Supplementary material

Chitosan with pendant (*E*)-5-((4-acetylphenyl) diazenyl)-6-aminouracil groups as antimicrobial agents

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Content

1. Anti- fungal Activity studies

1.1 Well diffusion method to check the Minimum Inhibition Concentration (MIC)

Processed sample (VB0, VB1, VB2, VB3, VB4, VB5 and VB6) were assessed for MIC property against organisms (*Aspergillus niger, Aspergillus flavus, Candida albicans* and *Trichophyton rubnum*)

1.2 Culture Preparation

Potato Dextrose Broth (PDB: Potato-200g, Dextrose-20g, Agar-20g, Distilled water-1000mL) 400mL of Broth was prepared by boiling 80g of Potato in 200mL distilled water and filtered. 8g of Dextrose was added into the filtrate and the volume was made upto 400mL with distilled water and 100mL of broth was transferred into 4 conical flasks respectively and autoclaved at 121°C for 15 mins. Later, the organism (*Aspergillus niger, Aspergillus flavus, Candida albicans* and *Trichophyton rubnum*) was inoculated respectively in 100mL sterilized PDB broth and incubated at 25˚ C for 72h.

1.3 Sample preparation

Different aliquots of processed sample (VB0, VB1, VB2, VB3, VB4, VB5, VB6) was prepared by pipetting 10µL (30µg), 20µL (60µg), 30µL (90µg), 40µL (120µg). Fluconazole 1mg dissolved in 1mL of DMSO as positive Control was prepared by pipetting 10µL (10µg), 20µL (20µg), 30µL (30µg), 40µL (40µg) and the final volume was made upto 50µL by adding Dimethyl sulfoxide (DMSO)

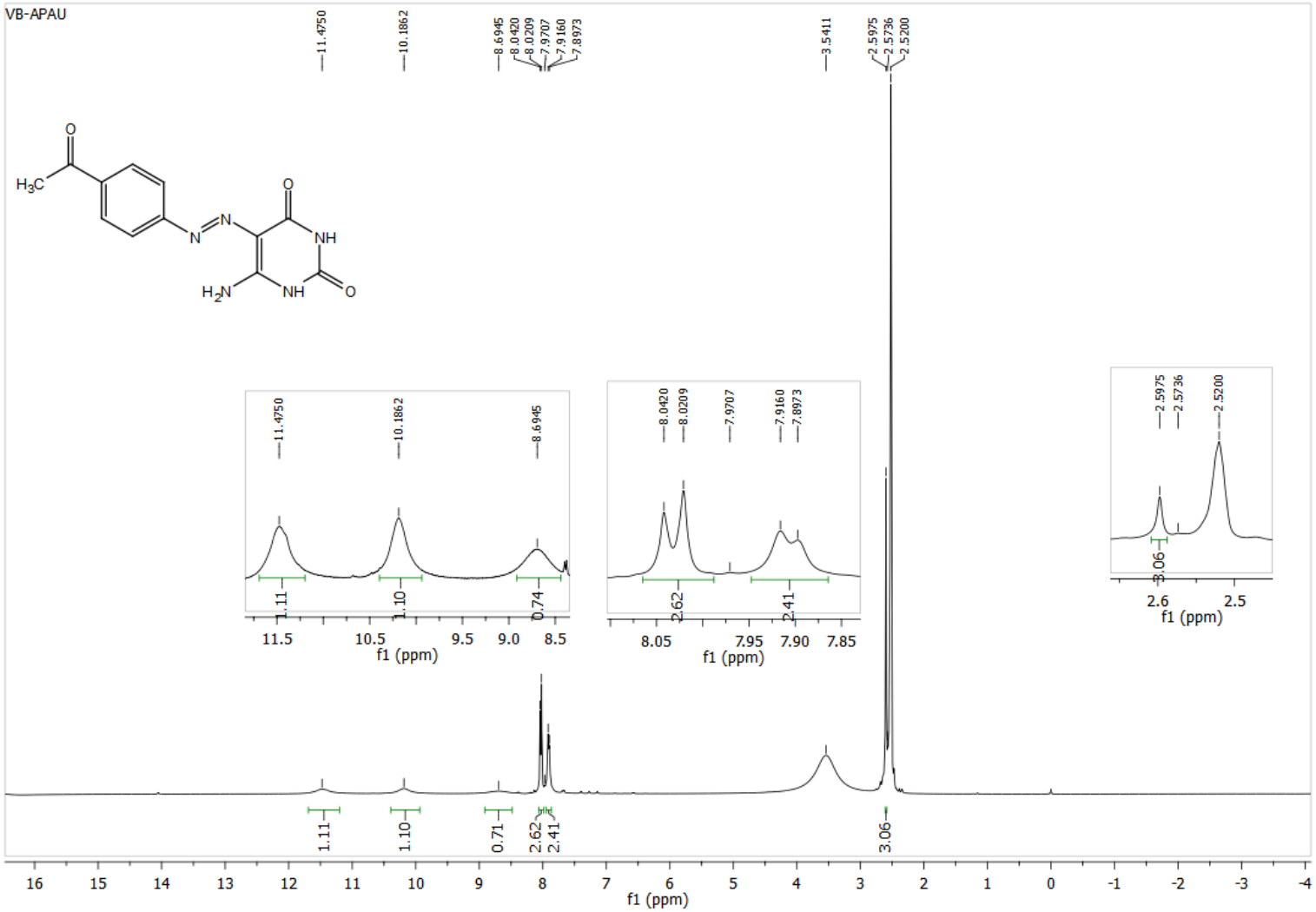
1.4 Media preparation for MIC

Potato Dextrose Agar (PDA: Potato-200g, Dextrose-20g, Agar-20g, Distilled water-1000mL) 800mL of media was prepared by boiling 160g of Potato in 500mL distilled water and filtered. 16g of Dextrose, 16g of Agar was added into the filtrate and the volume was made upto 800mL with distilled water. Autoclaved at 121°C for 15 mins.

1.5 Plating for MIC against organisms.

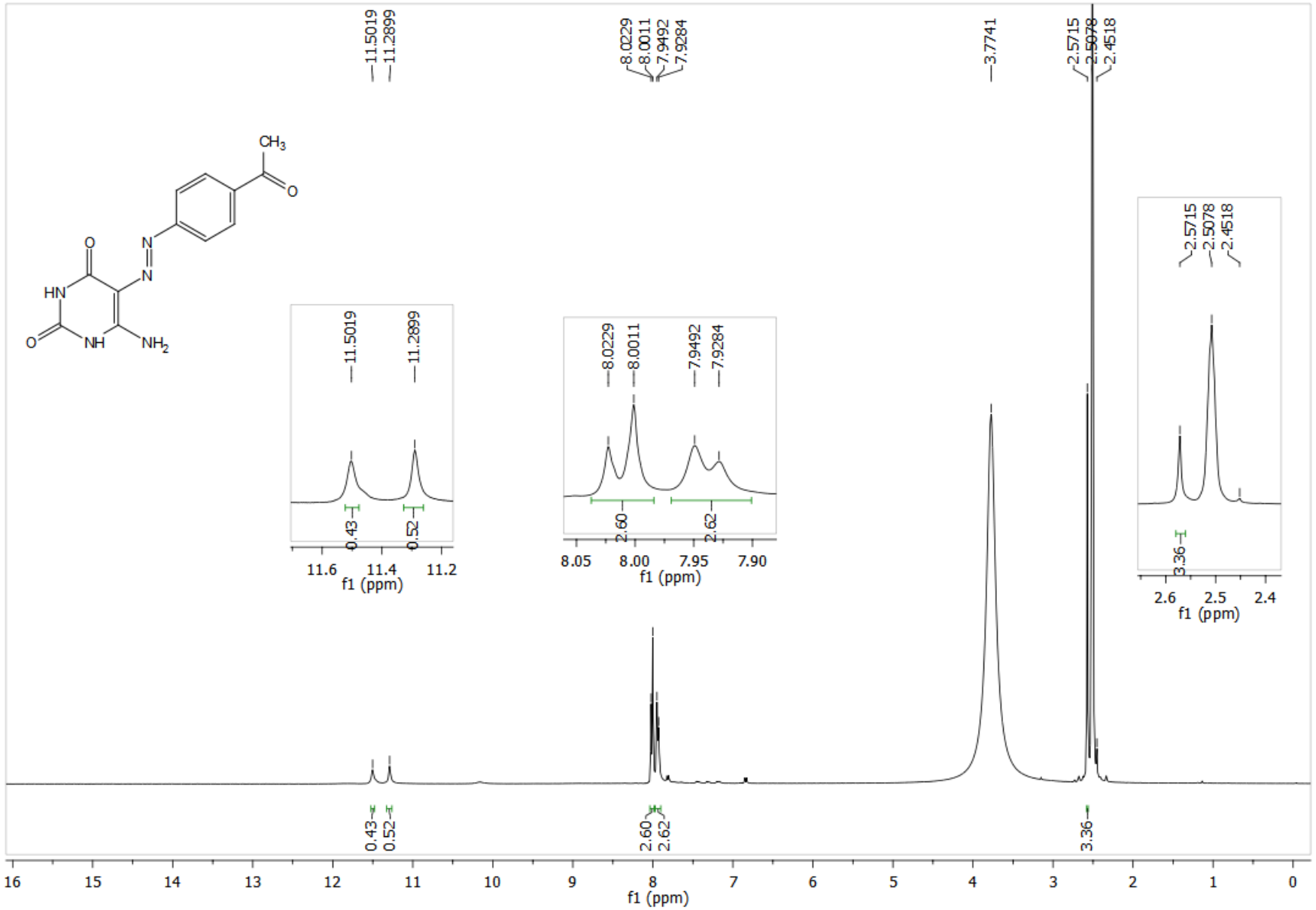
Approximately 25mL of PDA was poured into a sterilized petriplates and allowed it to solidify. 200µL inoculum from a 10^6 CFU/ml concentration stock *(Aspergillus niger, Aspergillus flavus, Candida albicans* and *Trichophyton rubnum)* was poured respectively on a PDA plates and spread thoroughly using a plate spreader. Five wells measuring 0.6cm was made in each plates using the borer and 50µL of sample (VB0, VB1, VB2, VB3, VB4, VB5 and VB6) containing 30µg, 60µg, 90µg, 120µg were loaded into the respective wells and 50µL of DMSO was loaded in the middle well as control blank. The fungal plates incubated at 25°C for 72h. Later, zone of inhibition was recorded in mm (Millimeter).

2. Figure S1 ^{1}H NMR of APAU in DMSO D_6

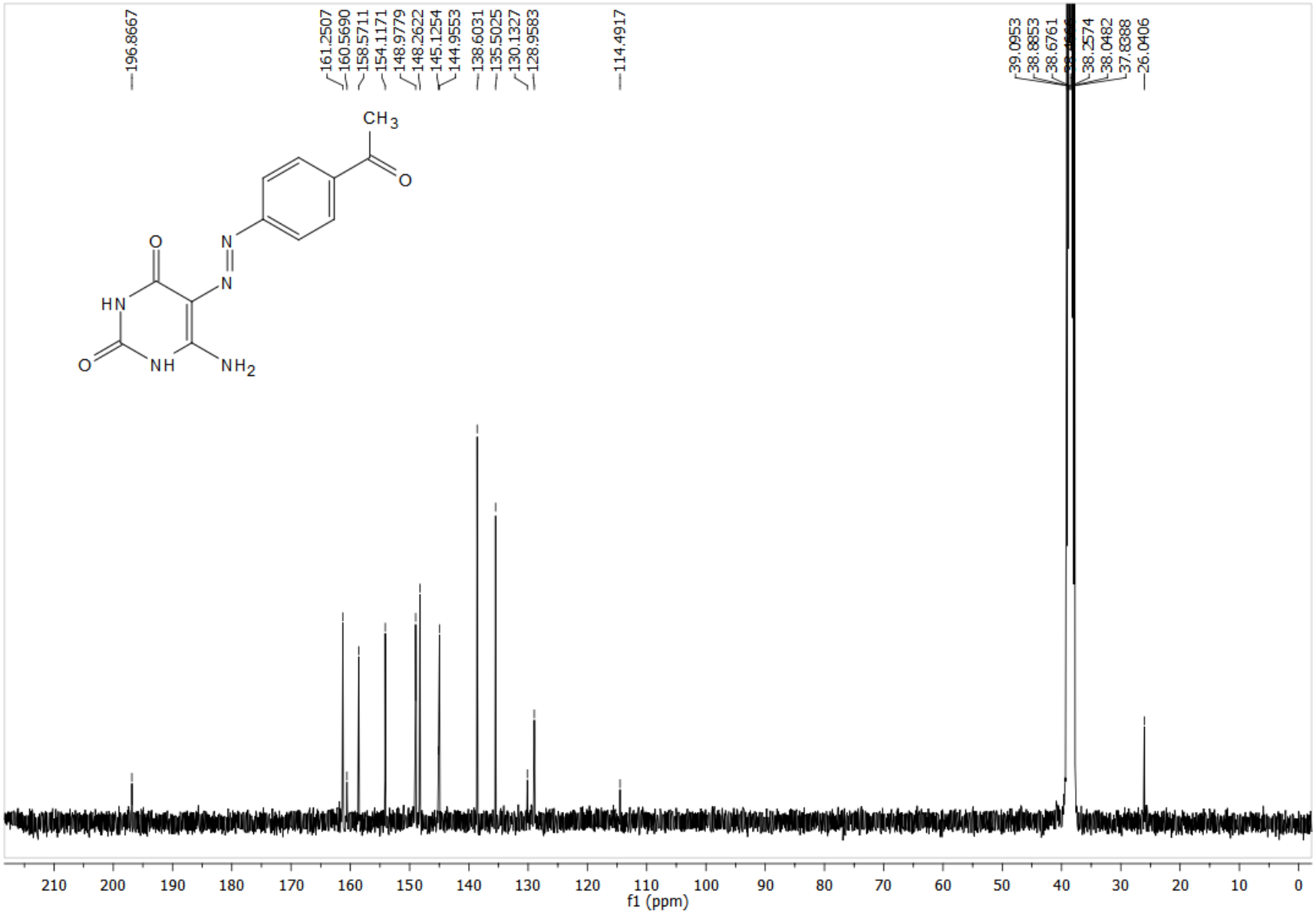


(E)-5-((4-acetylphenyl) diazenyl)-6-aminouracil: Yellow solid, yield: 95 %, ^{1}H NMR (400 MHz, DMSO-d6) δ 11.48 (s, 1H), 10.19 (s, 1H), 8.69 (s, 1H), 8.03 (d, *J* = 8.5 Hz, 3H), 7.91 (d, *J* = 7.5 Hz, 2H), 2.60 (s, 3H).

3. Figure S2 ^{1}H NMR of APAU in DMSO D_6 with added D_2O

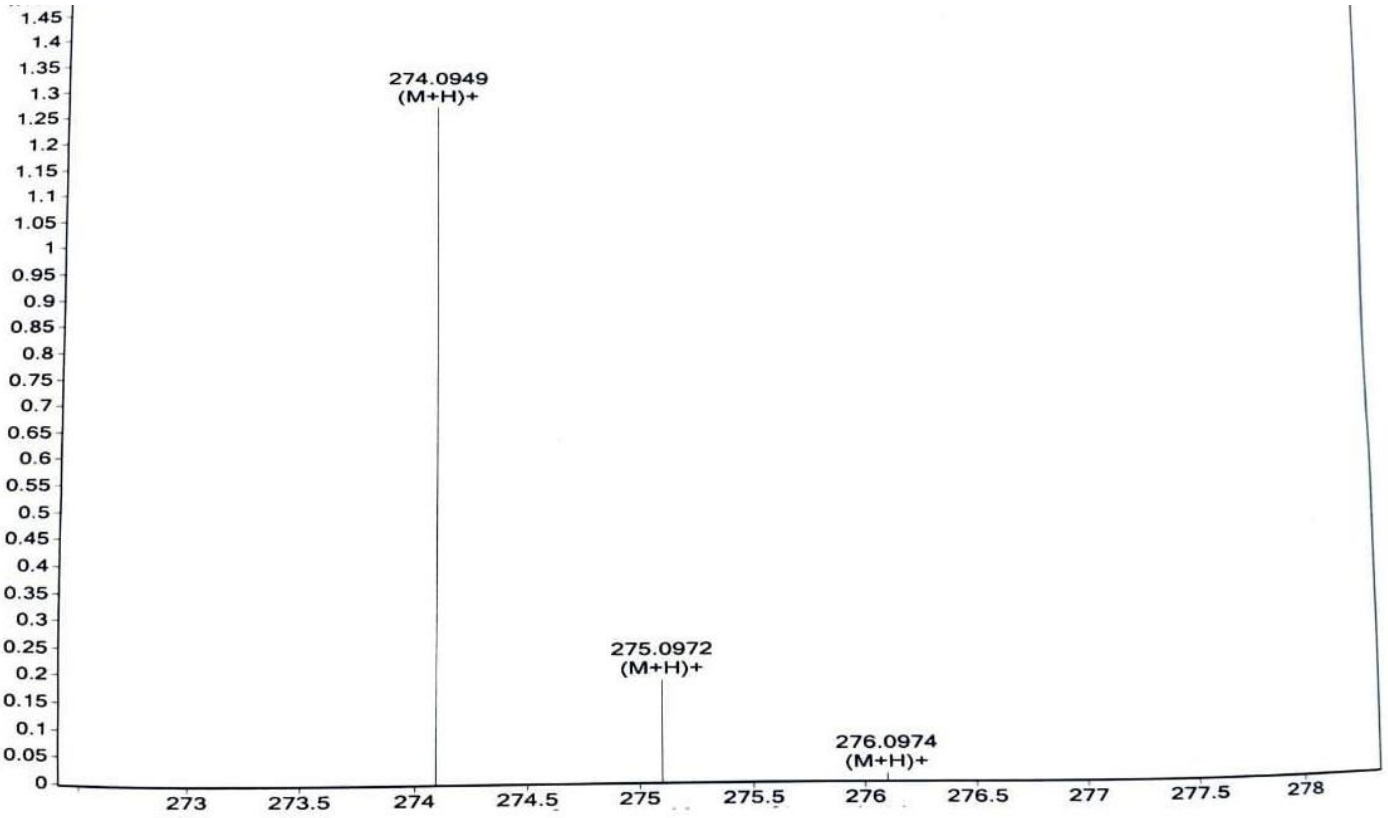


4. Figure S3 ^{13}C NMR of APAU in DMSO D_6



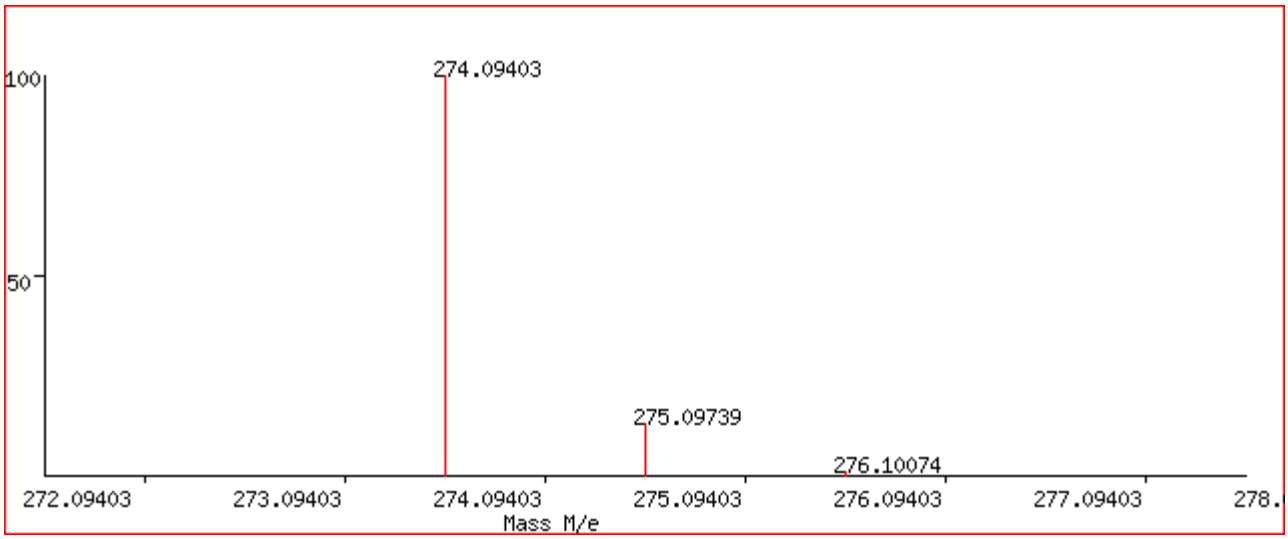
^{13}C NMR (101 MHz, DMSO-d6) δ 196.87 (s), 161.25 (s), 160.57 (s), 158.57 (s), 154.12 (s), 148.98 (s), 148.26 (s), 145.04 (d, J = 17.1 Hz), 138.60 (s), 135.50 (s), 130.13 (s), 128.96 (s), 114.49 (s), 26.04 (s).

5. Figure S4 Experimental ESI-MS ionization peak pattern of APAU

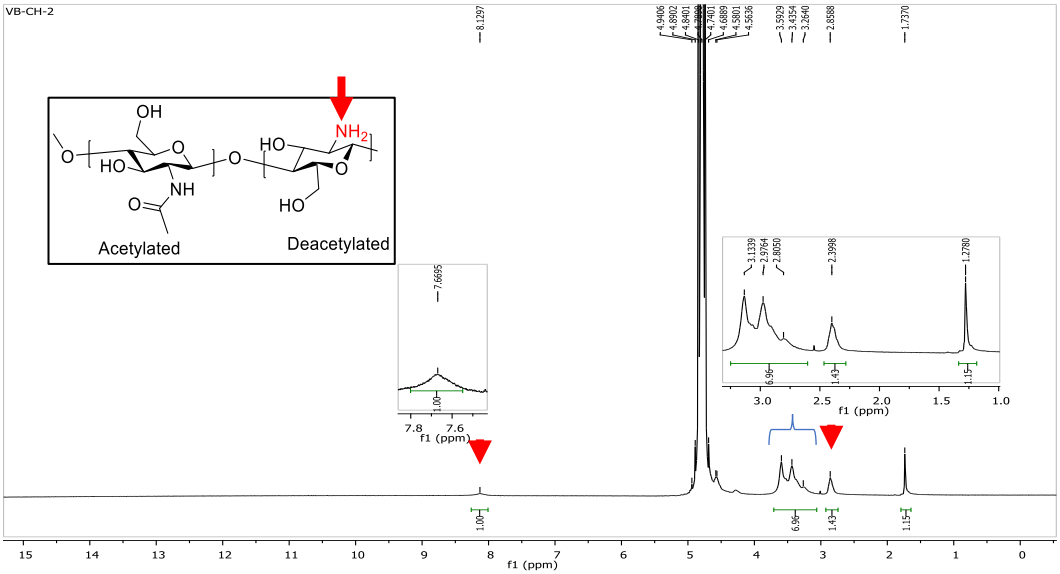


The isotopic distribution pattern for molecular ion peaks using ESI-MS corresponding to $[APAU + H]^+$ at *m/z* = 274.0949 matches with the theoretically predicted isotopic distribution pattern (Scientific Instrument Services tool https://www.sisweb.com/mstools/isotope.htm).

6. Figure S5 Theoretically predicted ESI-MS ionization peak pattern of APAU (predicted using https://www.sisweb.com/mstools/isotope.htm)

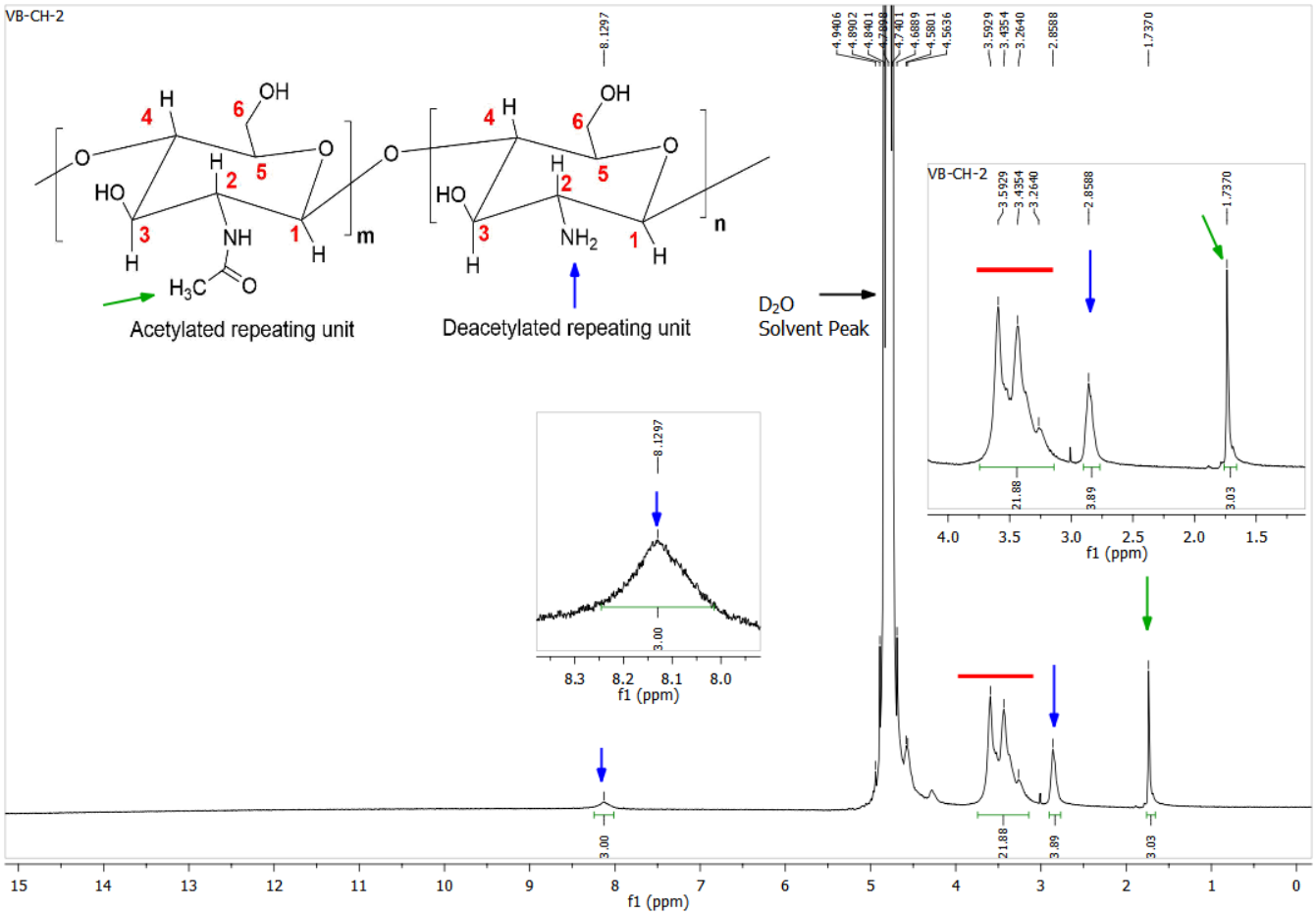


7.1. Figure S6a ^{1}H NMR of Chitosan in D_2O with DCl

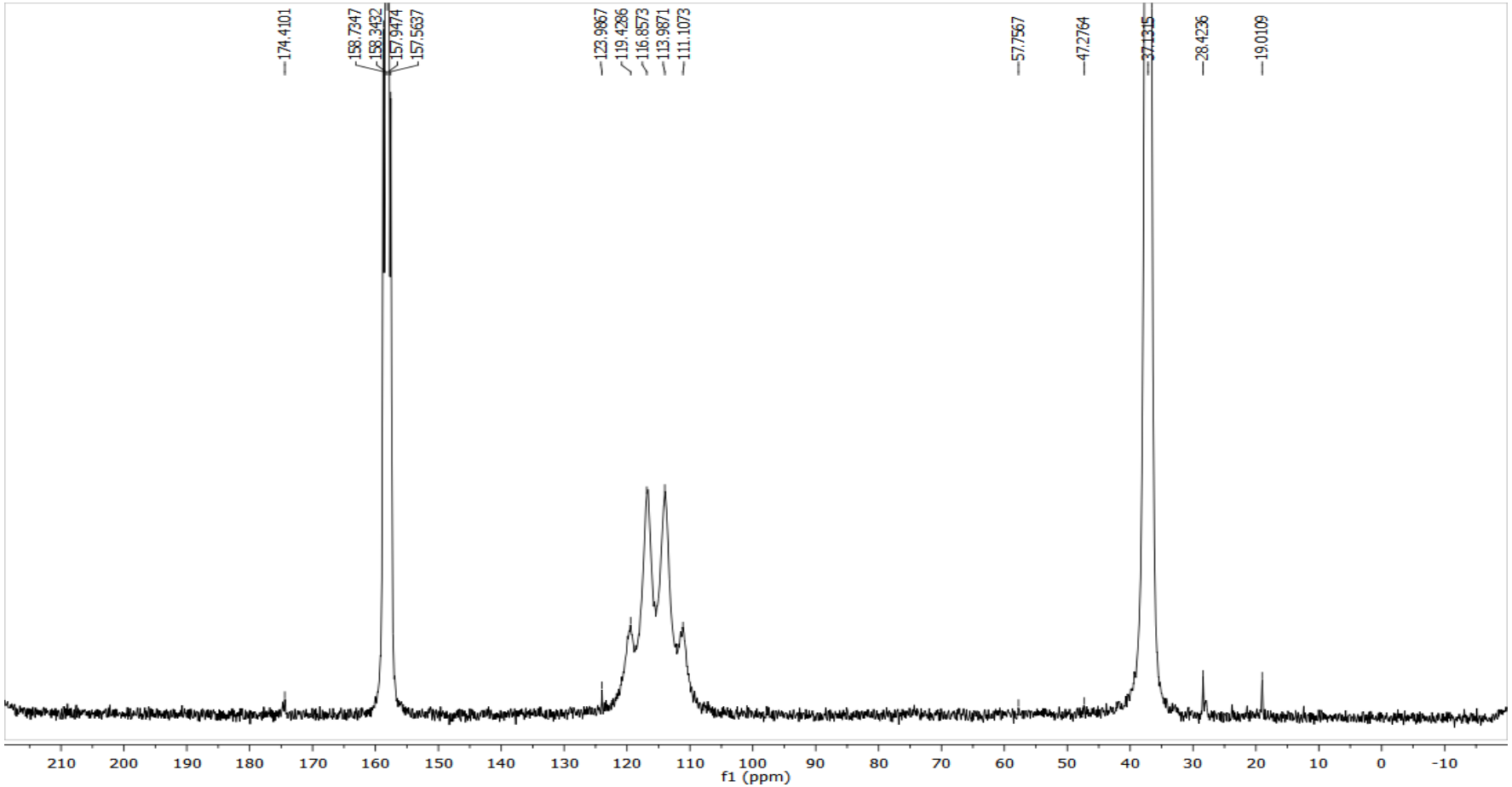


Initial consideration of NH proton for degree of deacetylation calculation is set aside due to possible proton exchange with deuterium. Hence, the same spectrum is reinterpreted considering area under methyl peak as part of an alternate equation for Degree of Deacetylation calculation. The analysis is given in Figure S6b as shown below.

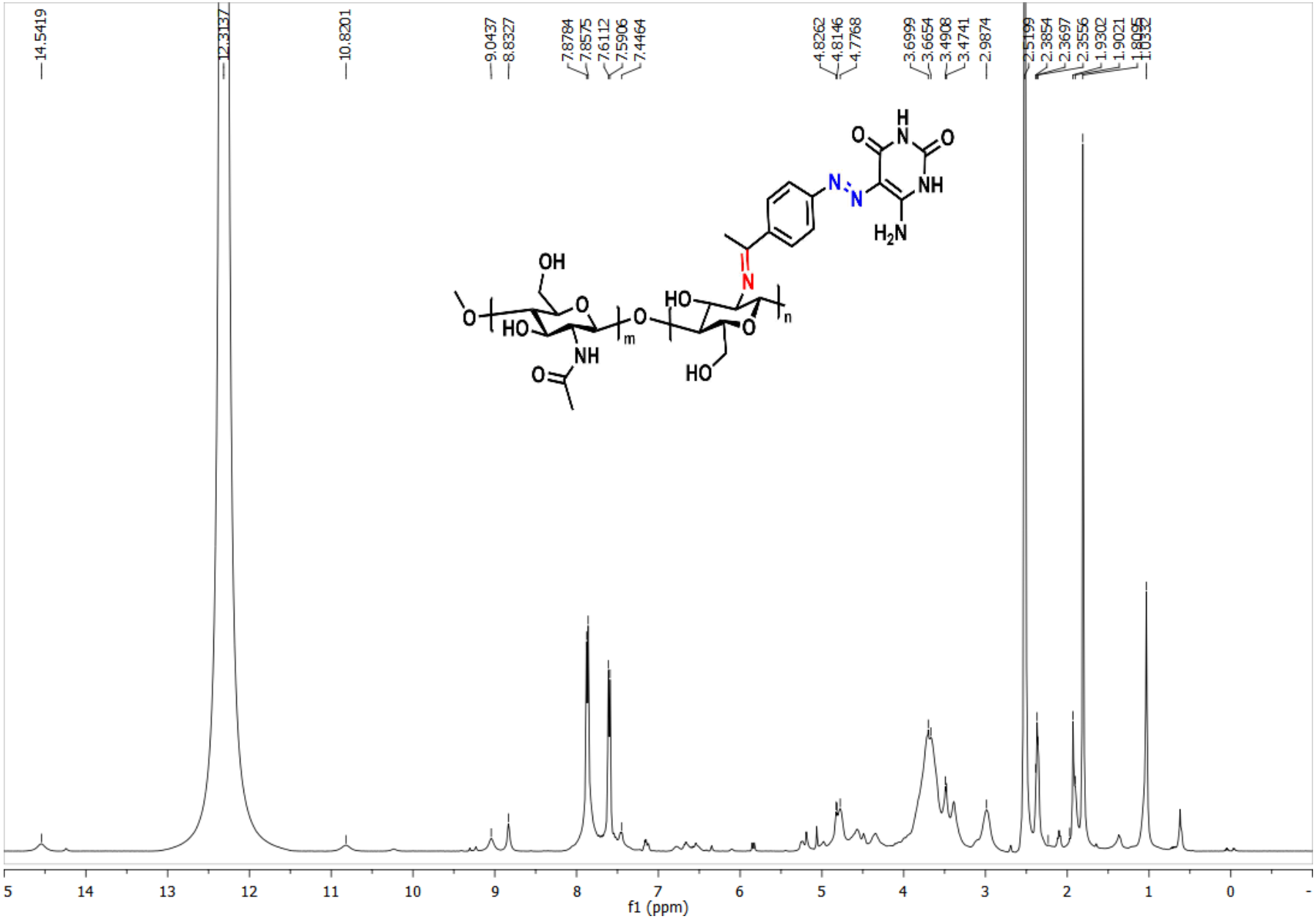
7.2. Figure S6b ^{1}H NMR of Chitosan in D_2O with DCl for Degree of deacetylation calculation



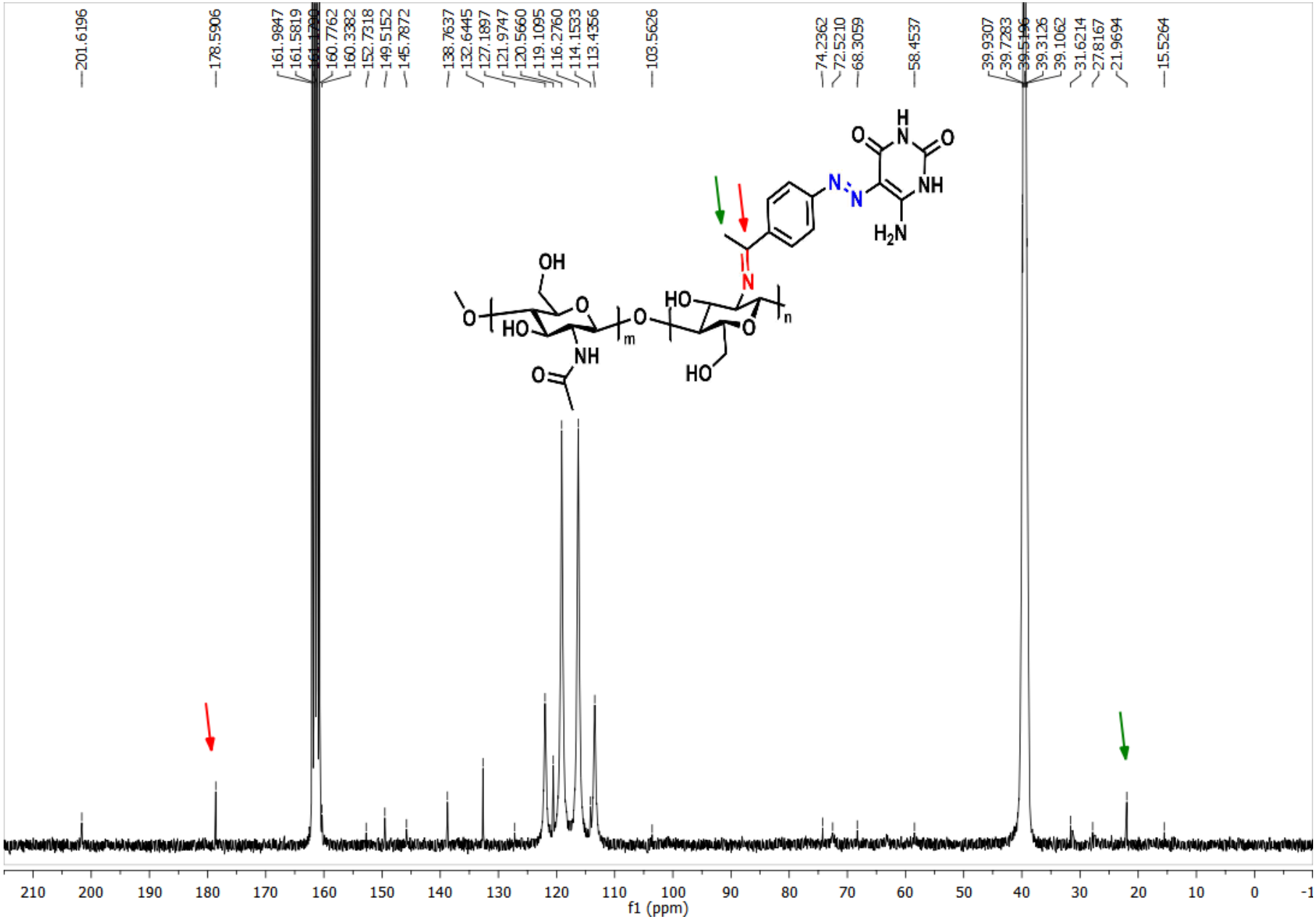
7.3. Figure S6c ^{13}C NMR Spectrum of Chitosan in DMSO-d6 and CF_3COOD



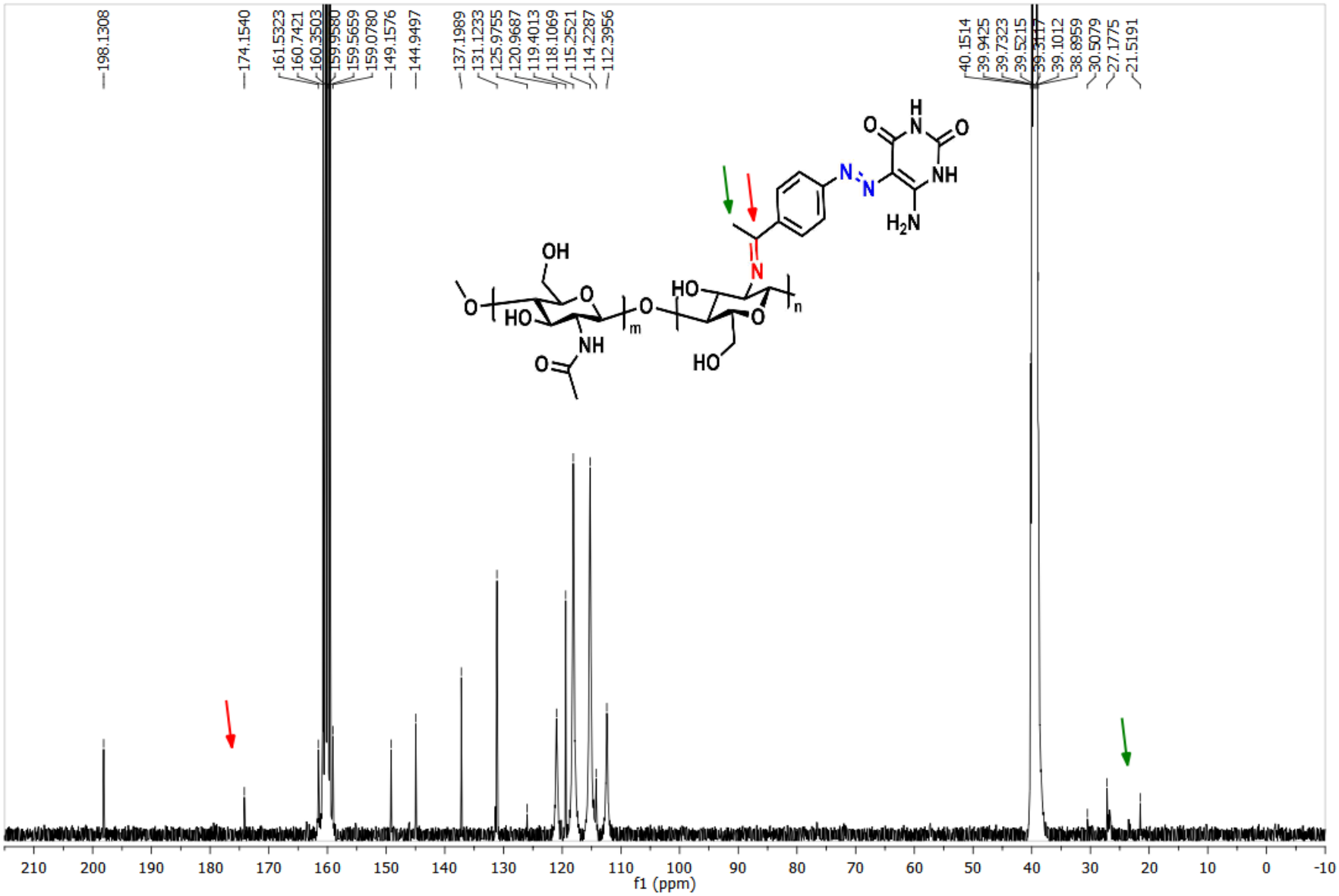
7.4. Figure S6d ^{1}H NMR Spectrum of CA40 in DMSO-d6 and CF_3COOD



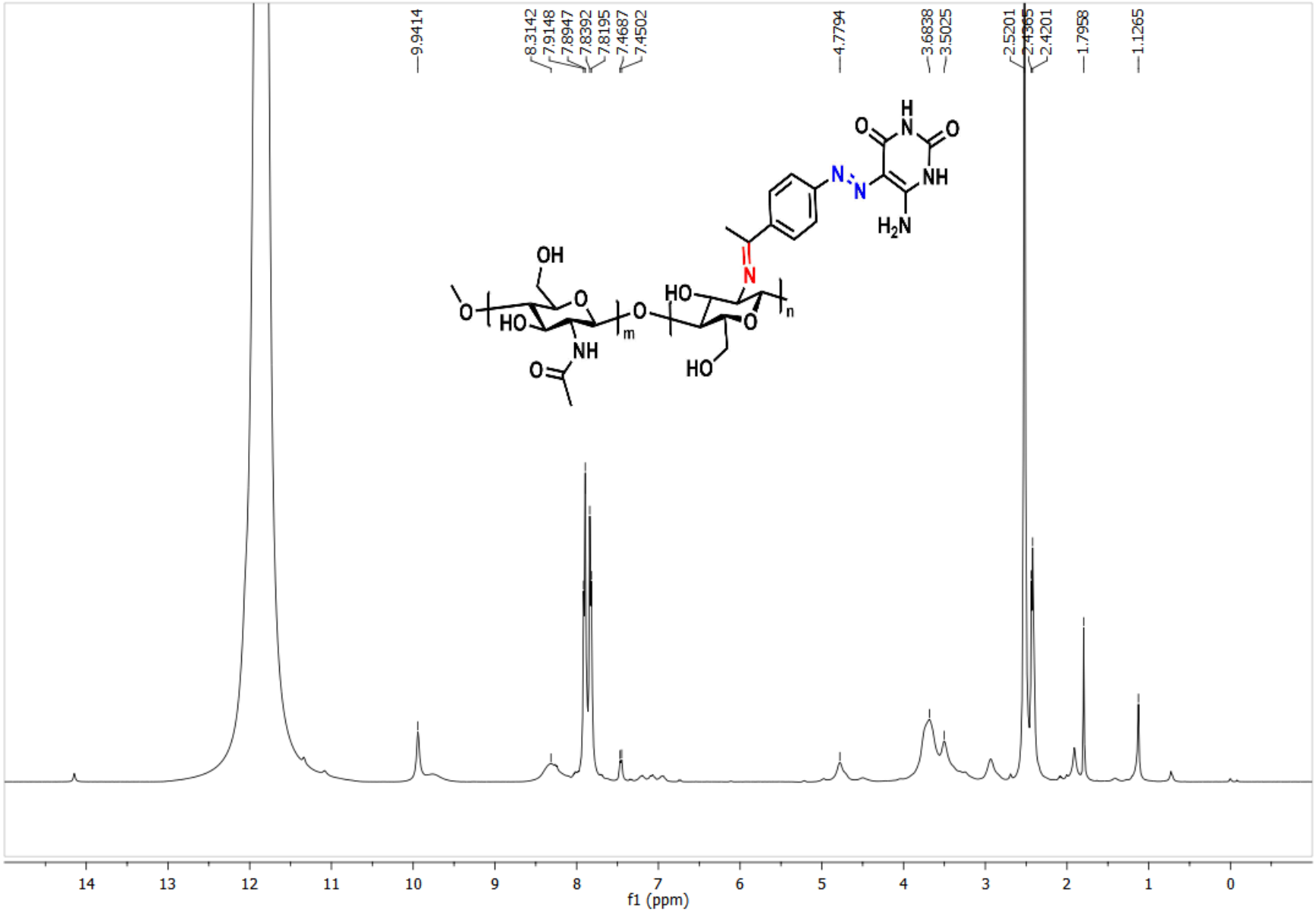
7.5. Figure S6e ^{13}C NMR Spectrum of CA40 in DMSO-d6 and CF_3COOD



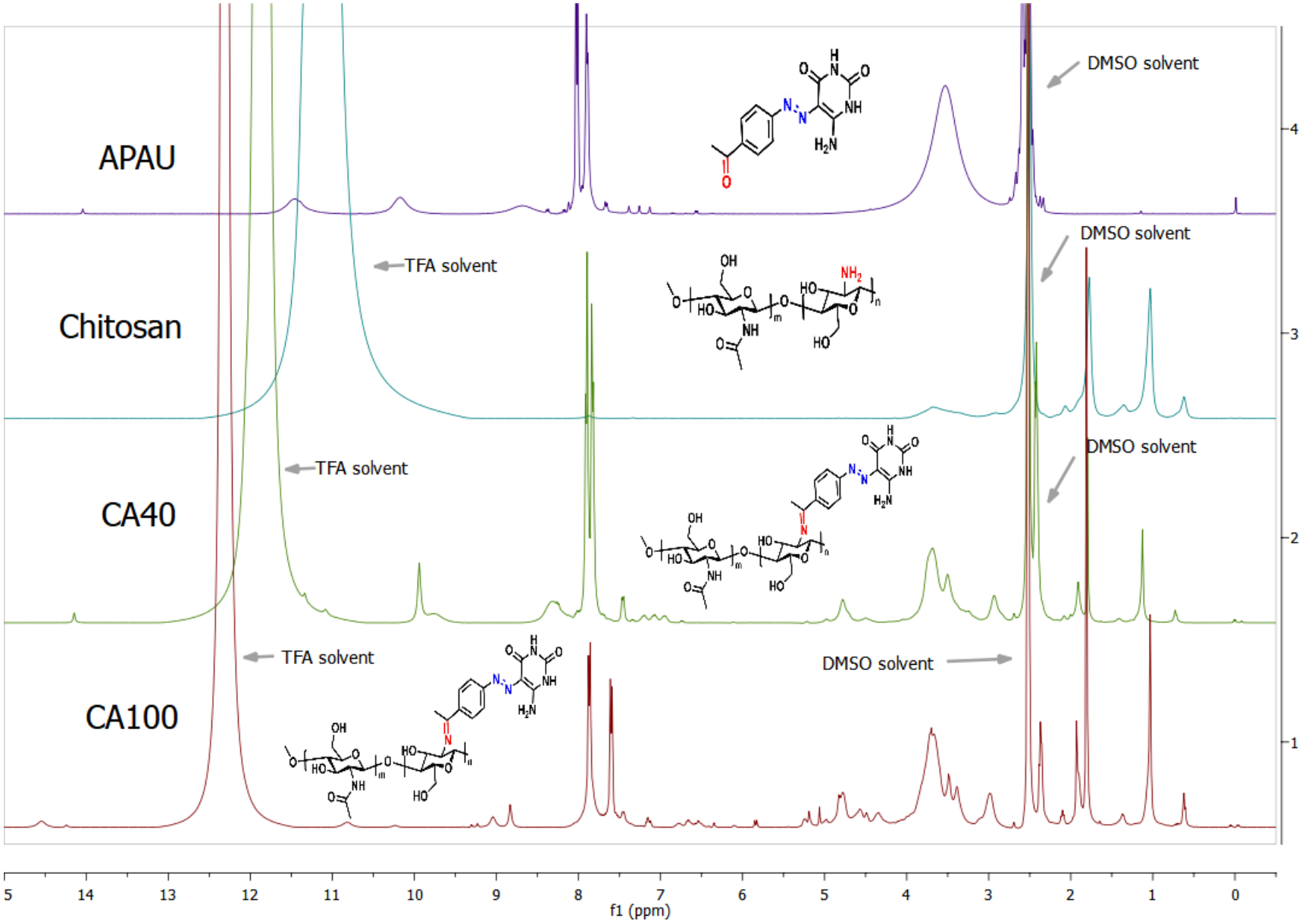
7.6. Figure S6f ^{1}H NMR Spectrum of CA100 in DMSO-d6 and CF_3COOD.



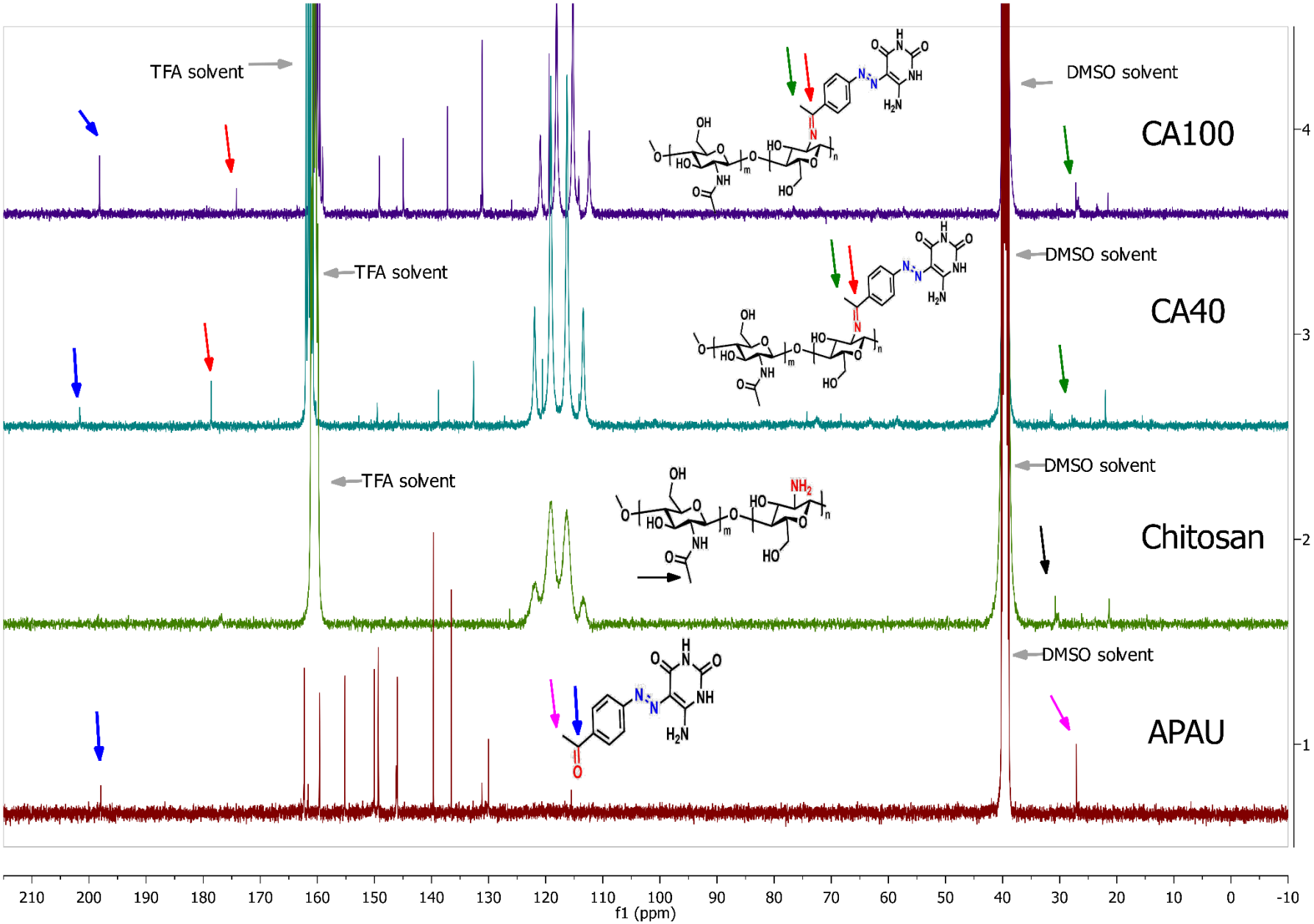
7.7. Figure S6g ^{13}C NMR Spectra of CA100 in DMSO-d6 and CF_3COOD



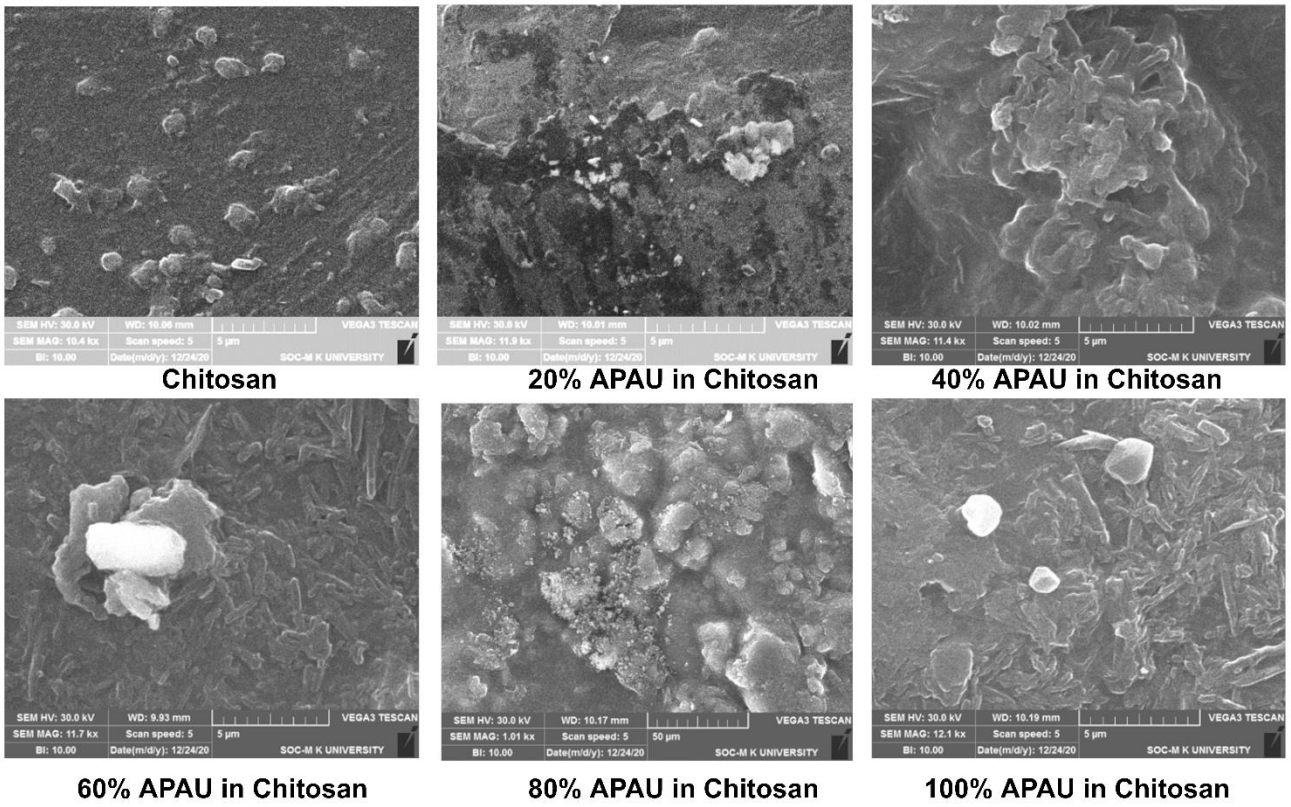
7.8. Figure S6h stacked ^{1}H NMR Spectra of Chitosan, CA40, CA100 in DMSO-d6 and CF_3COOD, and APAU in DMSO-d6.



7.9. Figure S6i stacked ^{13}C NMR Spectra of APAU, Chitosan, CA40 and CA100 in DMSO-d6 and CF_3COOD



8. Figure S7 SEM micrographs of biopolymers

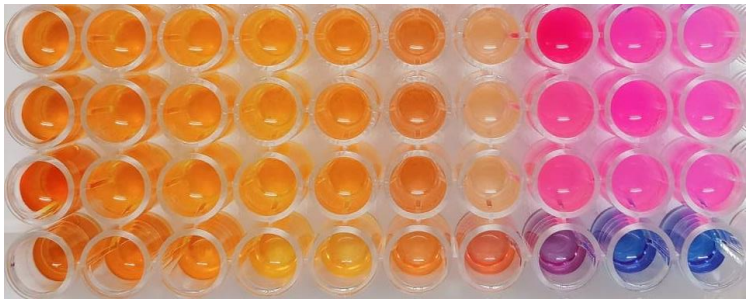


9. Table S1 Feed ratio for modified polymer synthesis

Code	Glucosamine in chitosan to be converted to imine pendant groups*		APAU
	(%)	(mmol)	(mmol)
CA20	20	0.538	0.538
CA40	40	1.076	1.076
CA60	60	1.615	1.615
CA80	80	2.153	2.153
CA100	100	2.691	2.691

*Chitosan feed mass was kept constant allowing for 2.691 mmol of glucosamine per reaction.

10. Figure S8 Representative image of well plate assays



	1	2	3	4	5	6	7	8	9	10
A										-ve control
B										-ve control
C										-ve control
D								+ve control	+ve control	+ve control

A1-A9, B1-B9 and C1-C9 contain successive dilutions of the compound to be tested.
Cells D1-D7 do not contain cell culture and are used to account for colored nature/absorbance of the compound in successive dilutions.

11. Antimicrobial activity study images

The working codes of samples in the images correspond to the sample codes in the table below to avoid any confusion from labelling in the following images.

Working code	**Corresponding Sample code**
VB6	APAU
VB5	CA100
VB4	CA80
VB3	CA60
VB2	CA40
VB1	CA20
VB0	Chitosan

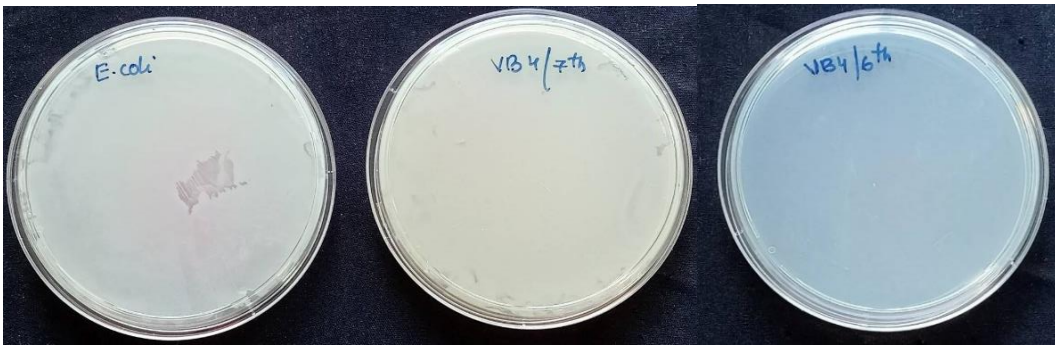
11.1. Antimicrobial imaging against *E. coli*

11.1.1 Antimicrobial study of *E. coli* against CA100 and subsequent subcultures of *E. coli* from MIC and MBC indicative wells.

In well plate, column 7 is MIC and Column 6 is MBC, subsequent subcultures are shown below alongside control cultures.

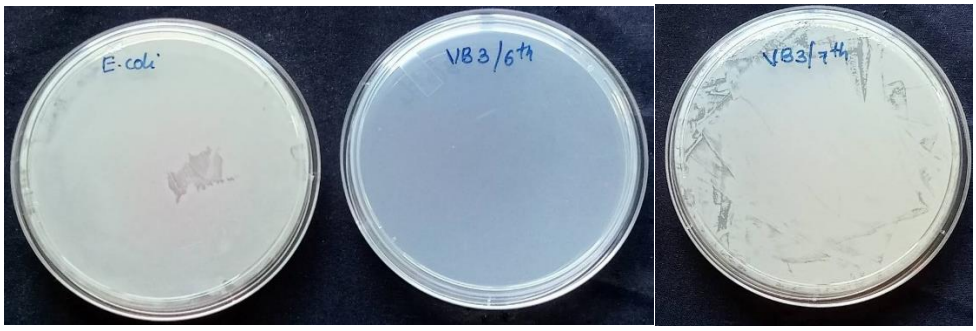
11.1.2. Antimicrobial study of *E. coli* against CA80 and its subsequent subcultures of *E. coli* from MIC and MBC indicative wells.

In well plate, Column 7 is MIC Column 6 is MBC, subsequent subcultures are shown below alongside control cultures.



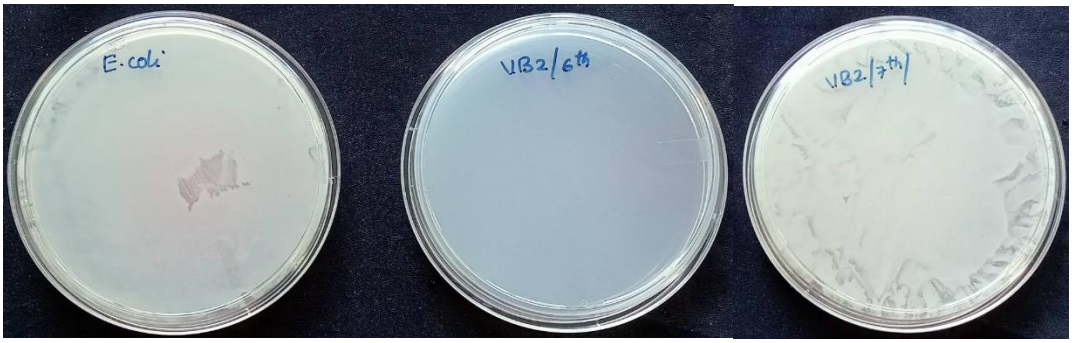
11.1.3. Antimicrobial study of *E. coli* against CA60 and its subsequent subcultures of *E. coli* from MIC and MBC indicative wells.

In well plate, Column 7 is MIC and column 6 is MBC, subsequent subcultures are shown below alongside control cultures.



11.1.4. Antimicrobial study of *E. coli* against CA40 and its subsequent subcultures of *E. coli* from MIC and MBC indicative wells.

In well plate, Column 7 is MIC and column 6 is MBC, subsequent subcultures are shown below alongside control cultures.

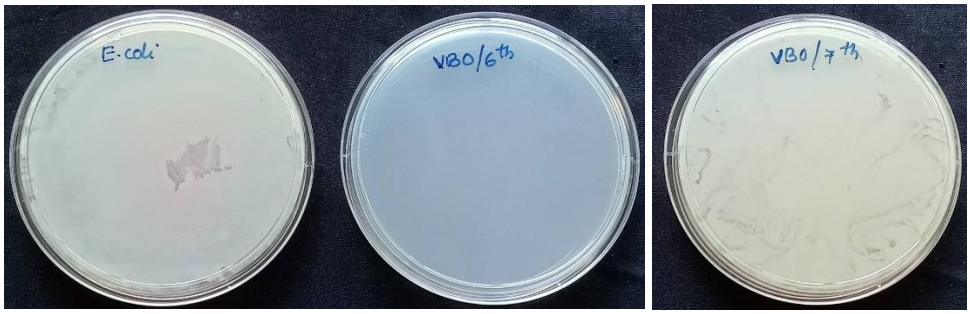


11.1.5. Antimicrobial study of *E. coli* against CA20 and its subsequent subcultures of *E. coli* from MIC and MBC indicative wells.

In well plate, Column 7 is MIC and column 6 is MBC, subsequent subcultures are shown below alongside control cultures.

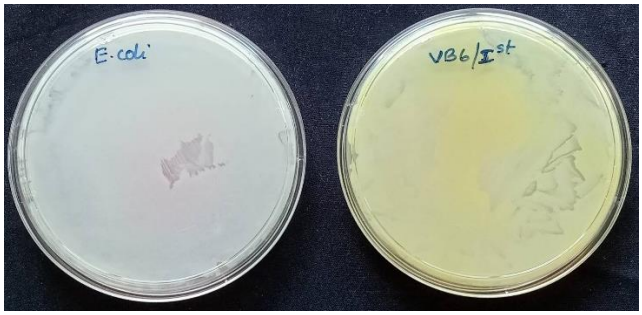
11.1.6. Antimicrobial study of *E. coli* against Chitosan and its subsequent subcultures of *E. coli* from MIC and MBC indicative wells.

In well plate, Column 7 is MIC and column 6 is MBC, subsequent subcultures are shown below alongside control cultures.



11.1.7. Antimicrobial study of *E. coli* against APAU and its subsequent subcultures of *E. coli* from MIC and MBC indicative wells

No inhibition was seen, subsequent subculture is shown below alongside control.



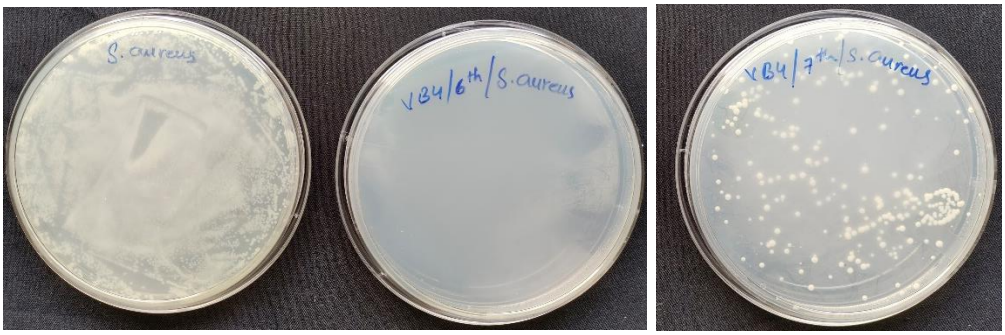
11.2. Antimicrobial imaging against *S. aureus*

11.2.1. Antimicrobial study of *S. aureus* against CA100 and its subsequent subcultures of *S. aureus* from MIC and MBC indicative wells

In well plate, Column 7 is MIC and column 6 is MBC, subsequent subcultures are shown below alongside control cultures.

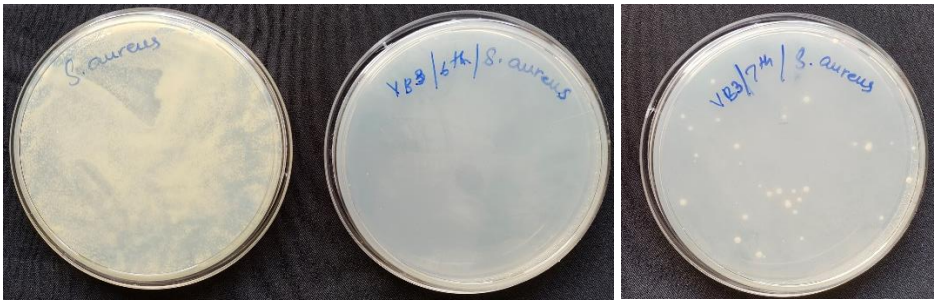
11.2.2. Antimicrobial study of *S. aureus* against CA80 and its subsequent subcultures of *S. aureus* from MIC and MBC indicative wells

In well plate, Column 7 is MIC and column 6 is MBC, subsequent subcultures are shown below alongside control cultures.



11.2.3. Antimicrobial study of *S. aureus* against CA60 and its subsequent subcultures of *S. aureus* from MIC and MBC indicative wells

In well plate, Column 7 is MIC and column 6 is MBC, subsequent subcultures are shown below alongside control cultures.



11.2.4. Antimicrobial study of *S. aureus* against CA40 and its subsequent subcultures of *S. aureus* from MIC and MBC indicative wells.

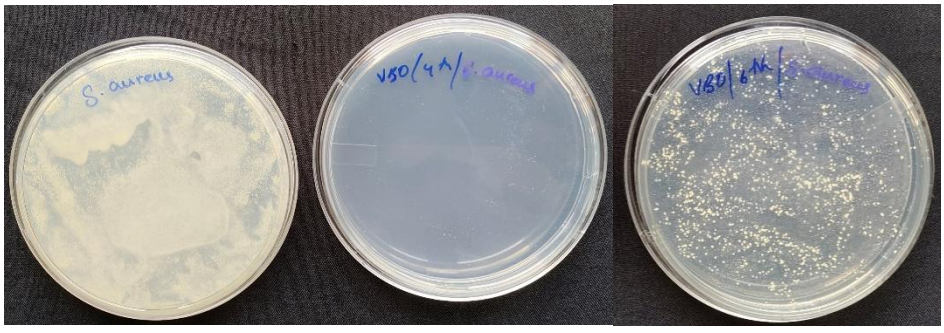
In well plate, Column 7 is MIC and column 6 is MBC, subsequent subcultures are shown below alongside control cultures.

11.2.5. Antimicrobial study of *S. aureus* against CA20 and its subsequent subcultures of *S. aureus* from MIC and MBC indicative wells.

In well plate, Column 7 is MIC and column 5 is MBC, subsequent subcultures are shown below alongside control cultures.

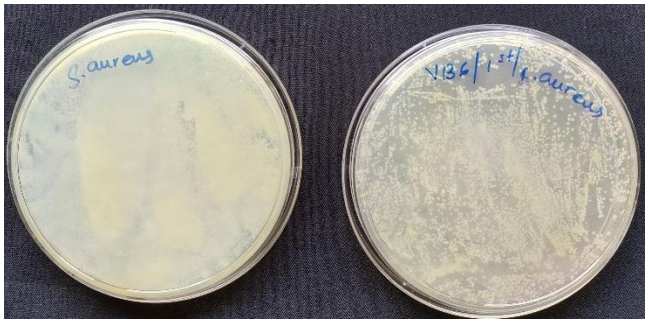
11.2.6. Antimicrobial study of *S. aureus* against Chitosan and its subsequent subcultures of *S. aureus* from MIC and MBC indicative wells

In well plate, Column 6 is MIC and column 4 is MBC, subsequent subcultures are shown below alongside control cultures.



11.2.7. Antimicrobial study of *S. aureus* against APAU and its subsequent subcultures of *S. aureus* from MIC and MBC indicative wells.

No inhibition was seen even at highest concentration in column, subsequent subcultures are shown below alongside control cultures.



12. Raw Antimicrobial activity study data against *K. pneumonia* with standard deviation and error

Sample			MEAN	SD	STD ERROR
Chitosan	0.151	0.104	0.1275	0.0235	0.135
CA20	0.114	0.084	0.099	0.015	0.086
CA40	0.431	0.375	0.403	0.028	0.1616
CA60	0.265	0.249	0.257	0.008	0.0461
CA80	0.262	0.237	0.2495	0.0125	0.0721
CA100	0.462	0.467	0.4645	0.0025	0.014
APAU	0.0151	0.034	0.024	0.00945	0.0545
CTRL	0.015	0.074	0.044	0.0295	0.17

13. Raw Antimicrobial activity study data against *E.coli* with standard deviation and error

Sample			Mean	SD	STD EROR
Chitosan	0.02	0.032	0.026	0.006	0.034
CA20	0.107	0.129	0.118	0.011	0.063
CA40	0.208	0.149	0.1785	0.0295	0.1703
CA60	0.186	0.179	0.1825	0.0035	0.0202
CA80	0.493	0.489	0.491	0.002	0.0115
CA100	0.462	0.432	0.447	0.015	0.0866
APAU	0.105	0.081	1085	0.012	0.016
CTRL	0.111	0.137	0.124	0.013	0.075

14. Cell cytotoxicity analysis against Mo7e and BA/F3 cells

14.1. Cell culture

The human megakaryocyte cell line Mo7e (ACC 104) and Murine pro B cell line BA/F3 (ACC 300) were obtained from German Collection of Microorganisms and Animal Cell Cultures, DSMZ, Germany. Mo7e and BA/F3 cells were maintained in RPMI 1640 medium (Gibco, Waltham, MA USA) with 10 % fetal calf serum (HiMedia, India) in the presence of 20 ng/ml human IL-3 and 10 ng/ml murine IL-3 (Peprotech Asia, Rehovot, Israel) respectively.

14.2. Cytotoxicity Assay

Cell cytotoxicity assay was performed using WST-1 (Roche, Basel, Switzerland) according to manufacturer's protocol. Mo7e and BA/F3 cells were plated in 96-well tissue culture plate at a concentration of 20,000 cells per well in 100 µL of media and stimulated with different concentrations of compounds APAU, CA-80 and CA-100 for 24 hours. Cells were also treated only with phosphoric acid (PA) to exclude solvent induced cytotoxicity. After incubation, 10 µL of WST-1 reagent was added and absorbance was measured against a background control as blank using microplate (ELISA) reader at 440 nm. Statistical analysis was performed using Graphpad Prism (GraphPad Software, Inc., CA, US). *P* value of <0.05 are considered significant.