SUPPORTING INFORMATION

Antimicrobial peptide shows enhanced activity and reduced toxicity upon grafting to chitosan polymers

Priyanka Sahariah,[a] Kasper K. Sørensen,[b] Martha Á. Hjálmarsdóttir,[c] Ólafur E. Sigurjónsson,[d,e] Knud J. Jensen,[b] Már Másson[a,*] and Mikkel B. Thygesen[b,*]

[a]Faculty of Pharmaceutical Sciences, School of Health Sciences, University of Iceland, Hofsvallagata 53, IS-107 Reykjavík, Iceland; [b]Department of Chemistry, Faculty of Science, Centre for Carbohydrate Recognition and Signalling, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Fredriksberg C, Copenhagen, Denmark; [c]Department of Biomedical Science, Faculty of Medicine, University of Iceland, Stapi, Hringbraut 31, 101 Reykjavík, Iceland; [d]The REModeL Lab, The Blood Bank, Landspitali University Hospital, Snorrabraut 60, 105 Reykjavik, Iceland; [e]Institute of Biomedical and Neural Engineering, Reykjavik University, Menntavegur 1, 101, Reykjavik, Iceland.

E-mail: mmasson@hi.is, mbth@chem.ku.dk

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Materials and analysis

Chitosan polymer (TM-3863) used was obtained from Primex ehf (Siglufjordur, Iceland). All other chemicals, including 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), 2-(*N*-morpholino)ethanesulfonic acid (MES), ethylenediamine tetraacetic acid (EDTA), 1-hydroxybenzotriazole (HOBt), and *N*,*N*-diisopropylethylamine (DIEA), were purchased from Sigma-Aldrich and used without any further purification. A sample of Dialysis membranes (RC, Spectra/Por, MW cut-off 3.5 kDa) and Float-A-Lyzers (Spectra/Por, MW cut-off 3.5–5 kDa, 5 mL sample volume) were purchased from Spectrum Laboratories Inc. (Rancho Dominguez, USA). *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were obtained from the American Type Culture Collection. Mueller-Hinton broth and blood agar (heart infusion agar with 5% (v/v) defibrinated horse blood) were purchased from Oxoid (Hampshire, UK).

Characterization of chitosan compounds was performed using ^{1}H, COSY and HSQC NMR, and IR spectroscopy. ^{1}H, COSY and HSQC spectra were recorded at 300 K using a Bruker Avance 300 MHz spectrometer equipped with a BBO probe, a Bruker Avance 400 MHz spectrometer, and a Bruker 500 MHz instrument equipped with an Observe cryoprobe. Chemical shifts were referenced to the *N*-acetyl peak at 2.08 ppm in D_2O. A sample of 2-(2-azido)acetamido-2-deoxy-D-glucose used as a reference substance for NMR assignments was kindly provided by Klaus Villadsen. FT-IR measurements were performed using an AVATAR 370 FT-IR instrument (Thermo Nicolet Corporation, Madison, USA) with 32 scans and resolution of 4 cm^{-1} and a Specac compressor (Specac Inc., Smyrna, USA). Trace element analysis of samples was performed using an Agilent ICP-MS 7500ce (Agilent technologies, UK) equipped with a PFA micro-flow nebulizer. LCMS analyses of peptides were performed on a Dionex Ultimate 3000 HPLC instrument coupled to a Bruker Daltonics micrOTOF-Q II mass spectrometer. Analytical HPLC was performed using a Gemini C18 (3 μm, 50×4.6 mm) column and a linear gradient flow of CH_3CN-H_2O (0.1% formic acid). High resolution mass spectrometry (HR-MS) of peptides was performed on a Micromass LCT time-of-flight instrument by direct injection with ionization in positive electrospray mode.

The average molecular weight (M_w) of the chitosan polymer was calculated to be 94.07 kDa using size exclusion chromatography. Its degree of deacetylation was found to be 89% (degree of

acetylation, DA= 11%) as obtained from the integrals of ^{1}H-NMR spectroscopy.[1] Equivalent quantities of reagents were calculated on the basis of one glucosamine unit. The degrees of substitution (DS) for the *N*-substituted chitosan derivatives were determined on the basis of the integral values from ^{1}H NMR spectra by applying the ratios between the *N*-acetyl signal (2.08 ppm in D_2O) and the substituted acyl CH_2CO signals for *N*-(2-azidoacetyl) chitosan (DS_{Az}) or *N*-(2-aminooxyacetyl) chitosan (DS_{Ao}). The percentage of unsubstituted glucosamine units (DS_{NH2}) was calculated from DS_{Az} (or DS_{Ao}) and DA values as follows: DS_{NH2}= 100%–DS_{Az}–DA. The peptide grafting ratios (DS_{pept}) for anoplin-chitosan conjugates, **6a-c** and **7a-c**, were determined from a combination of the ratios between the *N*-acetyl signal (2.08 ppm) and the triazole CH, *N*-triazoloacetyl CH_2CO, and aliphatic peptide CH_3 and CH_2/CH signals on the basis of the integral values from ^{1}H NMR spectra.

General procedures for peptide synthesis

All materials were obtained from commercial suppliers; the resins (TentaGel S RAM and TentaGel S NH2) were purchased from Rapp Polymere (Germany). Acetonitrile, formic acid, 4-pentynoic acid, propargylamine, and dichloromethane (CH_2Cl_2) were from Sigma-Aldrich, while N^{α}-9-fluorenylmethoxycarbonyl (Fmoc) amino acids, *N*,*N*-dimethylformamide (DMF), *N*-methyl-pyrrolidone (NMP), *N*-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methyl-methanaminium hexafluorophosphate *N*-oxide (HBTU), 1-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA), piperidine and *N*,*N*-diisopropylethylamine (DIEA) were from Iris Biotech GmbH. 5-(2-Formyl-3,5-dimethoxyphenoxy)pentanoic acid (*o*-PALdehyde) was obtained from Peptides international Inc. Milli-Q (Millipore) water was used for LC-MS analysis. Fmoc-amino acids contained the following side-chain protecting groups: *tert*-butyl for Thr, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) or di-*tert*-butyloxycarbonyl $(Boc)_2$ for Arg, and *tert*-butyloxycarbonyl (Boc) for Lys. All coupling reactions were performed with amino acids (4 equiv) preactivated for five minutes with HBTU (3.7 equiv), HOBt (4 equiv), and DIEA (7.2 equiv) in NMP (6 mL) prior to transferring to the resin. Coupling with 4-pentynoic acid was performed as for amino acid couplings. The washing procedure in all cases was DMF (3×8 mL), followed by CH_2Cl_2 (3×8 mL), and finally DMF (3×8 mL). Fmoc removal was performed by shaking the resin with piperidine/DMF (1:4) for 5 minutes followed by shaking of the resin with fresh piperidine/DMF (1:4) reagent for 15 minutes. Purification of

peptides was performed on a preparative Dionex Ultimate 3000 HPLC equipped with a Phenomenex Gemini-NX (5 μm 110 Å, 100×21.20 mm) C18 column. CH_3CN-H_2O (0.1% TFA) was used as eluent with a flow rate of 15 mL/min, and with a gradient from 25% to 55% CH_3CN to ensure maximum separation.

N-Terminal 4-pentynoylated anoplin peptide (HC≡CCH_2CH_2CO-Gly-Leu-Leu-Lys-Arg-Ile-Lys-Thr-Leu-Leu-NH_2) (1)

The N-terminal, 4-pentynoyl derivatized peptide **1** was synthesized on TentaGel S RAM resin (loading 0.25 mmol/g). The resin (0.2 mmol) was placed in a 20 mL syringe with a Teflon filter. Swelling and washing of the resin was followed by removal of the Fmoc group protecting the Rink linker. The resin was washed again and the first Fmoc-Leu-OH was then coupled for 18 hours while shaking to ensure complete anchoring of the first amino acid. The resin was washed followed by removal of the Leu Fmoc group and washing of the resin. The rest of the peptide including the N-terminal 4-pentynoic amide was then synthesized with subsequent coupling reactions (coupling time was two hours), washings, deprotections, and washings until the peptide was fully elongated. Fmoc-Arg(Pbf)-OH was used for introduction of Arg. The peptide was then released and the side-chain protecting groups simultaneously removed by treating the resin with TFA-H_2O-triethylsilane (95:2.5:2.5) for two hours. The TFA was evaporated under a nitrogen flow. The resulting peptide was precipitating by diethyl ether and purified by preparative HPLC. The peptide was lyophilized. Yield: 30 mg (12%). HR-MS (ESI): m/z calcd. for $C_{59}H_{108}N_{16}O_{12}$: 1233.8405 $[M+H]^+$; found: 1233.8414. ^{1}H NMR (300 MHz, D_2O): δ 4.45–4.29 (m, 8 H, 8×α-CH), 4.24–4.15 (m, 2 H, α-CH (Ile)+β-CH (Thr)), 4.00 (app. d, *J*=2.5 Hz, 2 H, CH_2C≡C), 3.95 and 3.88 (2×app. d, *J*=16.9 Hz, 2 H, CH_2CH_2C≡C), 3.86 (s, 2 H, α-CH_2 (Gly)), 3.22 (br, t, *J*=6.9 Hz, 2 H, $CH_2NC(N)NH_2$ (Arg)), 3.01 (br, t, *J*=7.5 Hz, 4 H, 2×CH_2NH_2 (2×Lys)), 2.64 (t, *J*=2.5 Hz, 1 H, C≡CH), 1.93–1.33 (m, 31 H, 13×CH_2+5×CH), 1.22 (d, *J*=6.4 Hz, 3 H, CH_3 (Thr)), 0.99–0.85 (m, 30 H, 10×CH_3 (4×Leu+Ile)) ppm.

C-Terminal propargylated anoplin peptide (H-Gly-Leu-Leu-Lys-Arg-Ile-Lys-Thr-Leu-Leu-Gly-NHCH$_2$C≡CH) (2)

Solid phase synthesis of the C-terminal propargyl derivatized peptide **2** was performed using the backbone amide linker (BAL) strategy[2] on TentaGel S NH2 resin (loading 0.29 mmol/g). The resin (0.2 mmol) was washed with DMF (3×8 mL), CH_2Cl_2 (3×8 mL) and DMF (3×8 mL). A mixture of *o*-PALdehyde (0.4 mmol, 2 equiv), HBTU (0.4 mmol, 2 equiv), HOBt (0.4 mmol, 2 equiv), and DIEA (0.6 mmol, 3 equiv) in DMF (6 mL) was allowed to preactivate for 5 min. The solution was then added to the resin and shaken for 18 h. The resin was then washed, and treated with acetic anhydride-CH_2Cl_2 (1:4, 2×10 min) to cap any unreacted amines. Following washing of the resin, reductive amination was performed on the resin using propargylamine (0.4 mmol, 2 equiv) and sodium cyanoborohydride (2 mmol, 10 equiv) in DMF-AcOH (99:1, 10 mL) under microwave heating at 60 °C for 10 min. The resin was then washed with NMP (3×8 mL) followed by DMF-AcOH (99:1, 3×8 mL), and the reaction was repeated with fresh reagents under same conditions. After completion the resin was washed with DMF (3×8 mL), CH_2Cl_2 (3×8 mL) and dried. The first Fmoc-Gly-OH (2 mmol, 10 equiv) was preactivated using diisopropyl carbodiimide (1 mmol, 5 equiv) in CH_2Cl_2-DMF (9:1, 6 mL) for 5 min, and after addition of the solution to the resin the mixture was heated in a microwave reactor at 60 °C for 10 min. The reaction was repeated using fresh reagents and the resin was placed in a 20 mL syringe with a Teflon filter and washed. The rest of the peptide was synthesized using alternating Fmoc removals and couplings (coupling time was 45 minutes) with washings in between. Fmoc-Arg(Pbf)-OH was used for introduction of Arg. The peptide was then released and the side-chain protecting groups simultaneously removed by treating the resin with TFA-H_2O-triethylsilane (95:2.5:2.5) for two hours. The TFA was evaporated under a nitrogen flow. The resulting peptide was precipitating using diethyl ether and purified by preparative HPLC. The peptide was lyophilized. Yield: 128 mg (54%). HR-MS (ESI): m/z calcd. for $C_{59}H_{109}N_{17}O_{12}$: 1248.8514 $[M+H]^+$; found: 1248.8508.

3,6-*O*-Di-*tert*-butyldimethylsilyl chitosan (TBDMS chitosan) (3)

TBDMS chitosan **3** was synthesized via chitosan mesylate salt**,** according to our previously published procedure.[3] Briefly, chitosan mesylate (7 g, 27.28 mmol on glucosamine unit basis) was dissolved in dry DMSO (100 mL). A solution of imidazole (31.5 g, 463.7 mmol) and TBDMS-Cl

(20.5 g, 136.4 mmol) in dry DMSO (100 mL) was added dropwise, and the resulting mixture was stirred at 25 °C for 24 h. The solid gel-type material obtained was filtered off and washed with H_2O (5×150 mL), and CH_3CN (3×100 mL). The material was then dried in vacuo at 40 °C for 18 h, to afford TBDMS chitosan (**3**) Yield: 9.6 g (89%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.30 (br s, 1 H, H-1), 4.00–3.50 (m, 3 H, H-4,6), 3.50–3.10 (m, 2 H, H-3,5), 2.71 (br s, 1 H, H-2), 0.89 (br s, 18 H, $(CH_3)_3C$), 0.05 (br s, 12 H, $(CH_3)_2Si$) ppm. FT-IR (KBr): ν 3442 (br, N–H), 2956–2857 (C–H), 1705 (C=O amide I), 1573 (amide II), 1472 (C–H), 1390–1361 (C–H *tert*-butyl), 1255 (Si–CH_3), 1105 –1050 (C–O), 837–778 (Si–CH_3) cm^{-1}.

***N*-(2-Azidoacetyl)-3,6-*O*-di-TBDMS-chitosans (4a-c)**

Bromoacetic acid (0.173 g, 1.27 mmol, 1 equiv) and sodium azide (82.9 mg, 1.27 mmol, 1 equiv) were dissolved in DMF (12 mL) and stirred 18 h. EDC (0.244 g, 1.27 mmol, 1 equiv) and HOBt (0.172 g, 1.27 mmol, 1 equiv) were added to the solution and allowed to stir for 30 min. A solution of compound **3** (0.5 g, 1.27 mmol on glucosamine unit basis, 1 equiv) in DMF (8 mL) was added to the reaction mixture where after the colourless solution turned dark brown. The reaction was continued for 18 h at room temperature. The product was precipitated with water (20 mL), filtered off on a sintered funnel and washed with water (3×15 mL) and acetonitrile (3×15 mL). The product obtained was dried in vacuo for 10 h to obtain **4c** as a light yellow solid (0.53 g). The degree of substitution (DS) could not be obtained from ^{1}H NMR due to overlapping peaks. However, an isolated yield of 99% was calculated for compound **4c**, by applying the DS_{Az} of 24% from compound **5c** (see below). Products with lower degrees of substitution were prepared from 0.7 equiv reagents (**4b**, 0.52 g, 100% yield calculated from DS_{Az}= 15%, see compound **5b** below), or 0.5 equiv reagents (**4a**, 0.45 g, 89% yield calculated from DS_{Az}= 6%, see compound **5a** below). Compound **4c**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.8–4.1 (m, ~1.4 H, H-1 and CH_2), 4.1–3.0 (m, ~5.1 H, H-3–H-6), 2.67 (br s, ~0.7 H, H-2), 1.92 (br s, 0.33 H, NAc), 0.83 (br s, 18 H, $(CH_3)_3CSi$), 0.0 (br s, 12 H, $(CH_3)_2Si$) ppm.

***N*-(2-Azidoacetyl)-chitosans (5a-c)**

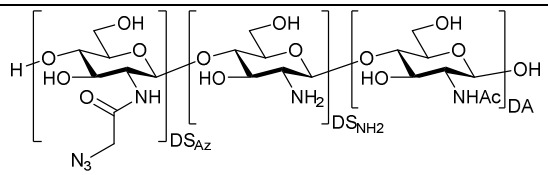
Compound **4c** (0.5 g, 1.2 mmol on glucosamine unit basis) was stirred in methanol (5 mL) and 1.25 M HCl in dioxane (10 mL) was added dropwise under cooling (10 °C). After stirring for 18 h at room temperature, the reaction mixture was diluted with water (20 mL), dialyzed against

water for 2 days and then freeze-dried for 2 days to get the product as yellow powder. Yield: **5c** (0.20 g, 93%, DS_{Az}= 24%). Compounds **5b** (0.23 g, 99% yield, DS_{Az}= 15%), and **5a** (0.22 g, 56% yield, DS_{Az}= 6%) were prepared from **4b** and **4a**, respectively. Compound **5c**: ^{1}H NMR (300 MHz, D_2O): δ 4.70 (br s, 1 H, H-1), 4.12 (br s, ~0.48 H, CH_2), 4.0–3.4 (m, ~5.2 H, H-3,4,5,6,H-2 (*N*-acylated)), 2.96 (br s, ~0.65 H, H-2 (glucosamine)), 2.08 (s, 0.33 H, NAc) ppm. FT-IR (KBr): ν 3418 (N–H, O–H), 2928 (C–H), 2115 (N_3), 1652 (C=O amide), 1521 (N–H), 1379 (C–N), 1079 (C–O) cm^{-1}.

General procedure for synthesis of anoplin-chitosan conjugates 6a-c and 7a-c using CuAAC

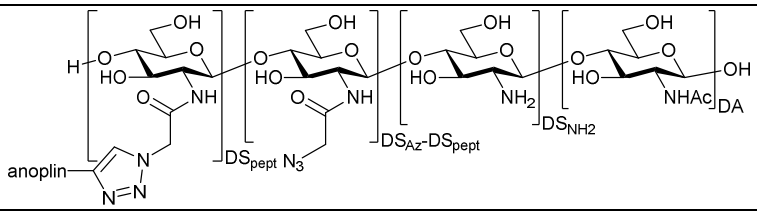
Compound **5c** (5 mg, 20.3 μmol, 1 equiv) and alkyne peptide (*N*-terminal **1**, or *C*-terminal **2**) (25 mg, 20.3 μmol, 1 equiv) were dissolved in MES buffer (150 mM, pH 6.0, 2 mL). A solution of $CuSO_4$ (0.5 M, 400 μL) in water was added to an ice-cold solution of sodium ascorbate (1 M, 400 μL) in water. The resulting Cu(I) solution was added to the reaction mixture when a yellow precipitate appeared. This mixture was then allowed to stir at room temperature for 18 h. After completion, EDTA (100 mM, 1 mL) was added to the blue coloured reaction mixture and stirred for 1 h. The reaction mixture was then purified by treating the sample with fresh EDTA (10 mL) followed by water (5×15 mL) in a centrifugal filter unit (3 kDa molecular weight cut-off). The purified sample was freeze-dried to get an off-white solid. Residual EDTA was removed using ion exchange, by stirring the products in 10% NaCl solution (2 mL) for 12 h. The solution was washed with water (6×10 mL) using a centrifugal filter unit (3 kDa molecular weight cut-off), followed by freeze-drying to obtain pure compounds **6c** and **7c**. Degrees of substitution (DS), conversions, and yields for **6a-c** and **7a-c** are given in Table 1 and Tables S1 and S2. Compound **6c**: ^{1}H NMR (300 MHz, D_2O): δ 7.96 (br s, ~0.19 H, triazole CH), 5.31 (br s, ~0.38 H, *N*-triazoloacetyl CH_2), 5.00–2.40 (m, partly overlapped by HOD), 2.08 (br s, 0.33 H, NAc), 2.00–1.30 (m, ~5.89 H, peptide CH_2/CH), 1.20 (br d, *J*=6.0 Hz, ~0.57 H, Thr CH_3), 1.00–0.76 (m, ~5.70 H, peptide CH_3) ppm. Compound **7c**: ^{1}H NMR (300 MHz, D_2O): δ 7.96 (br s, ~0.23 H, triazole CH), 5.31 (br s, ~0.46 H, *N*-triazoloacetyl CH_2), 5.00–2.90 (m, partly overlapped by HOD), 2.08 (br s, 0.33 H, NAc), 2.00–1.30 (m, ~7.13 H, peptide CH_2/CH), 1.20 (br d, *J*=6.0 Hz, ~0.69 H, Thr CH_3), 1.00–0.76 (m, ~6.90 H, peptide CH_3) ppm.

Table S1. Degree of substitution (DS) for chitosan derivatives before CuAAC reaction, as obtained by ^{1}H NMR.



Compound	DS_{Az} (%)	DS_{NH2} (%)	DA (%)	average MW per glucosamine unit (g/mol)
Chitosan	-	89	11	165.78
5a	6	83	11	170.77
5b	15	74	11	178.24
5c	24	65	11	185.72

Table S2. Degree of substitution (DS) for anoplin-chitosan conjugates after CuAAC reaction, as obtained by ^{1}H NMR.



Compound	DS_{pept} (%)	DS_{Az}-DS_{pept} (%)	DS_{NH2} (%)	DA (%)	average MW per glucosamine unit (g/mol)	Peptide mass, % of conjugate (%w/w)
6a	6	<1	83	11	244.78	28.3
6b	13	2	74	11	338.61	44.3
6c	19	5	65	11	420.10	52.2
7a	6	<1	83	11	242.26	28.6
7b	15	<1	74	11	356.98	48.5
7c	23	1	65	11	459.78	57.7

Synthesis of C-terminal anoplin peptide aldehyde (H-Gly-Leu-Leu-Lys-Arg-Ileu-Lys-Thr-Leu-Leu-Gly-NHCH$_2$CHO) (S1)

Solid phase synthesis of the *C*-terminal peptide aldehyde was initiated using the backbone amide linker[2] (BAL) anchor. TentaGel S NH_2 (689 mg, 0.2 mmol, 1 equiv) resin was washed with DMF (3×8 mL), CH_2Cl_2 (3×8 mL) and DMF (3×8 mL). A mixture of *o*-PALdehyde (113 mg, 0.4 mmol, 2 equiv), HBTU (151 mg, 0.4 mmol, 2 equiv), HOBt (54 mg, 0.4 mmol, 2 equiv), and DIEA (0.11 mL, 0.6 mmol, 3 equiv), in DMF was allowed to preactivate for 5 min. The solution was then added to the resin and shaken for 18 h. The resin was then washed with DMF (3×8 mL), CH_2Cl_2 (3×8 mL), treated with Ac_2O-CH_2Cl_2 (1:4, 2×10 min) and finally washed with DMF (3×8 mL), CH_2Cl_2 (3×8 mL) and DMF (3×8 mL). Reductive amination was performed on

the resin using aminoacetaldehyde dimethylacetal (0.05 mL, 0.4 mmol, 2 equiv) and sodium cyanoborohydride (125 mg, 2 mmol, 10 equiv) in DMF-AcOH (99:1, 10 mL) in a microwave reactor at 60 °C for 10 min. The resin was then washed with NMP (3×8 mL), DMF-AcOH (99:1, 3×8 mL) and the reaction was repeated with fresh reagents under same conditions. After completion the resin was washed with DMF (3×8 mL), CH_2Cl_2 (3×8 mL) and dried. The first amino acid Fmoc-Gly-OH (594 mg, 2 mmol, 10 equiv) was preactivated using diisopropyl carbodiimide (0.17 mL, 5 equiv) in CH_2Cl_2-DMF (9:1, 6 mL) for 5 min and after addition to the resin the mixture was heated in a microwave reactor at 60 °C for 10 min. The reaction was repeated once again using fresh reagents and the resin was washed with DMF (3×8 mL), CH_2Cl_2 (3×8 mL) and dried in vacuo. The rest of the peptide was synthesized using alternating Fmoc removals and couplings (coupling time was 45 minutes) with washings in between. Fmoc-Arg(Boc)$_2$-OH was used for introduction of Arg. The peptide was released and the side-chain protecting groups simultaneously removed by treating the resin with TFA-H_2O (95:5) for two hours. The TFA was evaporated under a nitrogen flow. The resulting peptide was precipitating using diethyl ether and purified by preparative HPLC. The peptide was lyophilized. Yield: 20 mg (8%). HR-MS (ESI): m/z calcd. for $C_{58}H_{109}N_{17}O_{13}$: 1252.8464 $[M+H]^+$; found: 1252.8455.

***N*-(2-(Boc-aminooxy)acetyl)-3,6-*O*-di-TBDMS-chitosans (S2a-c)**

To a solution of Boc-aminooxyacetic acid (0.24 g, 1.27mmol, 1 equiv) in dichloromethane (12 mL) was added EDC (0.24 g, 1.27 mmol, 1 equiv.) and HOBt (0.17 g, 1.27 mmol, 1 equiv) and the mixture was stirred for 30 min. A solution of compound **3** (0.5 g, 1.27 mmol, 1 equiv) in dichloromethane (8 mL) was then added to the reaction mixture followed by DIEA (0.56 mL, 2.55 mmol, 2 equiv) and stirred at room temperature for 18 h. After completion, the solvent was evaporated in vacuo and the residue was washed with acetonitrile (2×20 mL), water (2×20 mL) and acetonitrile (2×20 mL). The product obtained was dried in vacuo for 7 h to get **S2c** as a light yellow solid. Yield: **S2c** (0.58 g). The degree of substitution (DS) could not be obtained from ^{1}H NMR due to overlapping peaks. However, an isolated yield of 95% was calculated for compound **S2c**, by applying the DS_{Ao} of 25% from compound **S3c** (see below). Products with lower DS_{Ao} were prepared from 0.5 equiv reagents (**S2b**, 0.56, 87% yield calculated from DS_{Ao}= 15%, see compound **S3b** below), or 0.35 equiv reagents (**S2a**, 0.46g, 70% yield calculated from DS_{Ao}= 5%, see compound **S3a** below). Compound **S2c**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.29–3.21 (m,

~8.5 H, H-3–H-6, H-1, CH_2), 2.68 (br s, ~0.7 H, H-2), 2.00 (br s, 0.33 H, NAc), 1.47 (br s, ~2.2 H, $(CH_3)_3CO$), 0.88 (br s, 18 H, $(CH_3)_3CSi$), 0.06 (br s, 12 H, $(CH_3)_2Si$) ppm.

***N*-(2-aminooxyacetyl)-chitosans (S3a-c)**

Compound **S2c** (0.56 g, 0.67 mmol) was stirred in methanol (5 mL) and 1.25 M HCl in dioxane (10 mL) was added slowly under cooling at 10 °C. After stirring for 18 h at room temperature, the reaction mixture was diluted with water (20 mL), dialyzed against water for 2 days and then freeze-dried for 2 days to get the product **S3c** as yellow powder. Yield: **S3c** (0.07 g, 38% yield, DS_{Ao}= 25%). Compounds **S3b** (0.13 g, 63% yield, DS_{Ao}= 15%), and **S3a** (0.12 g, 58% yield, DS_{Ao}= 5%) were prepared from **S2b** and **S2a**, respectively. Compound **S3c**: ^{1}H NMR (300 MHz, D_2O): δ 4.70 (br s, 1 H, H-1), 4.29 (br s, ~0.50 H, CH_2), 4.00–2.95 (m, ~6.1 H, H-2–H-6), 2.08 (br s, 0.33 H, NAc) ppm. FT-IR (KBr): ν 3423 (N–H, O–H), 2925 (C–H), 1653 (C=O amide), 1544 (N–H), 1378 (C–N), 1070 (C–O) cm^{-1}.

Synthesis of chitosan-peptide conjugates using oxime formation

Compound **S3** (3.4 mg, 20.3 μmol, 1 equiv) and peptide aldehyde **S1** (25 mg, 20.3 μmol, 1 equiv) were dissolved in 100 mM aniline-acetate buffer (pH= 4.6, 2 mL). The reaction mixture was then heated to 60 °C for 18 h. The reaction mixture was subsequently treated with water (4×10 mL) in a centrifugal filter unit (3 kDa molecular weight cut-off). The purified sample was then freeze-dried to get an off-white solid. Table S3 provides an overview of tested reaction conditions and results.

Table S3. Attempted oxime coupling of *N*-(2-aminooxyacetyl) chitosans **S3** with aldehydes.[a]

Experiment No.	Starting material/ equivalents	Temperature (°C)	Time (h)	Result
1	*N*-(2-aminooxyacetyl)-chitosan **S3b** (DS_{Ao}= 15%, 1 equiv), aldehyde **S1** (0.3 equiv)	25	18	^{1}H-NMR showed traces of product and unreacted peptide aldehyde; reaction continued
		40	72	No changes in ^{1}H-NMR after continuation
2	*N*-(2-aminooxyacetyl)-chitosan **S3b** (DS_{Ao}= 15%, 1 equiv), aldehyde **S1** (0.5 equiv)	60	18	No product formation. Presence of excess unreacted peptide
3	*N*-(2-aminooxyacetyl)-chitosan **S3c** (DS_{Ao}= 25%, 1 equiv), aldehyde **S1** (1 equiv)	60	18	^{1}H-NMR showed partial product formation, difficult to calculate DS due to the presence of excess unreacted peptide; reaction continued
		60	36	No changes in ^{1}H-NMR after continuation
4	*N*-(2-aminooxyacetyl)-chitosan **S3c** (DS_{Ao}= 25%, 1 equiv), glyceraldehyde (1.2 equiv)	25	1	^{1}H-NMR showed product formation, degree of substitution= 11%

[a]All experimets were performed in 100 mM aniline in 100 mM acetate buffer, pH 4.6.

Trace element analysis

Analysis of residual copper content as well as other trace elements for representative anoplin-chitosan conjugates was performed by ICP-MS with assistance of assoc. prof. Thomas Hesselhøj Hansen, Department of Plant and Environmental Sciences, University of Copenhagen. Results are shown in Table S4.

Table S4. Trace element analysis by ICP-MS.[a]

Compound	Cr	Mn	Fe	Co	Ni	Cu	Zn	Cd	Na	K	Ca	Si	P	S
7a	ND	ND	ND	1	ND	**14**	ND	ND	45	ND	ND	3	19	7
7c	ND	ND	ND	2	ND	**30**	ND	ND	11	ND	ND	4	2	2

[a] Content in ppm; ND: not detected.

Molecular weight determination

Molecular weight (M_w) determinations were carried out using gel permeation chromatography (GPC) following a previously reported procedure.[4] GPC measurements were performed using the Polymer Standards Service (PSS) (GmbH, Mainz, Germany), Dionex Ultimate 3000 HPLC system (Thermo Scientific-Dionex Softron GmbH, Germering, Germany) equipped with a

Shodex RI-101 refractive index detector (Shodex/Showa Denko Europe GmbH, Munich, Germany) and a PSS ETA-2010 viscometer. WINGPC Unity 7.4 software (PSS GmbH, Mainz, Germany) was used for data collection and processing.

Table S5. Average molecular weight (M_w) for chitosan derivatives by GPC and NMR.

Compound	Average molecular weight M_w (Da)[a]	Average chain length, DP_{Av}[b]	Average number of azido groups/chain[b]
Chitosan	94,071	567	-
Chitosan mesylate salt	42,232	255	-
5a	35,066	205	12.3
5b	35,444	199	29.8
5c	32,501	175	42.0

[a]Measured by GPC; [b]Calculated from GPC data for **5a-c** by using DS_{Az} values from NMR.

Table S6. Calculated average molecular weight (M_w) for anoplin-chitosan derivatives from GPC and NMR.[a]

Compound	Calculated average molecular weight M_w (kDa)	Average chain length, DP_{Av}	Average number of anoplin peptides/chain
6a	50	205	12
6b	67	199	26
6c	74	175	33
7a	50	205	12
7b	71	199	30
7c	81	175	40

[a]Calculated by from GPC data for **5a-c** by using DS_{Az} and DS_{pept} values from NMR. Chitosan chain length was assumed not to be influenced by the CuAAC reaction.

Circular dichroism (CD) measurements

Samples were analyzed in water in absence and presence of sodium dodecyl sulfate (160 μM, SDS). CD spectra were measured over the range 190–250 nm, using a Jasco-810 spectropolarimeter (Jasco, Tokyo, Japan). Spectra were obtained at 25 °C using cells with a path length of 0.5 cm. Data points were recorded at a scan speed of 100 nm/min, bandwidth 1.0 nm, and 1 s response. Five repeat scans were accumulated to obtain the final averaged spectra. Following baseline correction, the observed ellipticity, θ (mdeg), was converted to mean residue ellipticity [Θ] (mdeg cm^2/dmol), using the relationship $[\Theta] = 100\theta/(l \times c \times n)$, where 'l' is the path length in centimeters, 'c' is the millimolar concentration, and 'n' is the number of residues in the peptide. Anoplin-chitosan conjugate samples were analyzed at concentrations in the range of 30 μg/mL to 70 μg/mL sample. The spectra were referenced to peptide concentration by using the peptide grafting ratios (DS) in Tables 1 and S2.

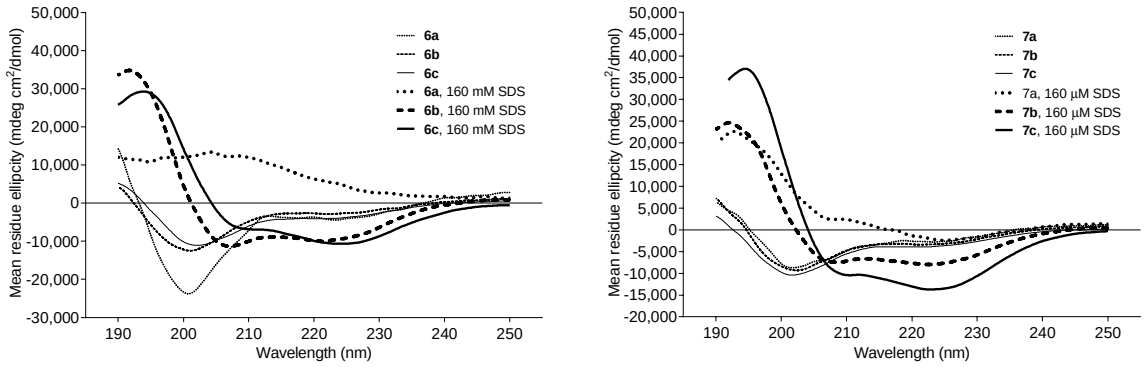


Figure S1. Circular dichroism spectra of anoplin-chitosan conjugates **6a-c** and **7a-c** in the absence or presence of 160 μM SDS. The spectra are referenced to mean peptide residue concentration using Table S2.

The α-helical structure content of **6a-c** and **7a-c** in the absence or presence of 160 μM SDS was predicted by using two different models: (i) assuming a two-state model according to Rohl and Baldwin,[5] as has been previously reported for anoplin,[6] or (ii) the K2D2 algorithm of Perez-Iratxeta and Andrade-Navarro.[7] Both of these models are based on globular protein structures. Results are summarized in Table S7.

Table S7. Prediction of α-helical secondary structure content from CD spectra.[a]

	In the absence of SDS							In the presence of 160 μM SDS						
Prediction	**6a**	**6b**	**6c**	**7a**	**7b**	**7c**	anoplin	**6a**	**6b**	**6c**	**7a**	**7b**	**7c**	anoplin
Two-state model	16	12	15	11	13	15	6[b]	NA[c]	35	36	8	28	47	55[b]
K2D2	10	8	8	9	8	7	-	3	44	24	10	31	40	-

[a] Content in %; [b] data from reference[6]; [c] NA: not applicable.

Antibacterial screen

The antibacterial tests were assayed, according to standard CLSI methods for antimicrobial dilution susceptibility tests[8] to measure the minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values.[4] The antibacterial activity was tested against four different bacterial species, that is, two Gram-positive bacteria *Staphylococcus aureus* (*S.aureus*, ATCC 29213) and *Enterococcus faecalis (E.faecalis*, ATCC 29212), and two Gram-negative bacteria *Escherichia coli (E.coli*, ATCC 25922) and *Pseudomonas aeruginosa (P.aeruginosa*, ATCC 27853), representing clinically important species and strains. The selection also represents one strain which is more susceptible (*S.aureus* and *E.coli*) and one strain which is more resistant (*E.faecalis* and *P.aeruginosa*) in each of the Gram-positive and Gram-negative groups. The broth microdilution method was used to determine the MIC values using Mueller–Hinton broth at pH 7.2. Blood agar was used to measure the MLC. Samples were prepared in sterile water at an initial concentration of 4,096 μg/mL, which was then serially diluted by twofold dilutions in a 96-well plate using Muller–Hinton broth. This gave a final concentrations varying from 2,048 to 1 μg/mL. *N*,*N*,*N*-Trimethyl-chitosan and gentamicin were used as positive controls during the test. A standard 0.5 McFarland suspension ($1–2\times10^8$ CFU/mL) was prepared by direct colony suspension in Mueller–Hinton broth and tested using McFarland turbidimetry meter (Densi CHEK plus). This suspension was further diluted to achieve a final test concentration of 5×10^5 CFU/mL in the wells in the microtiter plate. The microtiter plates were then incubated at 35 ºC for 18 h under moistened conditions. The MIC values were determined as the lowest concentrations of the antibacterial agent to completely inhibit the visible growth of the microorganism in the microtiter plate. For MLC measurements, 2×10 μL (of each of the dilutions that showed no visible growth) was placed on a blood agar plate and incubated at 35 ºC for 18 h. The MLC was determined as the lowest concentration that achieved a 99.9 % decrease in the viable cells. Results are shown in Table S8.

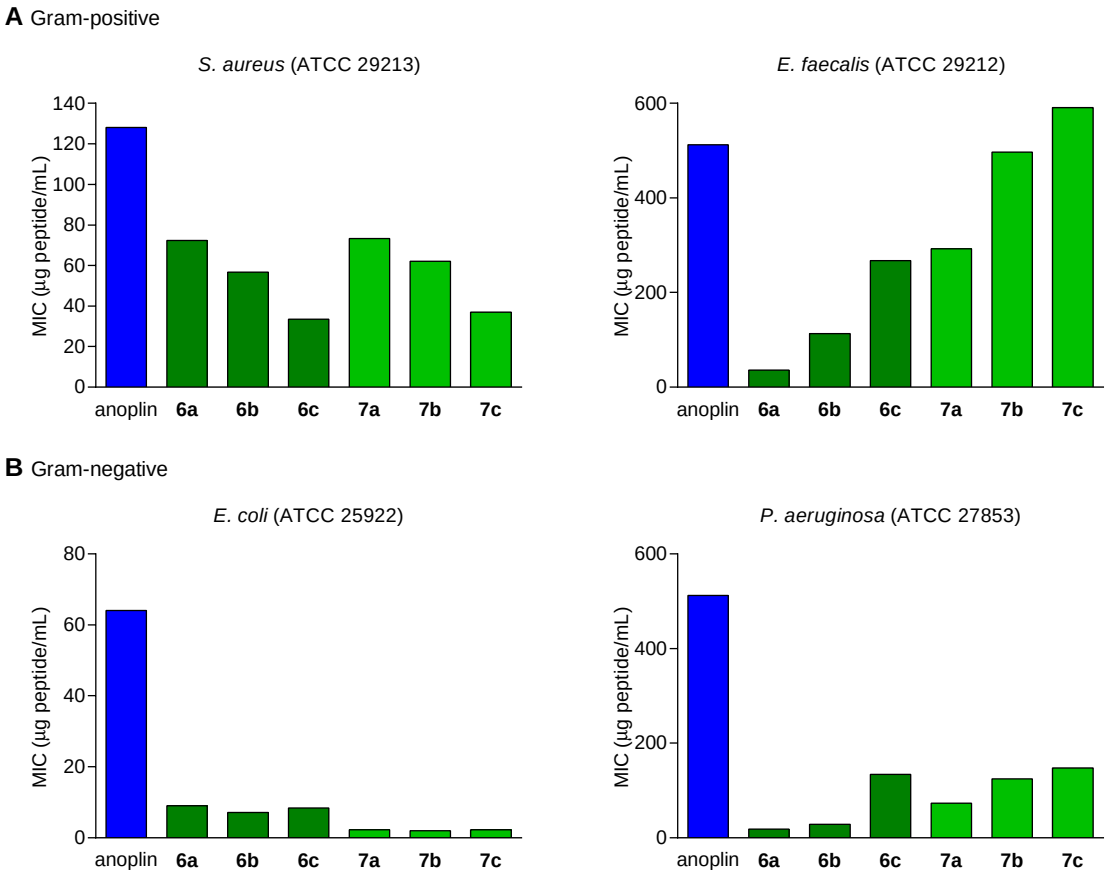


Figure S2. Graphical representation of data from Table 2 showing antimicrobial evaluation of anoplin-chitosan conjugates towards two Gram-positive (A) and two Gram-negative (B) bacterial strains as compared to anoplin. MIC values for **6a-c** and **7a-c** are referenced to peptide mass using Table S2.

Table S8. Minimum Lethal Concentration (MLC) of anoplin-chitosan conjugates.

Compound	*S. aureus* MLC (µg/mL)	*E. faecalis* MLC (µg/mL)	*E. coli* MLC (µg/mL)	*P. aeruginosa* MLC (µg/mL)
Anoplin	512	1,024	128	512
6a	1,024	≥1,024	16	512
6b	512	≥1,024	16	512
6c	64	≥1,024	8	512
7a	512	128	32	64
7b	256	256	32	64
7c	64	512	16	512
TMC	128	256	64	512

Hemolysis assay

Hemolysis assays were performed according to previously published procedures[4]. Human red blood cell (RBC) concentrate (3.40×10^{12} RBCs/L, total hemoglobin of 110 g/L, 0.04×10^{9} WBCs/L) was used for testing the hemolytic activity of the chitosan derivatives. RBCs (100 µL) were suspended in tris-buffered saline (TBS; 10 mL; pH=7.2). The polymer solutions were prepared in TBS at initial concentrations of 4,096 µg/mL and 32,768 µg/mL, which were serially diluted twofold in a 96-well plate, so as to have minimum concentrations of 2 µg/mL and 16 µg/mL. The RBC suspension (100 µL) was added to the polymer solutions (100 µL) and incubated at 37 °C with light shaking for 30 min. Cells treated with TBS and 2% TRITON-X were used as negative and positive controls, respectively. The cell suspensions were centrifuged at 1,500 rpm for 10 min, and the supernatant was used for measuring the absorbance of the released hemoglobin at 540 nm with the Thermo Scientific Multiscan Spectrum. The percentage hemolysis was calculated using the Equation (1):

$$\text{Hemolysis rate (\%)} = \frac{A - A_0}{(A_{100} - A_0)} \text{ x } 100\ \% \qquad (1)$$

where A is the absorbance of the polymer solutions, A_0 is the absorbance of the negative control, and A_{100} is the absorbance of the positive control.

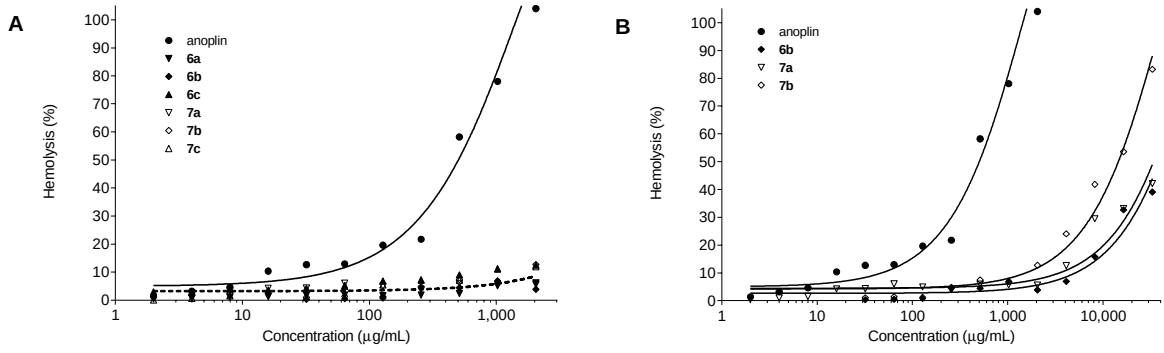
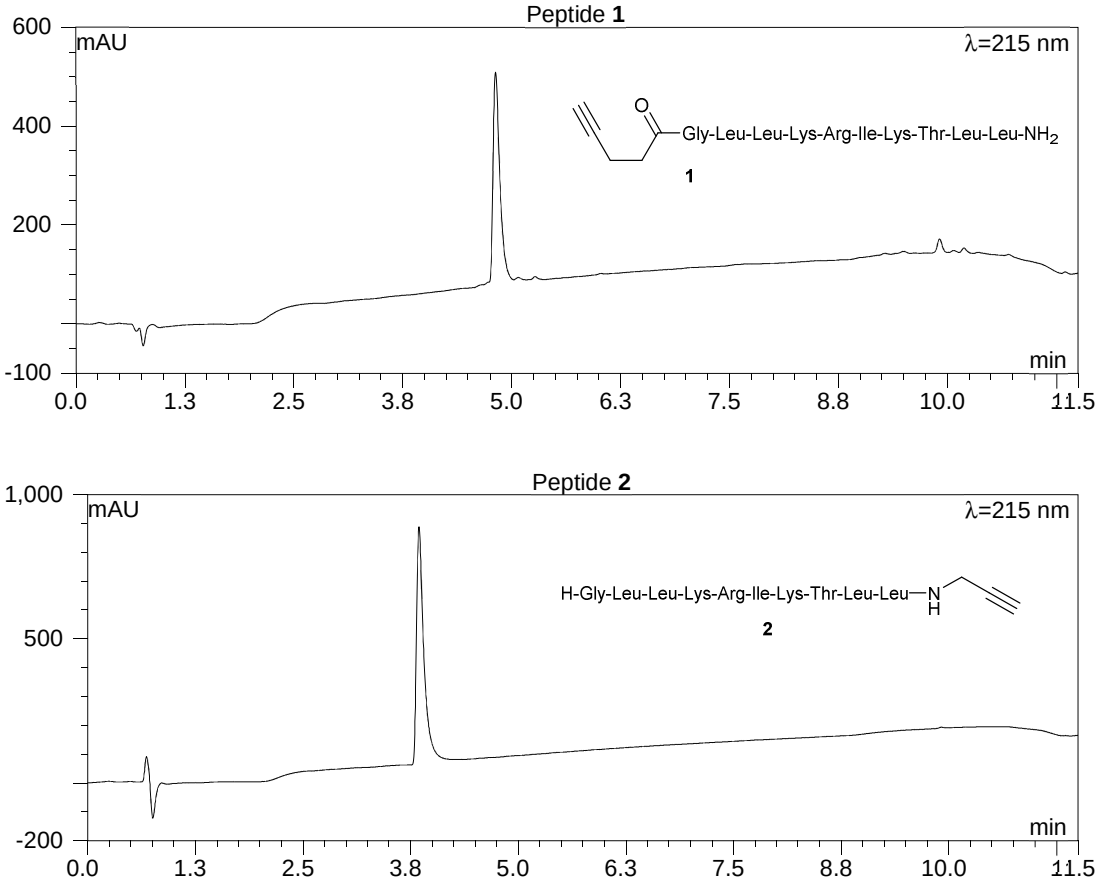


Figure S3. Hemolytic activity of anoplin-chitosan conjugates up to a conjugate concentration of 2,048 µg/mL. All conjugates are completely non-hemolytic in this concentration range (A). High-concentration hemolytic activity measurements were conducted with conjugates **6b**, **7a**, and **7b** up to 32.8 mg/mL showing that these conjugates were highly hemocompatible (B).

HPLC chromatograms for peptides 1 and 2



IR spectra for chitosan and *N*-(2-azidoacetyl)-chitosans 5a-c

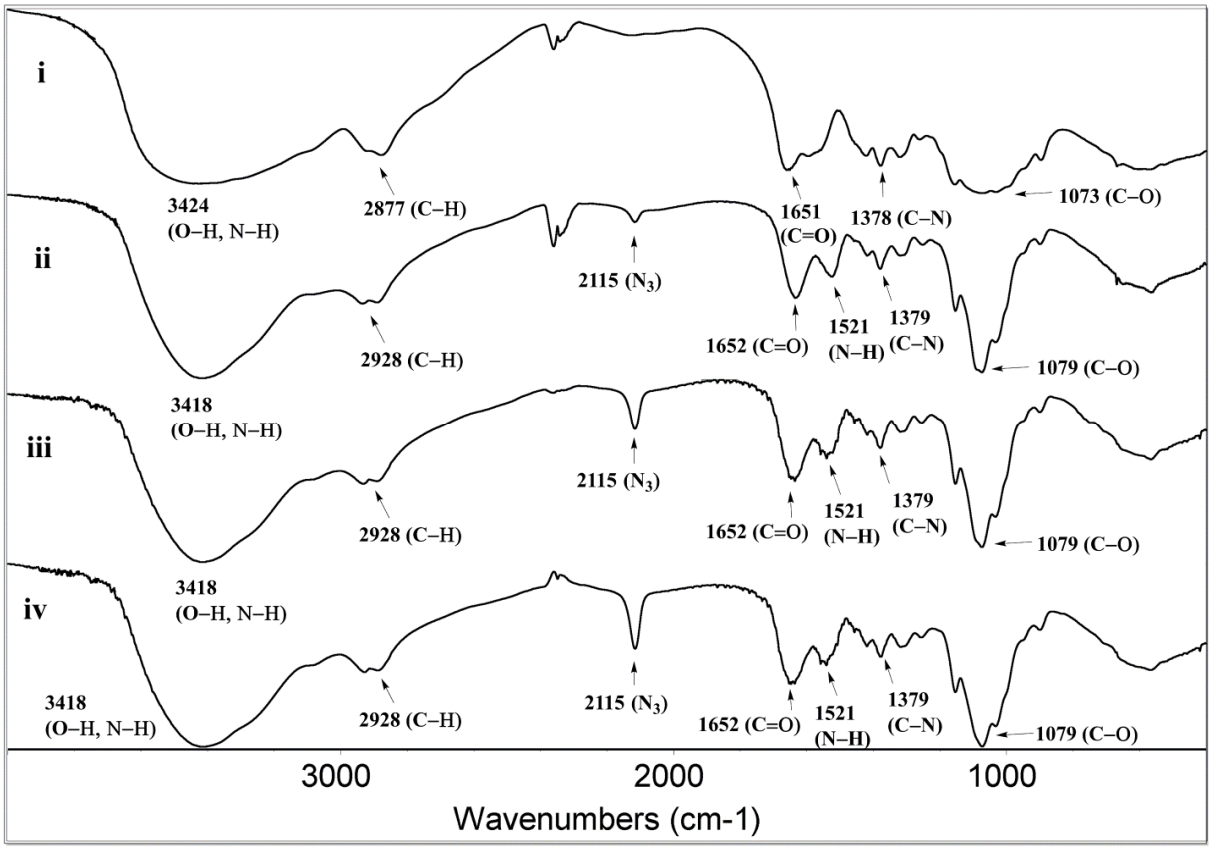
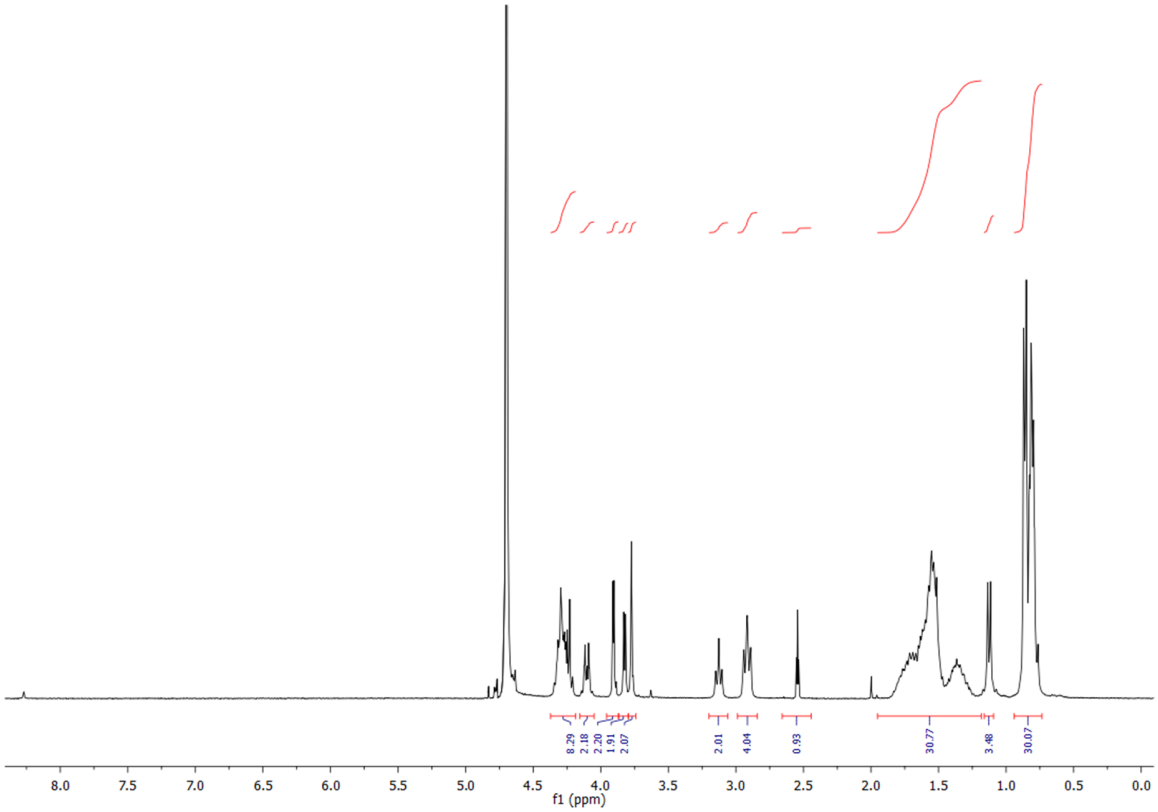
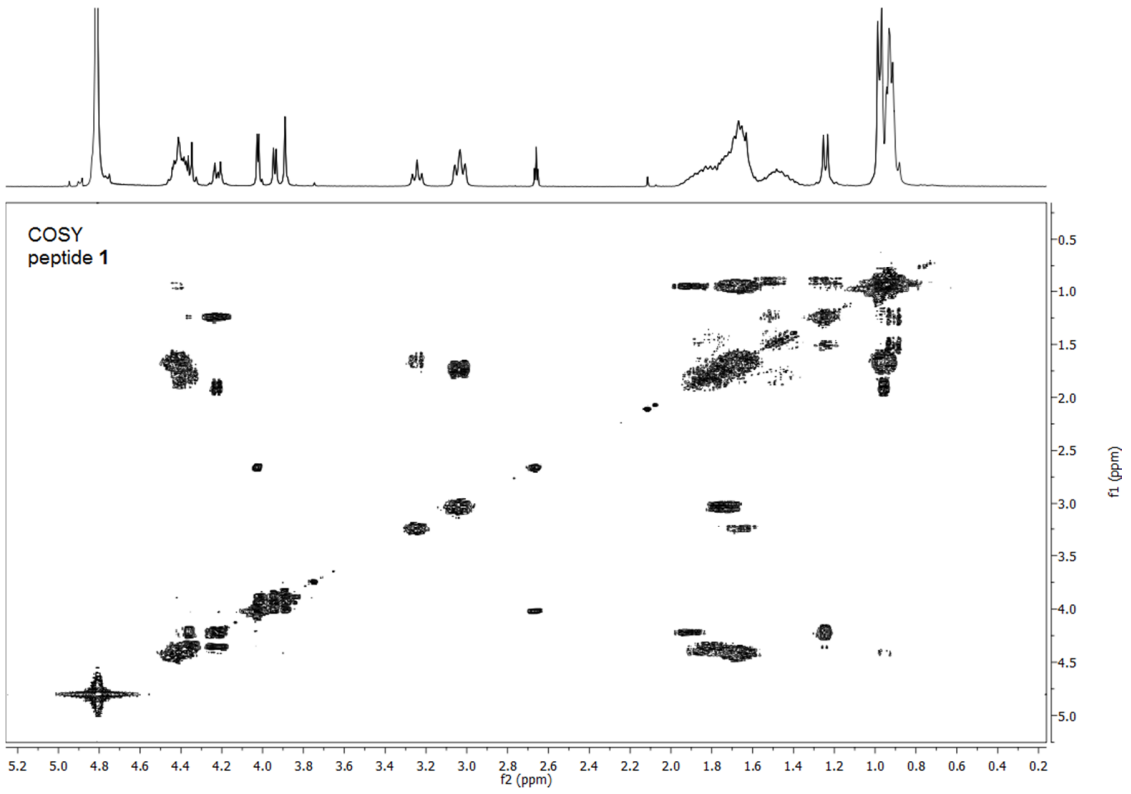
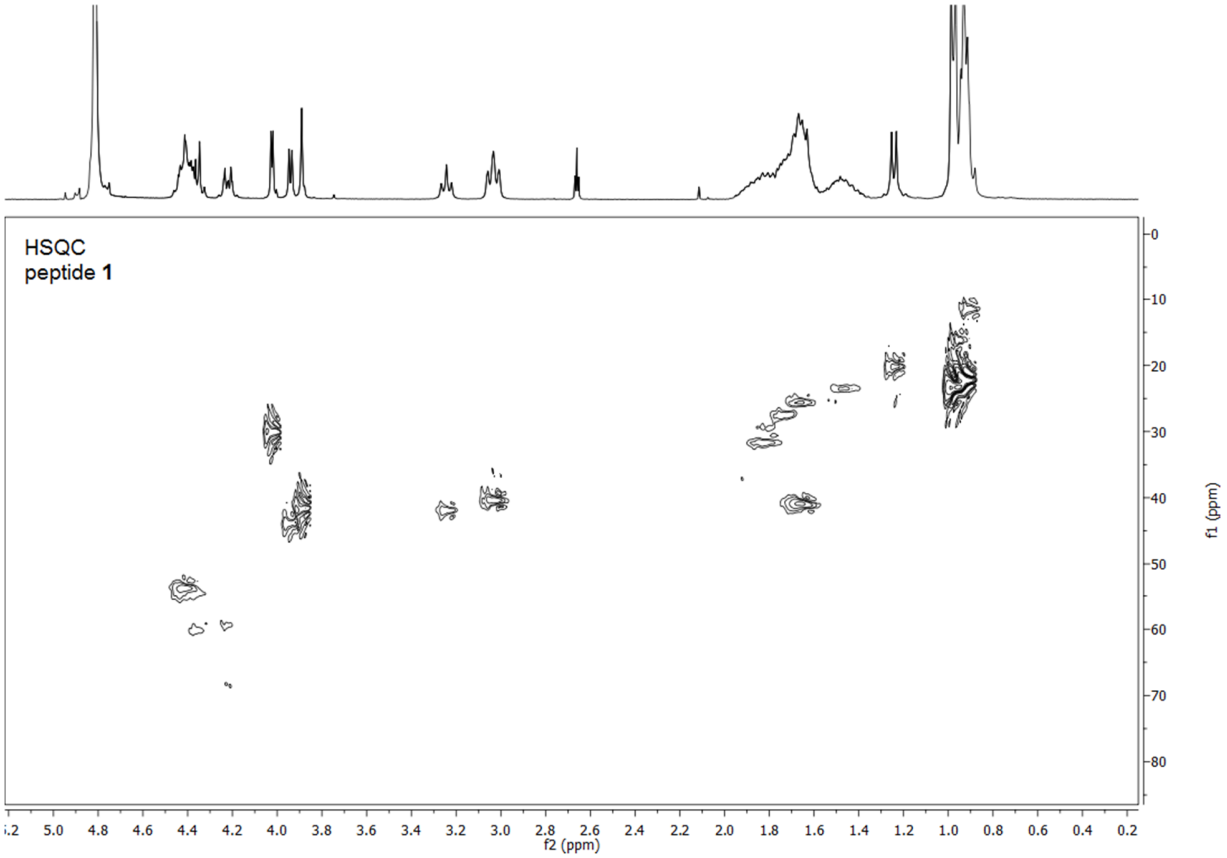


Figure S4. Stacked IR spectra. (i) chitosan, and *N*-(2-azidoacetyl) chitosans (ii) **5a**, (iii) **5b**, and (iv) **5c**. Compounds **5a-c** displays characteristic azido stretching vibration at 2115 cm^{-1} of increasing intensity with increasing degree of substitution, DS_{Az}.

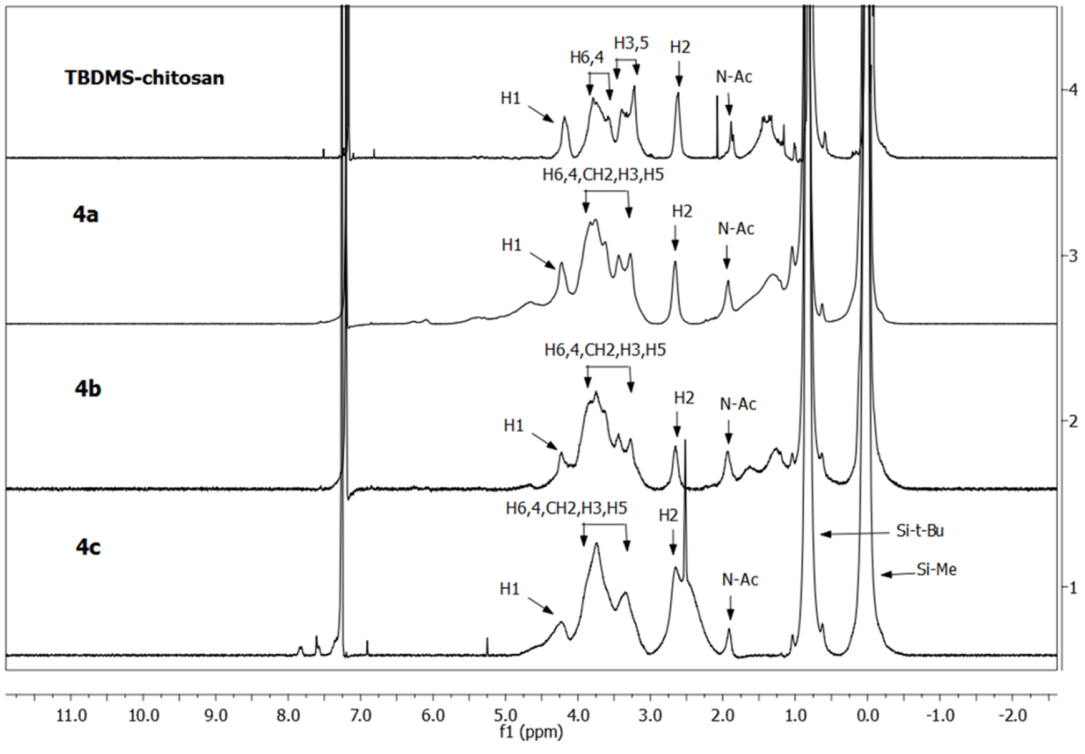
NMR spectra for peptide 1 in D_2O



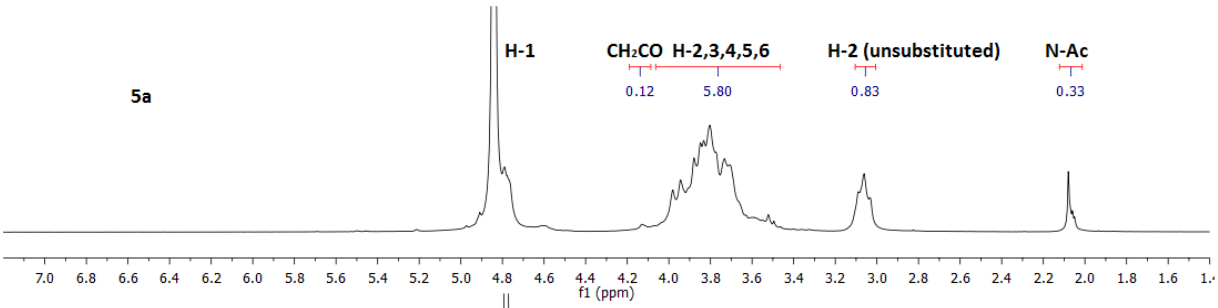


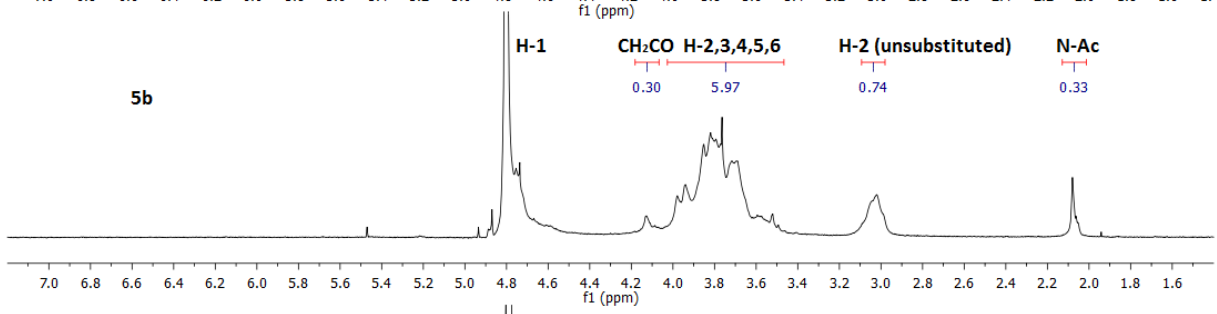


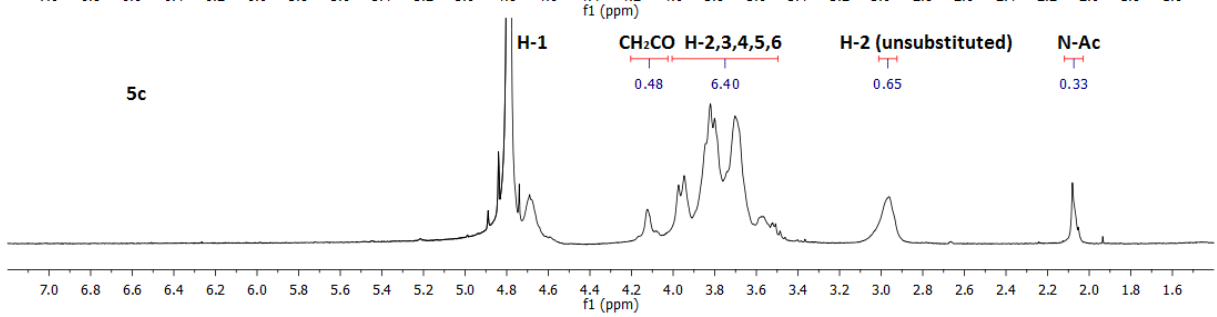
NMR spectra for chitosan derivatives 4a-c in $CDCl_3$

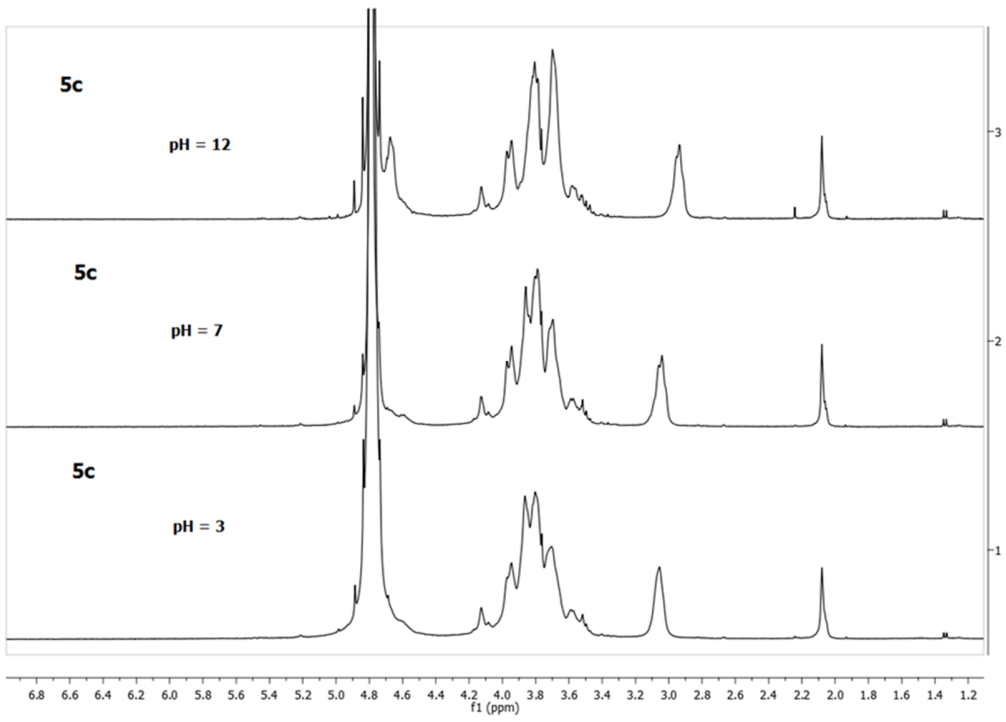


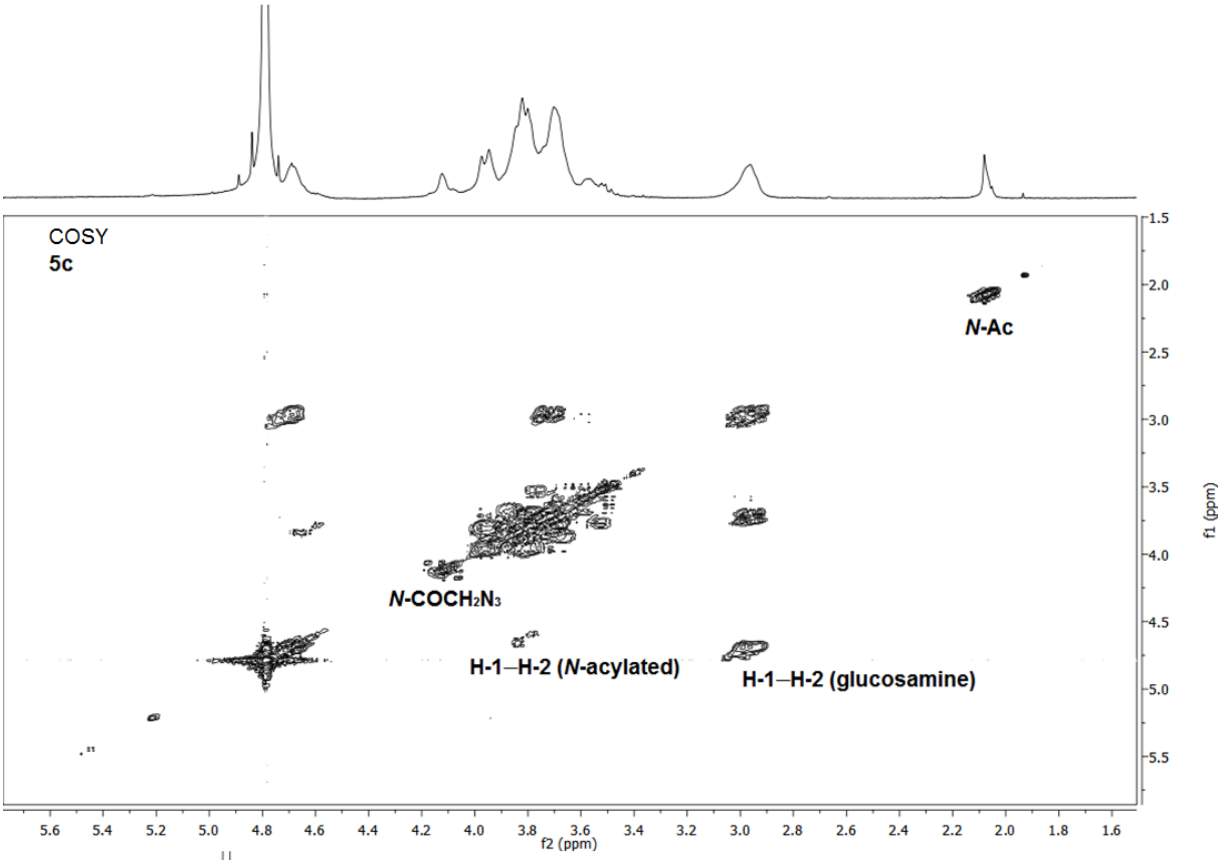
NMR spectra for *N*-(2-azidoacetyl)-chitosans 5a-c in D_2O

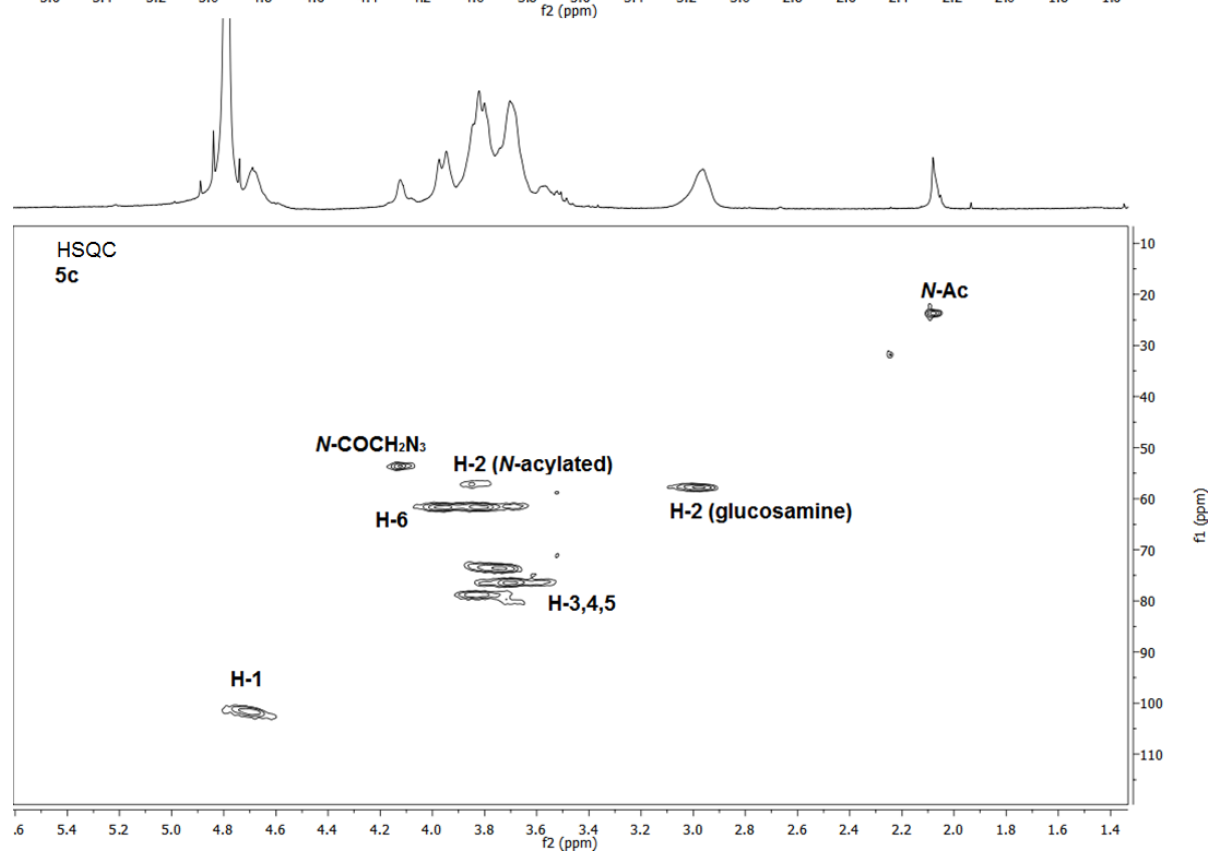




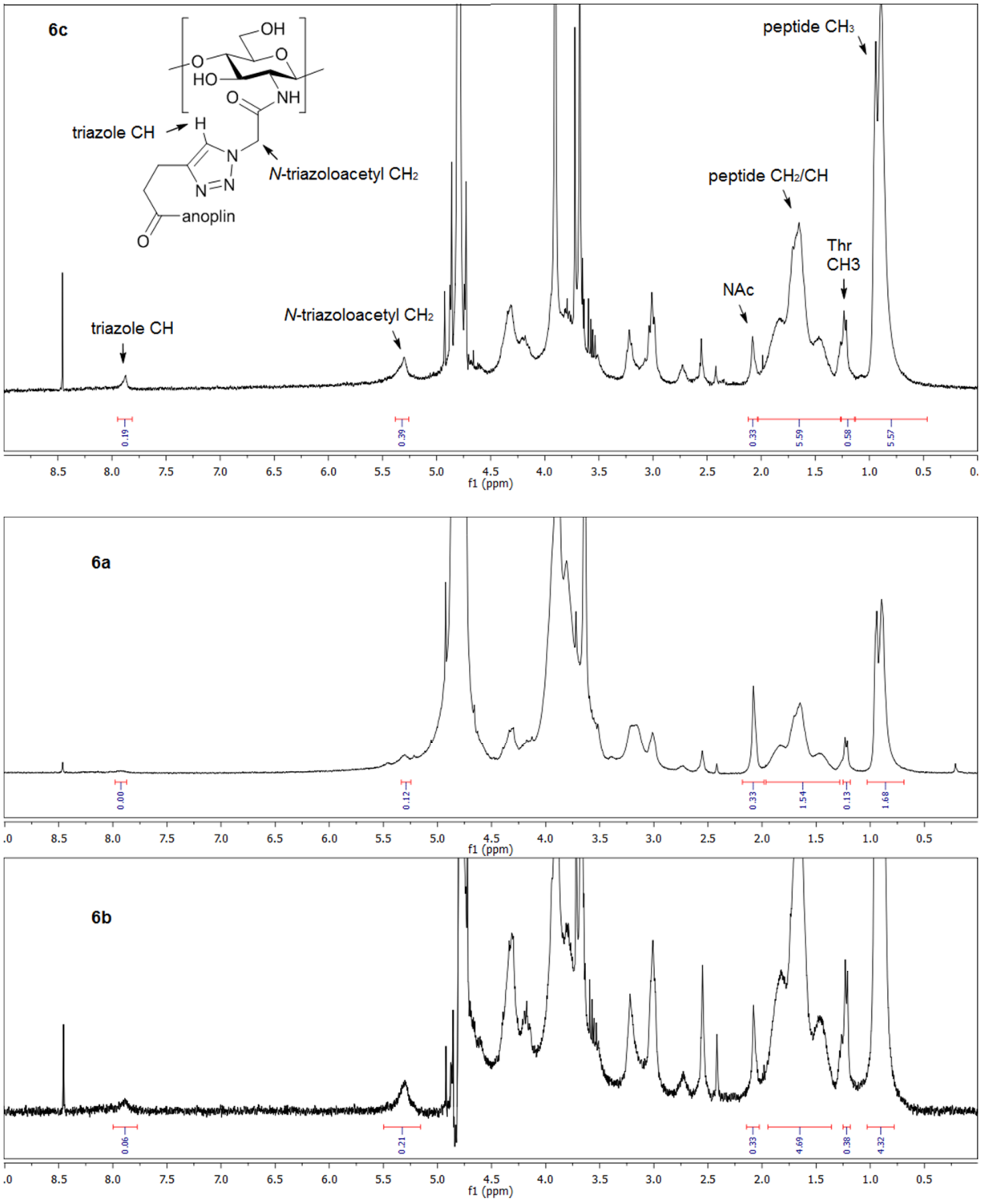


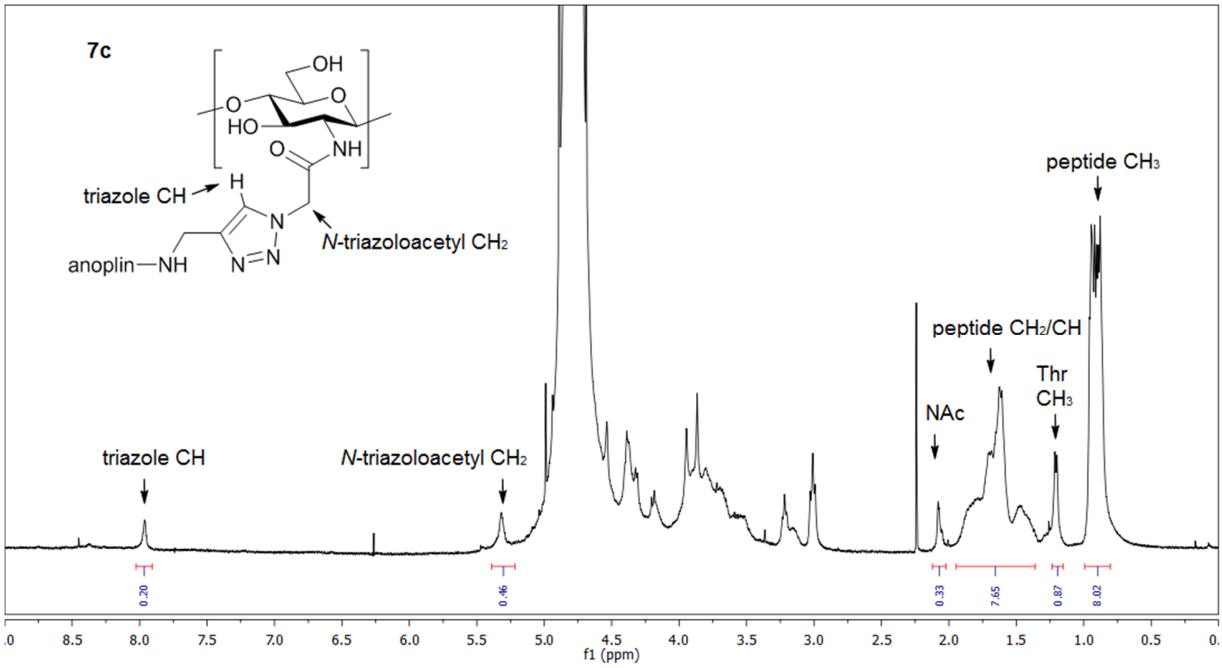


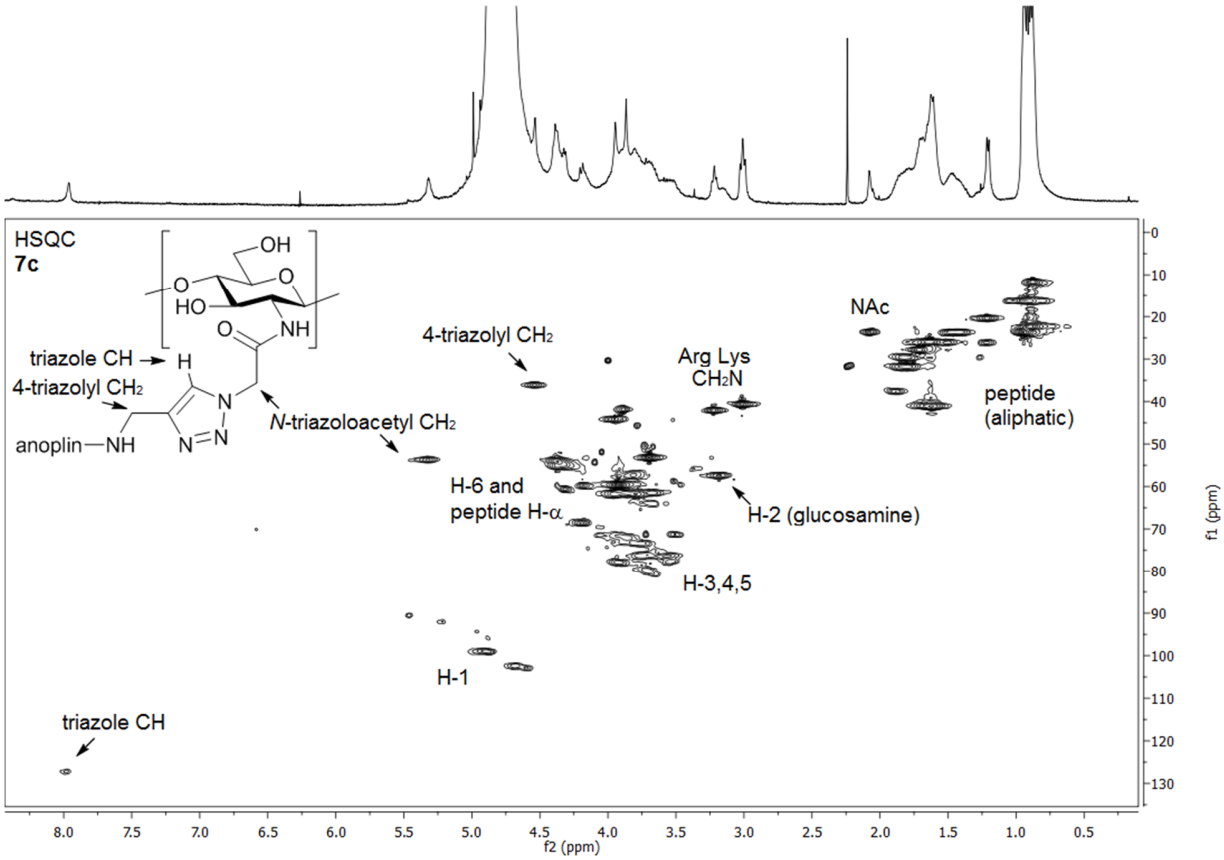

COSY
5c
N-Ac
N-COCH2N3
H-1–H-2 (N-acylated)
H-1–H-2 (glucosamine)
f1 (ppm)
f2 (ppm)

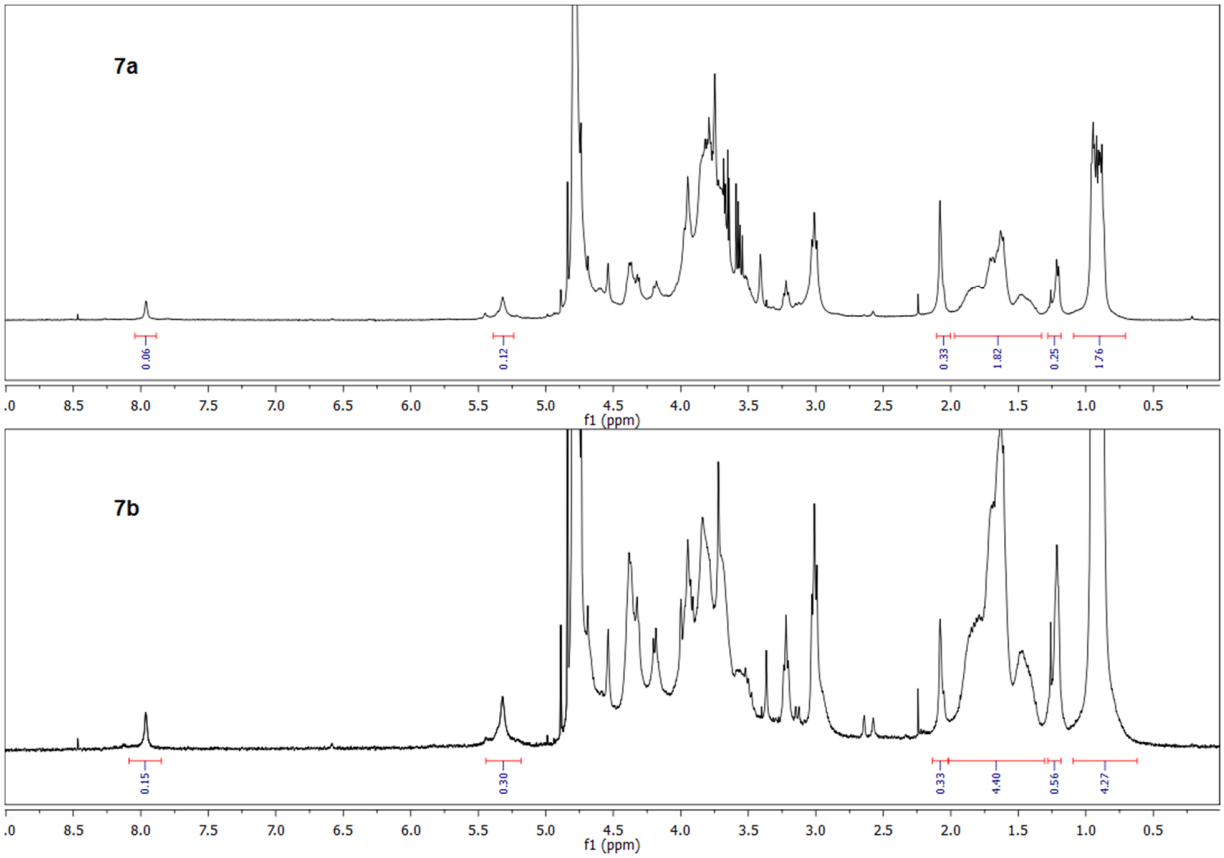

HSQC
5c
N-Ac
N-COCH2N3
H-2 (N-acylated)
H-6
H-2 (glucosamine)
H-3,4,5
H-1
f1 (ppm)
f2 (ppm)

NMR spectra for anoplin-chitosan conjugates, 6a-c and 7a-c, in D_2O

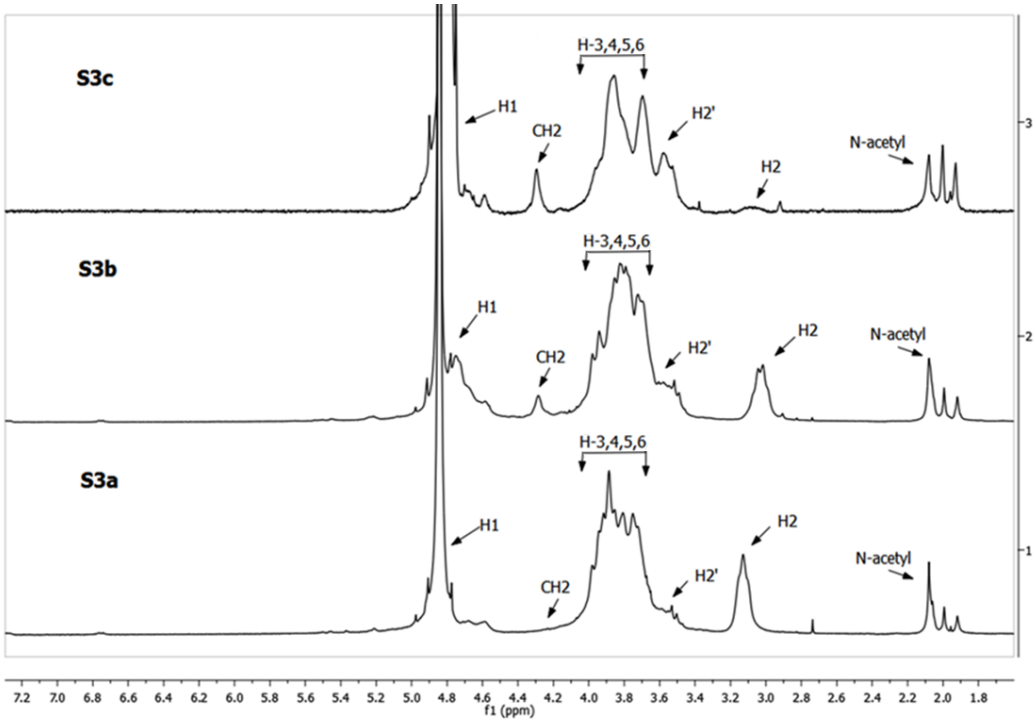



7c
triazole CH
N-triazoloacetyl CH2
anoplin—NH
peptide CH3
peptide CH2/CH
Thr CH3
NAc
triazole CH
N-triazoloacetyl CH2
0.20
0.46
0.33
7.65
0.87
8.02
f1 (ppm)


HSQC
7c
triazole CH
4-triazolyl CH2
anoplin—NH
N-triazoloacetyl CH2
4-triazolyl CH2
Arg Lys CH2N
NAc
peptide (aliphatic)
H-6 and peptide H-α
H-2 (glucosamine)
H-3,4,5
H-1
triazole CH
f2 (ppm)
f1 (ppm)



NMR spectra for *N*-(2-aminooxyacetyl) chitosans S3a-c in D_2O



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