined because the exact degrees of substitution of *O*-betainates were not determined. IR (KBr): ν 3600−3100 (O−H), 3100−3000 (C−H, trityl), 2980−2830 (C−H, pyranose), 1759 (C═O, *O*-betainate) 1687 (amide I, *N*-betainate), 1560 (amide II, *N*-betainate), 1491 (C═C, trityl), 1449 (C═C, trityl), 1203 (C−O, *O*-betainate), 1150−950 (C−O, pyranose), 765 (arom, trityl), 747 (arom, trityl), 703 cm^{-1} (arom, trityl).

Chitosan *N*-Betainate. 6-*O*-Triphenylmethyl protection was removed with 1 M HCl at room temperature with a 4 h reaction. 2.81 g of *N*-betainate-6-*O*-triphenylmethylchitosan (from the 1 equiv reaction) was reacted with 350 mL of 1 M HCl, 3.25 g of *N*-betainate-6-*O*-triphenylmethylchitosan (2 equiv) with 400 mL of 1 M HCl, and 4.45 g of *N*-betainate-6-*O*-triphenylmethylchitosan (4 equiv) with 560 mL of 1 M HCl. Solvents were evaporated from the reaction mixtures, and products were washed with methanol and ether. Products were dialyzed first against 0.05 M Tris buffer (pH 8.0) to unionize the unsubstituted amino groups and then against water.

Scheme 1. Synthetic Route for the Preparation of Chitosan *N*-Betainates[a]



[a] (A) Phthalic anhydride, DMF/water, 120 °C;[15] (B) triphenylchloromethane, pyridine, 90 °C;[16] (C) hydrazine monohydrate, water, 120 °C;[16] (D) *N*-chlorobetainyl chloride (1, 2, 4 equiv), pyridine, RT; (E) aqueous HCl, RT.

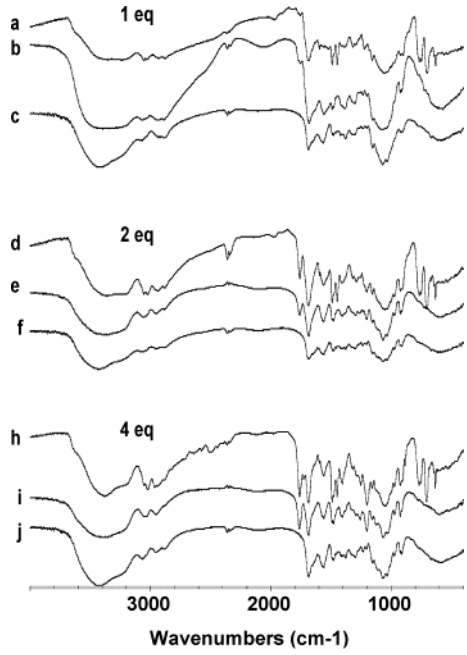


Figure 1. FT-IR spectra of *N*-betainate-6-*O*-triphenylmethylchitosans with 1 (a), 2 (d), and 4 (h) equivalents of *N*-chlorobetainyl chloride used in the acylation reaction and FT-IR spectra of chitosan *N*-betainate end products before (b, e, and i) and after dialysis (c, f, and j).

Finally, products were freeze-dried (ThermoSavant ModulyoD-230, Savant, Holbrook, NY). The yields of the products were 1.16 g (1 equiv), 1.38 g (2 equiv), and 1.75 g (4 equiv), resulting in relative yields of 71%, 68%, and 76%, respectively, for two consecutive steps. IR (KBr): ν 3600−3100 (O−H), 2980−2830 (C−H, pyranose), 1687 (amide I, *N*-betainate), 1560 (amide II, *N*-betainate), 1150−950 cm^{-1} (C−O, pyranose). Degrees of substitution calculated from ^{1}H NMR were 0.40, 0.80, and 0.90 when the equivalents of *N*-chlorobetainyl chloride used in the acylation reactions were 1, 2, and 4, respectively. Ds 0.40: ^{1}H NMR at 343 K (D_2O): δ 2.06 (CH_3, *N*-acetyl), 2.77 (H-2, when amino group unsubstituted), 3.34 (H-9, i.e., N^+-CH_3), 3.4−4.0 (H-6, H-6), 3.5−3.7 (H-5), 3.55−3.8 (H-4), 3.6−3.85 (H-3), 3.65−3.9 (H-2, substituted), 4.2 (H-8, i.e., $CO-CH_2-N^+$), 4.4−4.8 ppm (H-1). ^{13}C NMR at 343 K (D_2O): δ 24.94 (CH_3, *N*-acetyl), 57.27 (C-9, i.e., N^+-CH_3), 58.36 (C-2, substituted), 59.30 (C-2, unsubstituted), 63.16 (C-6), 67.44 (C-8, i.e., $CO-CH_2-N^+$), 74.62 (C-3, substituted), 76.57 (C-3, unsubstituted), 77.51 (C-5, substituted), 77.66 (C-5, unsubstituted), 81.17 (C-4 unsubstituted). 81.33 (C-4, substituted), 102.82 (C-1, substituted), 104,79 (C-1, unsubstituted), 167.21 (C═O, *N*-betainate), 176.98 ppm (C═O, *N*-acetyl). Ds 0.80: ^{1}H NMR at 343 K (D_2O): δ 2.05 (CH_3, *N*-acetyl), 2.78 (H-2, when amino group unsubstituted), 3.33 (H-9, i.e N^+-CH_3), 3.6−3.9 (H-6, H-6), 3.5−3.7 (H-5), 3.55−3.8 (H-4), 3.7−3.85 (H-3), 3.7−3.9 (H-2, substituted), 4.2 (H-8, i.e., $CO-CH_2-N^+$), 4.7 ppm (H-1). ^{13}C NMR at 343 K (D_2O): δ 24.92 (CH_3, *N*-acetyl) 57.27 (C-9, i.e., N^+-CH_3), 58.34 (C-2, substituted), 59.21 (C-2, unsubstituted), 63.08 (C-6), 67.44 (C-8, i.e., $CO-CH_2-N^+$), 74.54 (C-3, substituted), 76.17 (C-3, unsubstituted), 77.50 (C-5, substituted), 77.66 (C-5, unsubstituted), 81.31 (C-4, substituted), 102.81 (C-1, substituted), 103,90 (C-1, unsubstituted), 167.24 (C═O, *N*-betainate), 177.01 ppm (C═O, *N*-acetyl). Ds 0.90: ^{1}H NMR at 343 K (D_2O): δ 2.06 (CH_3, *N*-acetyl), 3.33 (H-9, i.e., N^+-CH_3), 3.5−3.9 (H-6, H-6), 3.5−3.7 (H-5), 3.6−3.75 (H-4), 3.7−3.85 (H-3), 3.7−3.9 (H-2), 4.2 (H-8, i.e., $CO-CH_2-N^+$), 4.7 ppm (H-1). ^{13}C NMR at 343 K (D_2O): δ 24.92 (CH_3, *N*-acetyl, 57.27 (C-9, i.e., N^+-CH_3), 58.34 (C-2), 63.08 (C-6), 67.43 (C-8, i.e., $CO-CH_2-N^+$), 74.57 (C-3), 77.51 (C-5), 81.32 (C-4), 102.83 (C-1), 167.21 (C═O, *N*-betainate), 176.98 ppm (C═O, *N*-acetyl).

Table 1. Substitution Degrees of End Products Determined by Comparing NMR Signals

^{1}H NMR	1 equiv	2 equiv	4 equiv
N^+-CH_3 protons[a] compared to pyranose protons[b] (298 K)	0.41	0.79	0.92
N^+-CH_3 protons[c] compared to hemiacetal proton[d] (343 K)	0.40	0.80	0.90

[a] 3.3 ppm. [b] 3.4−4 + 2.7 ppm. [c] 3.3 ppm. [d] 4.5−4.7 ppm.

Results and Discussion

The regioselective protection strategy developed by Kurita and co-workers[15,16] was applied to the five-step synthetic route presented in Scheme 1. The fact that chitosan is practically insoluble in most organic solvents makes the synthetic modifications of chitosan difficult. Solubilization of the rigid polysaccharide is critical in order to obtain high degrees of substitution and to have control over the chitosan modification reactions.[17] We started with attempts to acylate chitosan with betaine

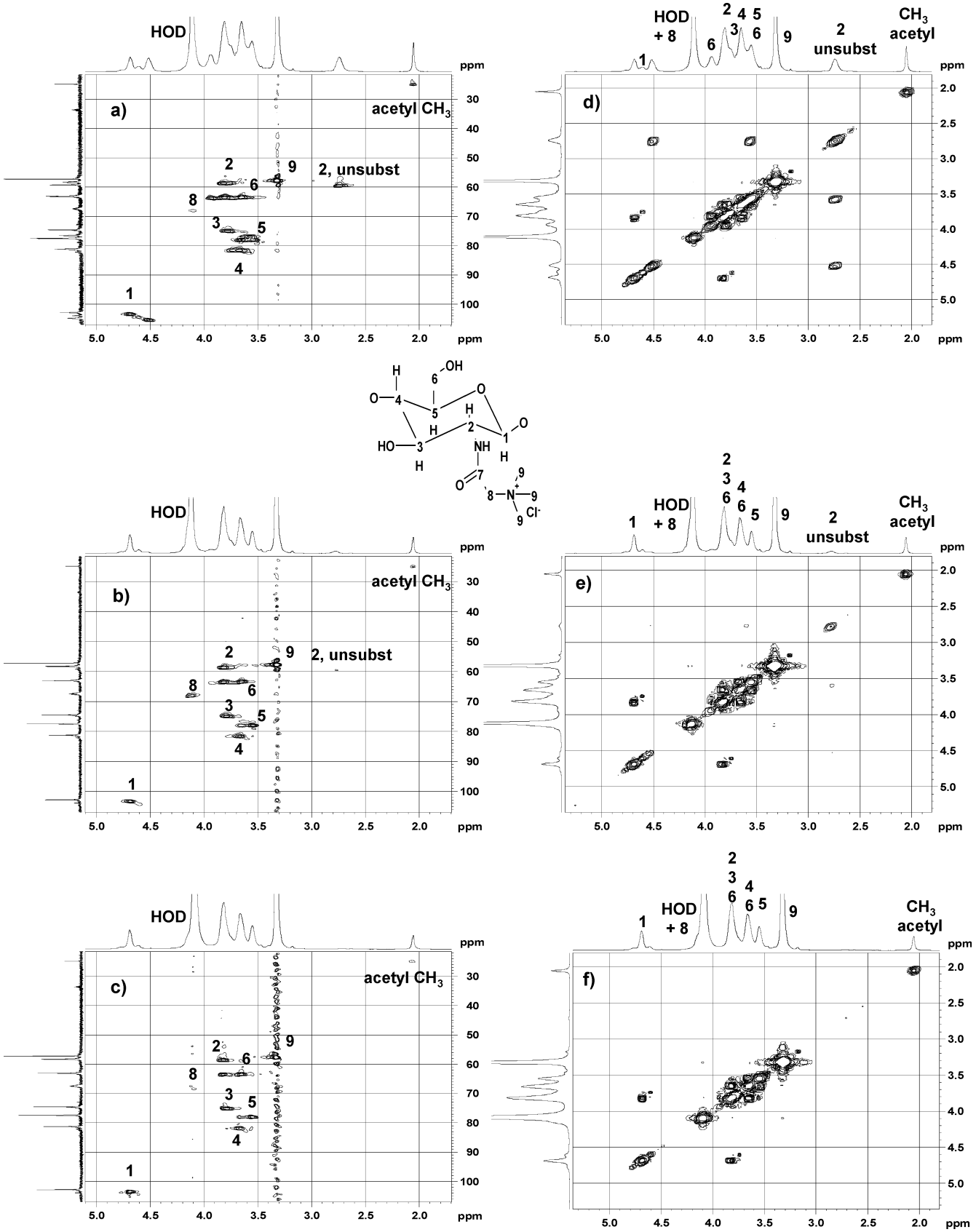


Figure 2. $^{13}C-^{1}H$ HSQC NMR spectra (a, b, and c) and $^{1}H-^{1}H$ COSY NMR spectra (d, e, and f) of chitosan *N*-betainates with ds 0.4, 0.8, and 0.9, respectively.

in aqueous acidic solutions by using widely used coupling reagents, but we did not obtain sufficient substitution degrees. The use of an organo-soluble 6-*O*-triphenylmethylchitosan intermediate as a starting material for *N*-acylation reactions enabled reactions in homogeneous reaction mixtures in organic solvents, resulting in high degrees of substitution and good control of the modification reaction.

Steps A, B, and C: *N*-Phthaloylation, 6-*O*-triphenylmethylation, and removal of the *N*-phthalimido moiety were accomplished as reported earlier.[15,16] However, complete substitution of *N*-phthaloylation[15] or 6-*O*-triphenylmethylation[16] was not achieved as reported in these references. The obtained substitution degrees was 0.81 for *N*-phthaloylation (i.e., 95% of the free amino groups were substituted) and 0.83 for 6-*O*-triphenylmethylation. However, the small amount of unsubstituted amino groups presented no problems in the process, as the amino groups cannot be tritylated by triphenylchloromethane because of the bulkiness of the trityl moiety. Although these intermediates are soluble enough to perform modification reactions in homogeneous organic solvent environments, their solubility does not enable sufficient liquid-state NMR characterization and quantification of degrees of substitution by ^{1}H NMR. Solid-state ^{13}C CP/MAS NMR was measured in order to characterize the structures as well as possible.

Step D: Addition of the *N*-Betainate. Some O-substitution was observed from the FT-IR spectra for the acylation reactions when reacted with 2 and 4 equiv of *N*-chlorobetainyl chloride (Figure 1). Both 3-*O*-esters and 6-*O*-esters were detected since the primary hydroxyl in the 6-*O*-triphenylmethylchitosan was not completely tritylated. The ester C═O bond stretches at 1759 cm^{-1} and the ester C−O bond at 1203 cm^{-1}. However, these unstable ester functions decomposed during the end-product purification by dialysis (Figure 1).

Step E: Chitosan *N*-Betainates. Complete removal of the 6-*O*-triphenylmethyl moiety was obtained. Chitosan *N*-betainate end products were highly soluble in water; e.g., NMR spectra were measured at 67 mg/mL in D_2O. Degrees of substitution of the end products were determined by comparing the ^{1}H NMR signal integrals. Two methods were applied: (1) signal integrals from CH_3 protons attached to quaternary nitrogen were compared to integrals of the polysaccharide backbone proton signals, and (2) signal integrals from CH_3 protons attached to the quaternary nitrogen were compared to the hemiacetal proton signal. These two distinct methods gave similar results (Table 1). Full N-substitution was obtained with 4 equiv of *N*-chlorobetainyl chloride. The amount of remaining *N*-acetyl group was 13%, as calculated from the ^{1}H NMR; i.e., no significant deacetylation occurred during the synthetic procedure. A substitution degree of 0.90 can be defined as full substitution when considering the degree of *N*-acetyl groups in the final products. Full substitution was also confirmed by ^{1}H NMR by the absence of the H-2 proton signal at 2.7 ppm. Two-dimensional ^{1}H−^{1}H COSY NMR and ^{13}C−^{1}H HSQC NMR confirm that this proton shifts to 3.8 ppm when the amino group in the glucosamine unit is substituted (Figure 2). These two-dimensional NMR techniques are clarifying and thus crucially important for characterizing chitosan and chitosan derivatives with overlapping proton signals.

An attempt to determine substitution degrees was also made by ^{13}C NMR by $MnCl_2$ addition, in an attempt to shorten the relaxation times. This, however, did not give reliable results.

Molecular Weights. GPC measurements show a significant decrease in molecular weights during the synthetic procedure (Table 2). This is mainly caused by the two reaction steps in an aqueous environment, i.e., *N*-phthalimido deprotection in basic and 6-*O*-trityl deprotection in acidic environments. Intermediate products were not characterized because they are not water-soluble and thus cannot be measured using the same method.

Table 2. Molecular Weights of End Products Determined by GPC

	M_n	M_w	M_w/M_n	d*n*/d*c*
chitosan	94 200	142 800	1.51	1.19
ds 0.40	11 900	21 100	1.76	1.19
ds 0.80	15 300	24 100	1.57	1.11
ds 0.90	18 000	34 100	1.89	1.06

It is extremely important to study the effect of modification reaction procedures on the molecular weight of polymers. Kurita and co-workers did not report any M_w determinations when they published the reaction procedures for steps A, B, and C.[15,16] Later on, however, some reports have been published that show a significant decrease in molecular weights with the protection and deprotection strategy applied here.[18−20] The use of absolute molecular weight determination techniques, such as GPC-LS, is necessary when studying the molecular weights of novel chitosan derivatives, as there are no appropriate polymer standards. It is not reliable to determine the molecular weights from the elution times, as these charged polymers can be vary considerable in hydrodynamic size. In this study, dextran standards were used to calibrate the light scattering detector.

Conclusions

The present synthetic procedure enables complete *N*-acylation of chitosan with the quaternary betaine moiety. Lower degrees of substitution can be produced simply by changing the equivalents of *N*-chlorobetainyl chloride in the acylation reaction. We also conclude the importance of exact characterization of novel chitosan derivatives. NMR is the primary method for characterization, and two-dimensional techniques are especially useful. ^{1}H NMR is more reliable than ^{13}C NMR in determining the substitution degrees. Equally important is the molecular weight and molecular weight distribution characterization of the final products. The polysaccharide backbone degrades during the presented procedure, which must be taken into consideration when preparing derivatives for different applications. The physicochemical, biological, and pharmaceutical properties of chitosan *N*-betainates will be studied and reported in the future.

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