Supporting Information

Multimerization of DAB-1 onto Au GNPs affords new potent and selective *N*-acetylgalactosamine-6-sulfatase (GALNS) inhibitors

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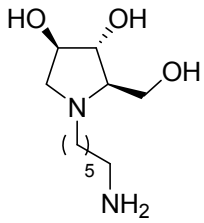
Table of contents



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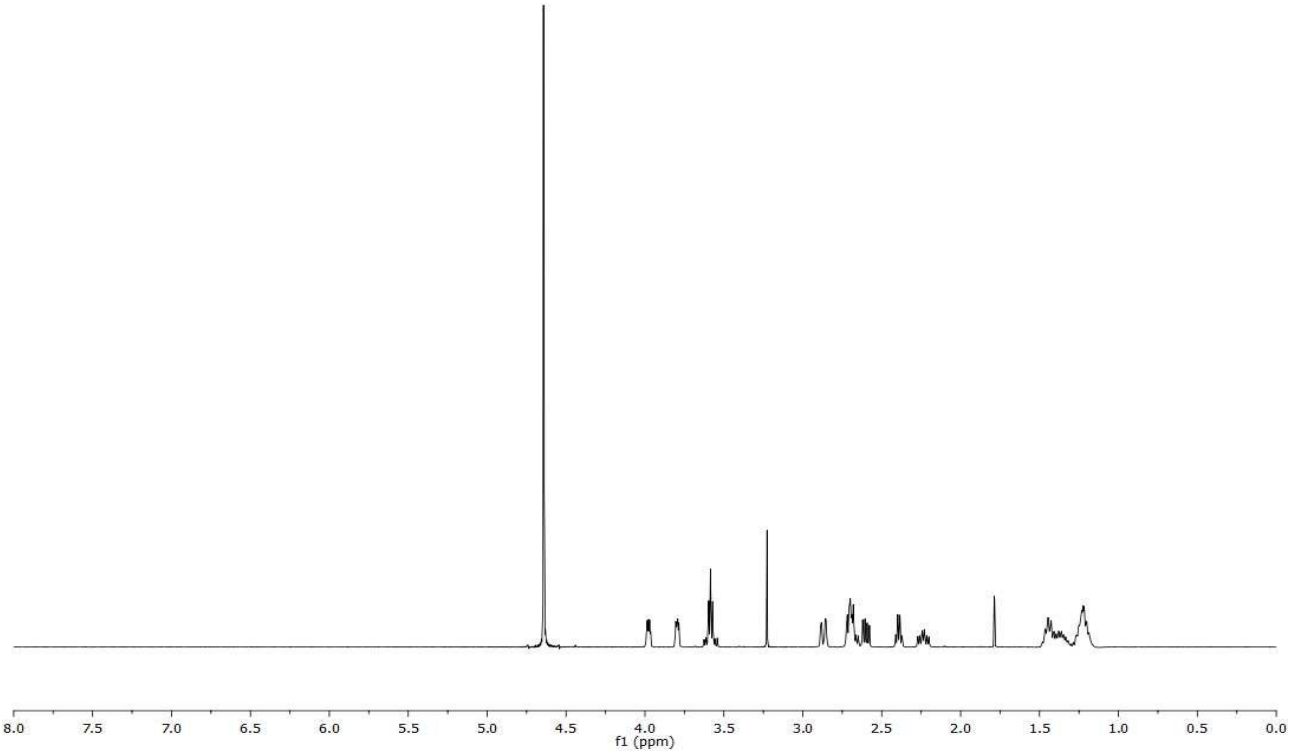
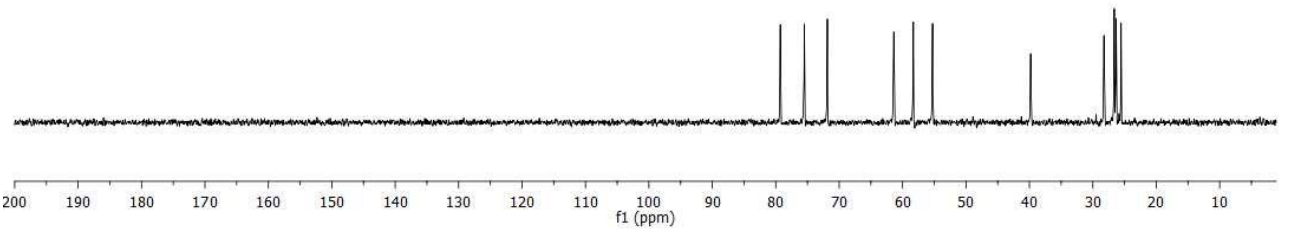


^{1}H NMR spectrum of compound 3 (400 MHz, D_2O)



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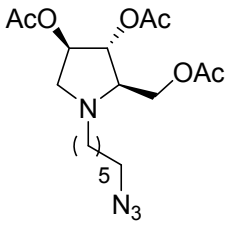

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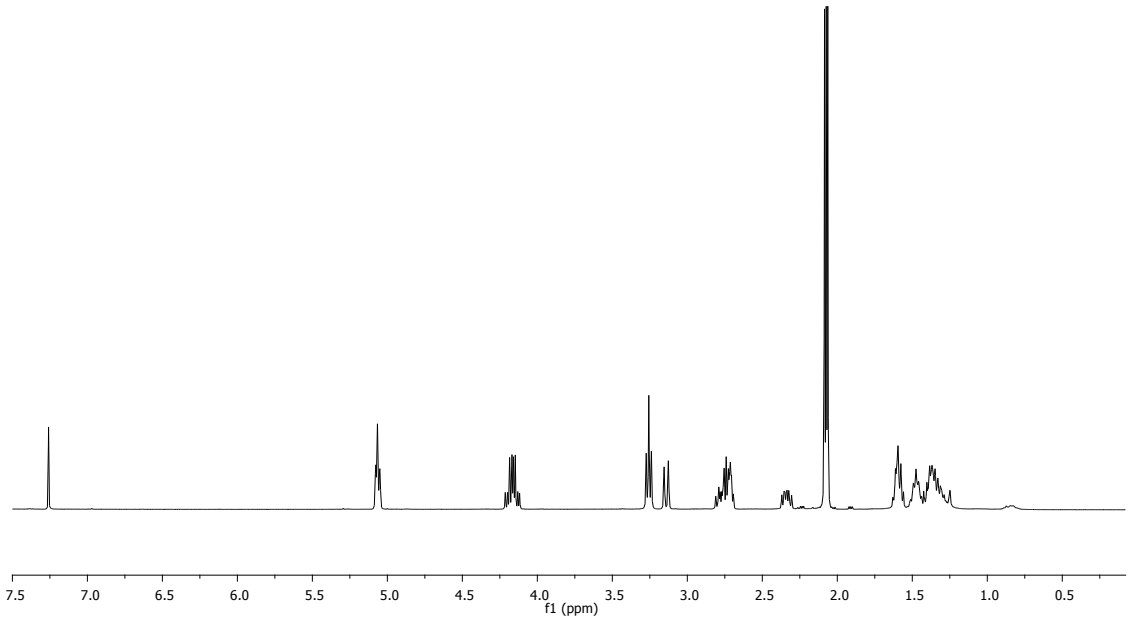
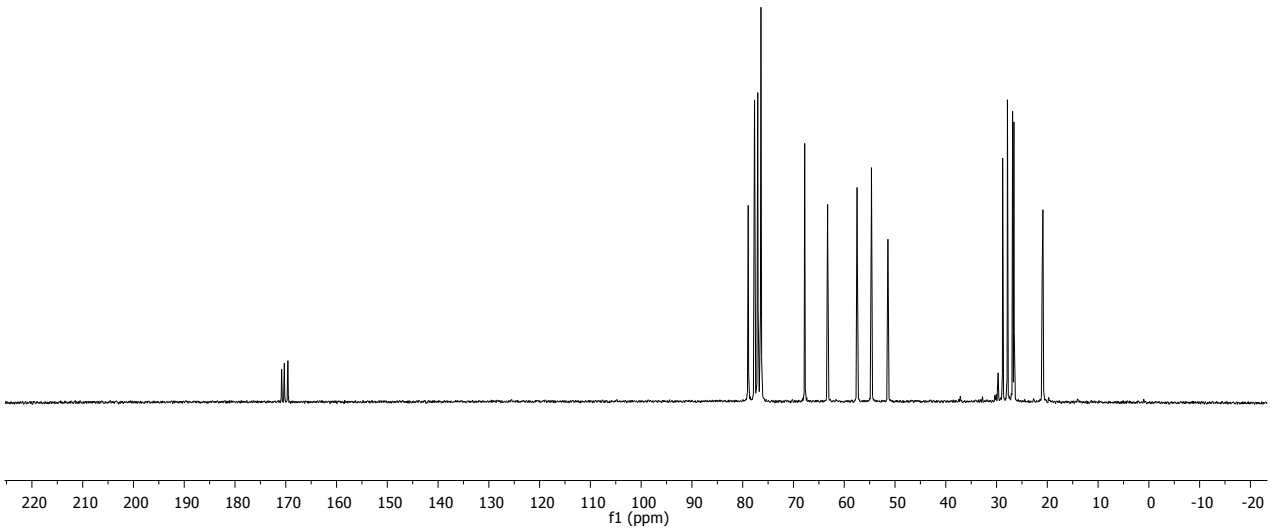
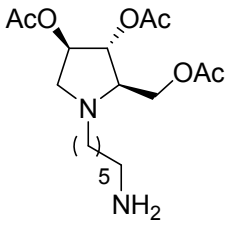


^{1}H NMR spectrum of compound 7 (400 MHz, $CDCl_3$)



^{13}C NMR spectrum of compound 7 (100 MHz, $CDCl_3$)



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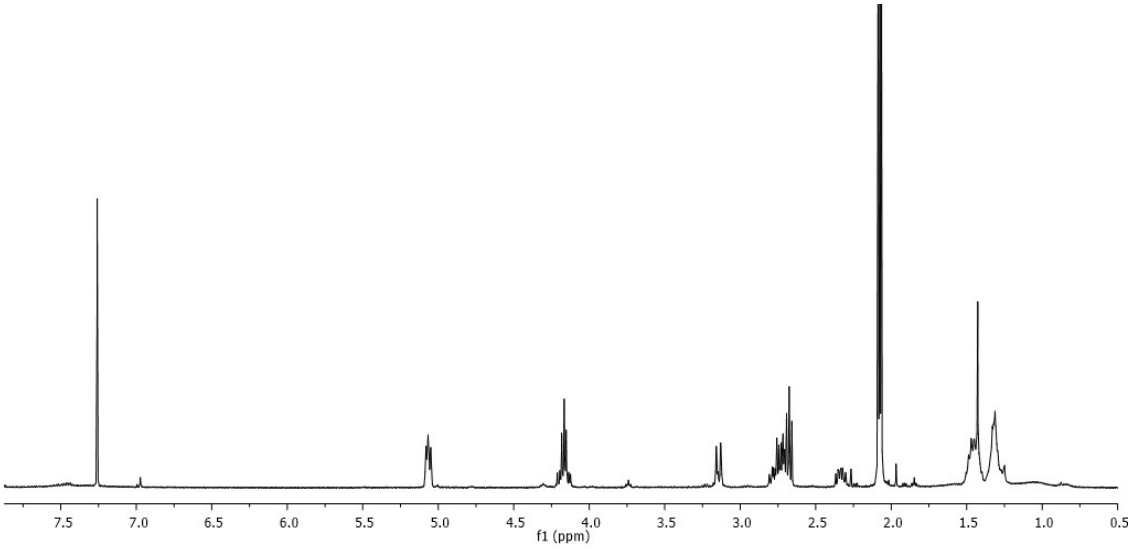

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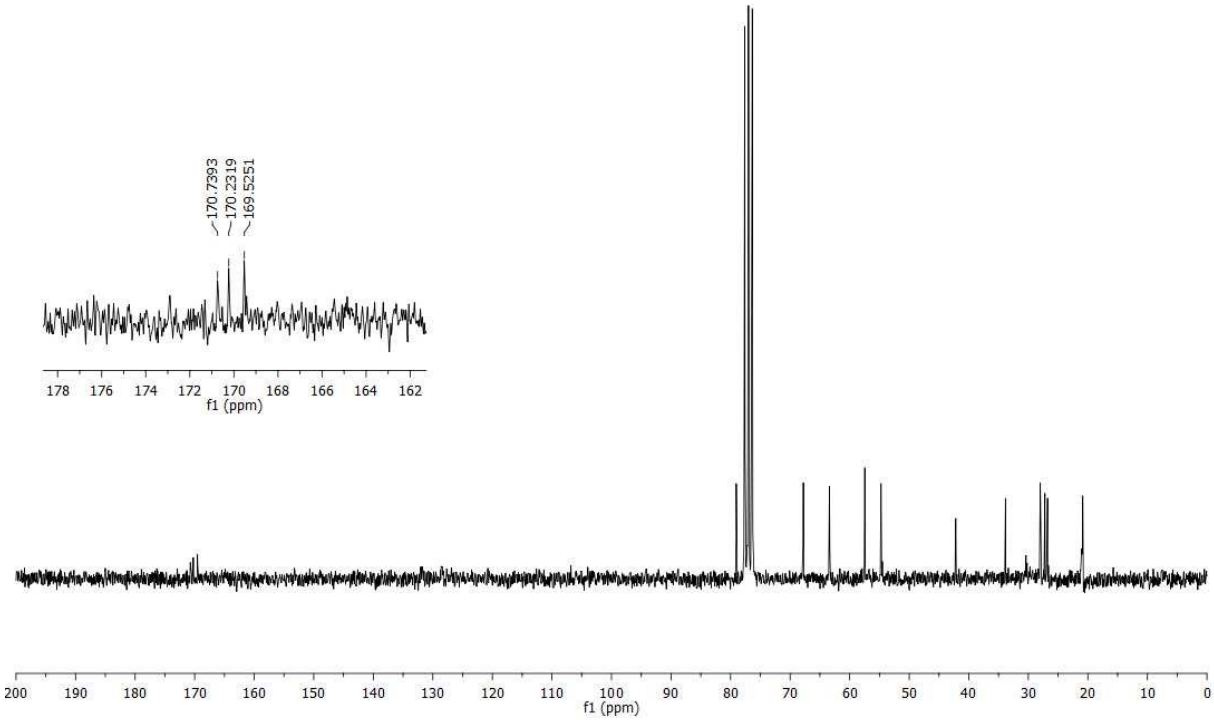

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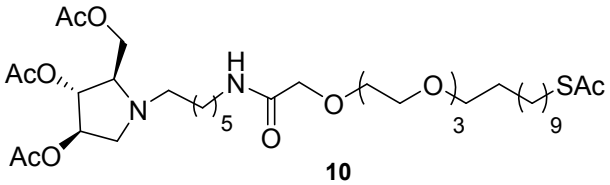

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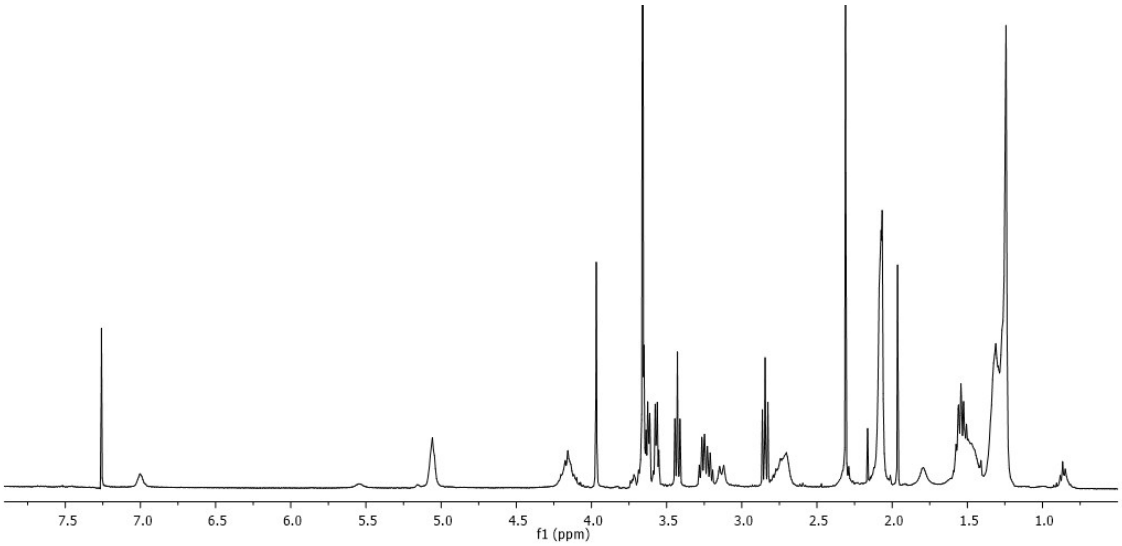

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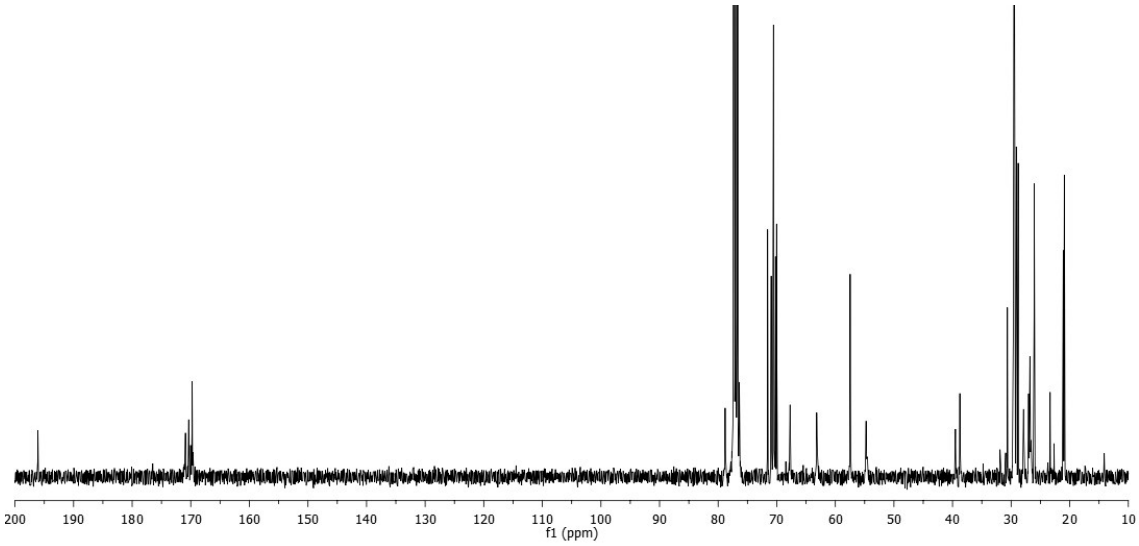
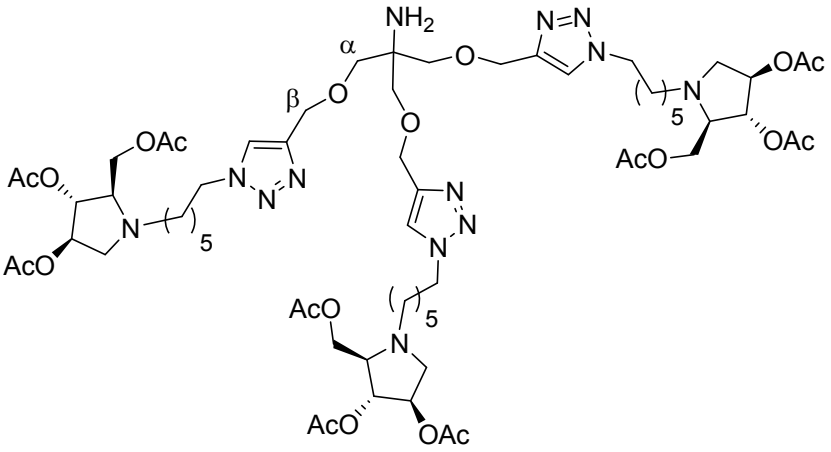


^{13}C NMR spectrum of compound 10 (100 MHz, $CDCl_3$)



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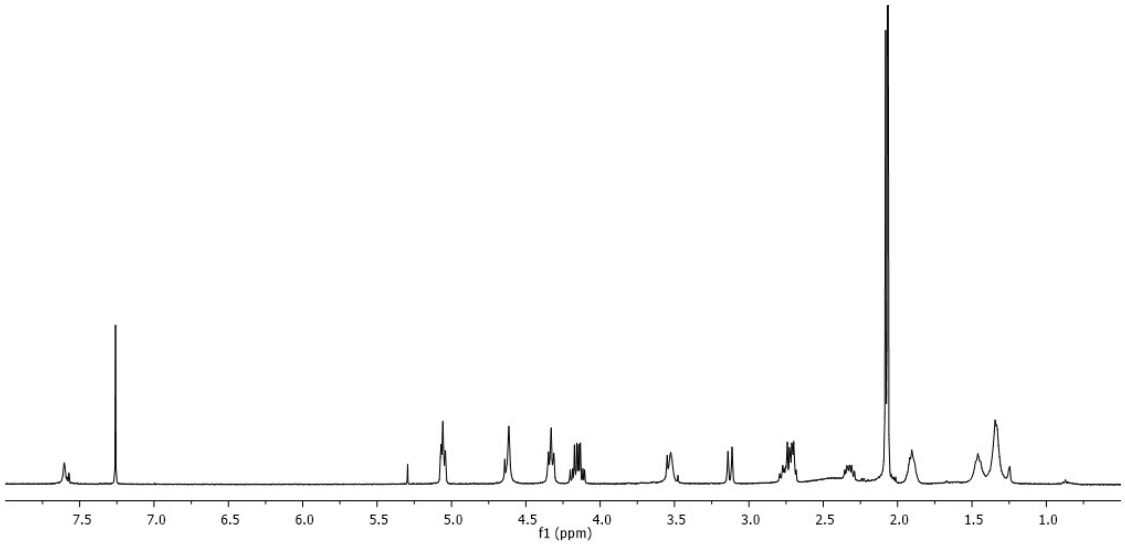

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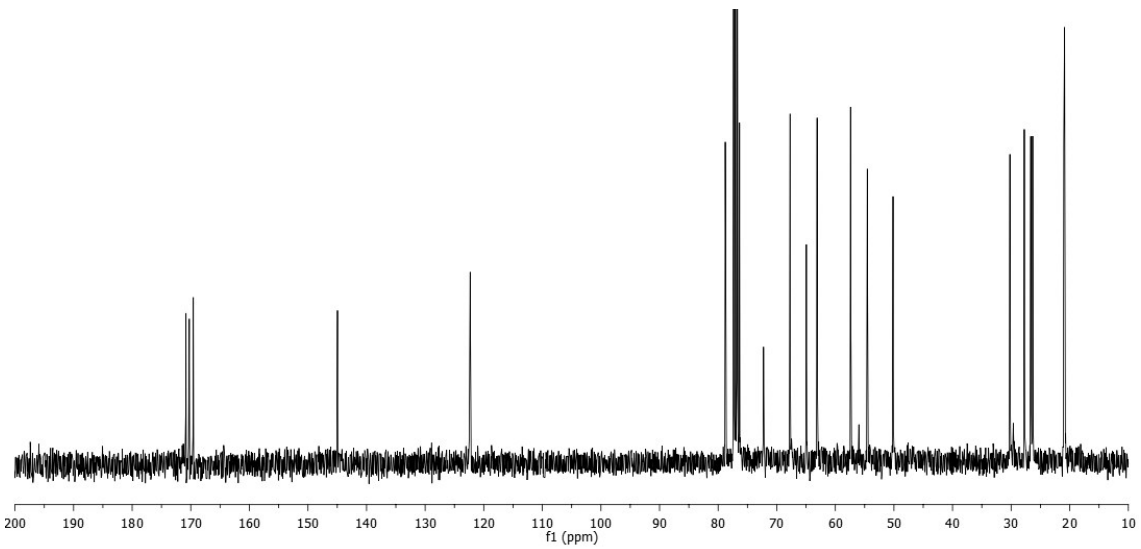
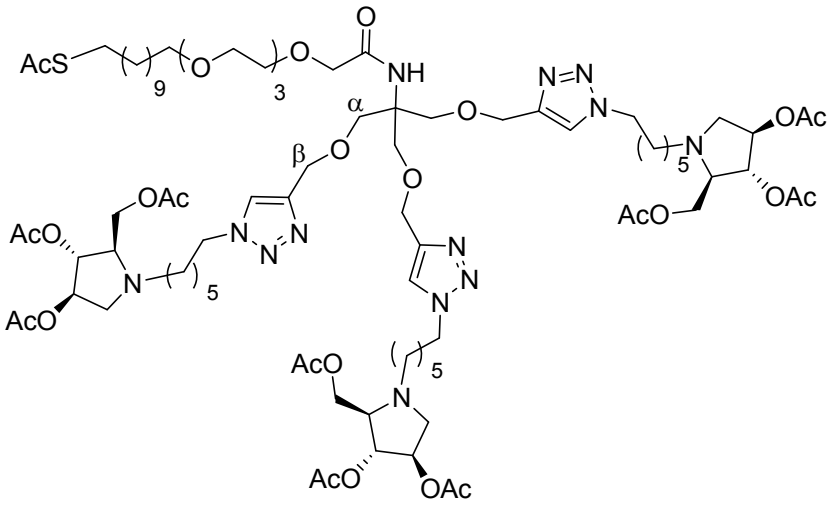


^{13}C NMR spectrum of compound 14 (100 MHz, $CDCl_3$)



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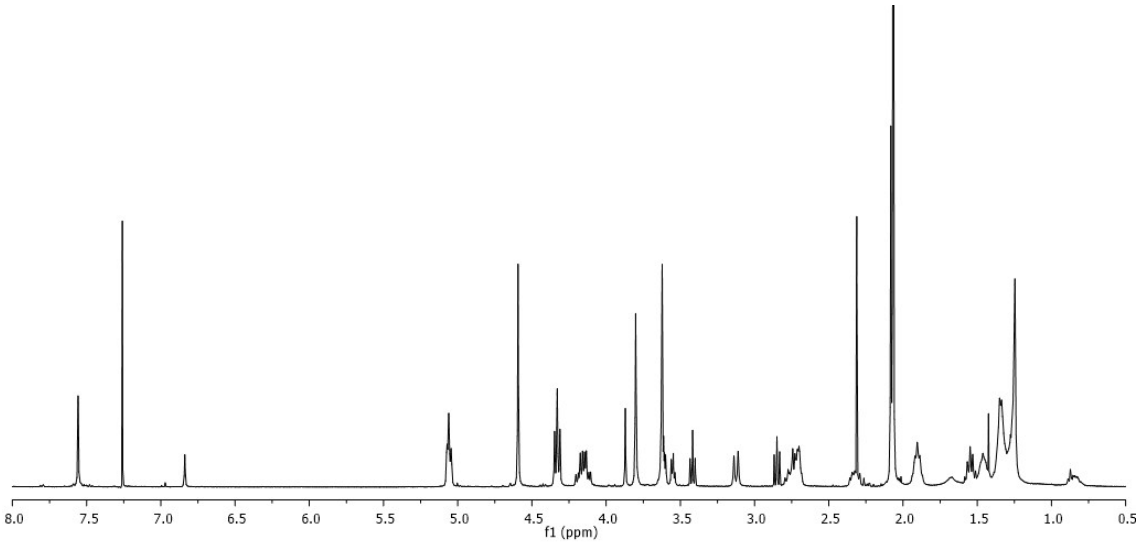
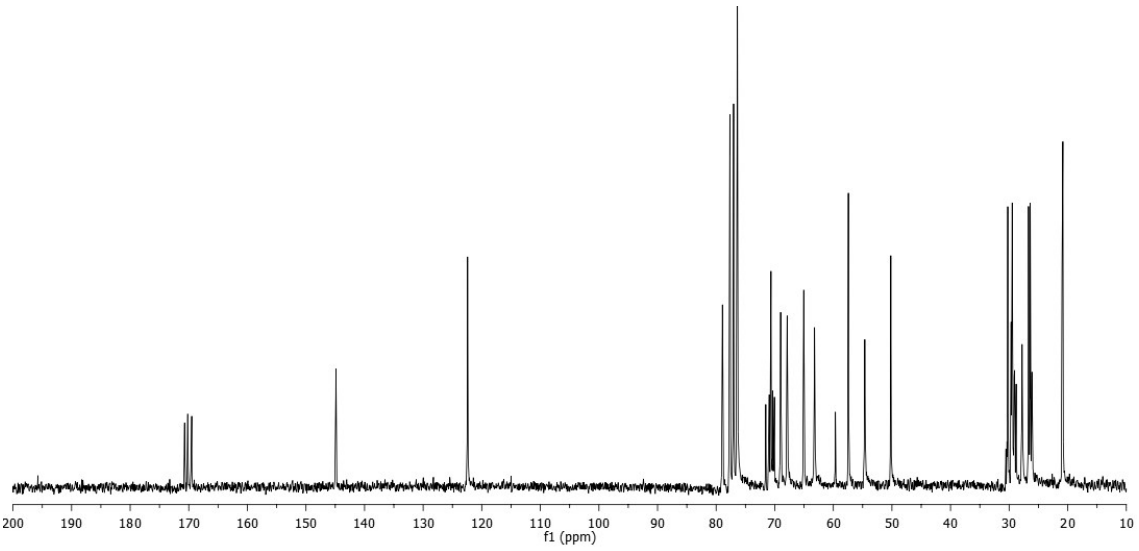


^{1}H NMR spectrum of compound 15 (400 MHz, $CDCl_3$)



^{13}C NMR spectrum of compound 15 (100 MHz, $CDCl_3$)

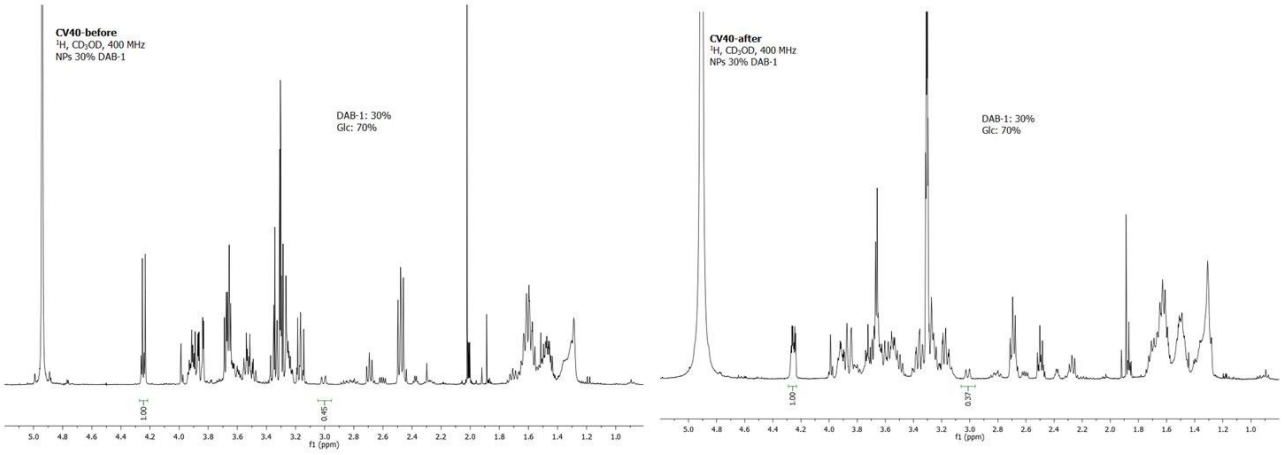
General Procedure for the "in situ" deprotection of *S*-acetyl conjugates 10 and 15: To a solution of **10** or **15** in CD_3OD (20 mg/ml and 10 mg/ml, respectively), 30 equivalents of NaOMe were added and the reaction mixture was left stirring for 2 hours at 25°C under nitrogen atmosphere. The complete disappearance of starting material was attested via ^1H NMR and the crude was directly used for the preparation of Au GNPs.

Preparation and characterization of Au GNPs: The Au GNPs coated with DAB-1 derivatives (mono and trivalent) and simple monosaccharide βGlcC_5S (DAB1-βGlc-Au NPs **12** and tri-DAB1-βGlc-Au NPs **16**) were prepared by reduction of an Au(III) salt using sodium borohydride in the presence of a mixture of thiol-ending iminosugar conjugate and βGlcC_5S **11** as ligands, in different ratios following a reported procedure.[1] For the analysis of the ratio between the iminosugar ligands and βGlcC_5S, ^1H NMR spectra of the initial mixture and of the supernatant after Au GNPs formation were recorded. The ligands loading on the Au GNPs was also evaluated by quantitative NMR (qNMR) using 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid (TSP-*d*$_4$) as an internal standard in the D_2O solution of the Au GNPs. The prepared Au GNPs were freeze-dried and stored at 4 °C. In these conditions, the Au- GNPs can be stored for months maintaining their biophysical properties. The Au GNPs **17**, coated only with the simple monosaccharide βGlcC_5S, were also prepared as previously described.[2]

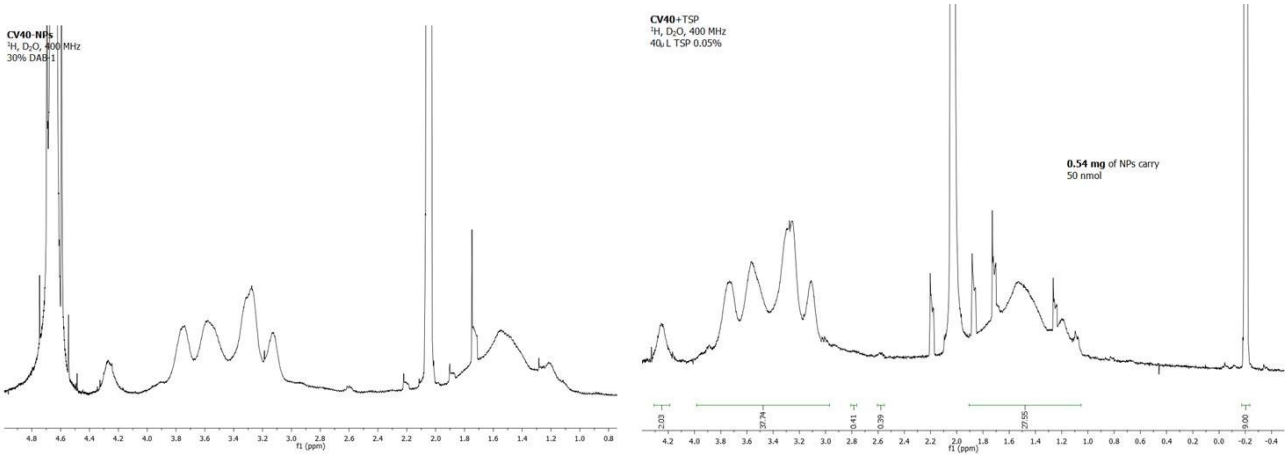
General Procedure for the preparation of Au GNPs coated with iminosugars: $HAuCl_4$ (25 mM, 1 equiv.) was added to a 12 mM methanolic solution of a suitable mixture of thiol-ending sugar and iminosugar (3 equiv. overall). An aqueous solution of $NaBH_4$ (1 M, 27 equiv.) was then added in four portions, with vigorous shaking. The black suspension formed was shaken for 2 hours at 25 °C. After that, the supernatant was removed and analysed by ^1H NMR to study the nanoparticle ligands composition. The residue was washed several times with MeOH. In order to well separate the nanoparticles from the supernatant a centrifugation (12000 rpm, 2 min) was performed. The residue was dissolved in a minimal volume of HPLC Gradient grade water and purified by dialysis (SnakeSkin® Pleated Dialysis Tubing, 10,000 MWCO and Slide-A-Lyzer® 10K Dialysis Cassettes, 10,000 MWCO). Iminosugar-coated Au GNPs were obtained as a dark-brown powder after freeze-drying and characterized via ^1H NMR, UV-Vis Spectroscopy and TEM analysis.

[1] C. Matassini, M. Marradi, F. Cardona, C. Parmeggiani, I. Robina, A. J. Moreno-Vargas, S. Penadés and A. Goti., *RSC Adv.*, **2015**, *5*, 95817.
[2] O. Martínez-Ávila, K. Hijazi, M. Marradi, C. Clevel, C. Campion, C. Kelly and S. Penadés, *Chem. Eur. J.*, **2009**, *15*, 9874.

Preparation of 30% DAB-1-βGlc NPs (12a): A 1: 2.3 mixture of thiol-ending **10** (7.83 mg, 12.9 µmol) and βGlcC$_5$S **11** (8.3 mg, 29.6 µmol) in CD_3OD (1.4 mL) was used to obtain 4.7 mg of AuGNPs **12a**. TEM (average diameter): 1.7 ±0.4 nm. Quantitative ^{1}H NMR (400 MHz, D_2O containing 0.05 wt. % of 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid, sodium salt as an internal standard): 0.54 mg of **12a** were dissolved in 120 µL of D_2O and 40 µL of D_2O containing 0.05 wt.% TSP were added and 50 nmoles of DAB-1 conjugate were found.[3] Significant peaks: δ = 4.24 (br s, from βGlcC5S), 3.95-2.95 (m), 2.82-2.70 (m, 1H, from DAB-1 conjugate) 2.62-2.53 (m, 1H, from DAB-1 conjugate), 1.90-1.00 ppm (m).

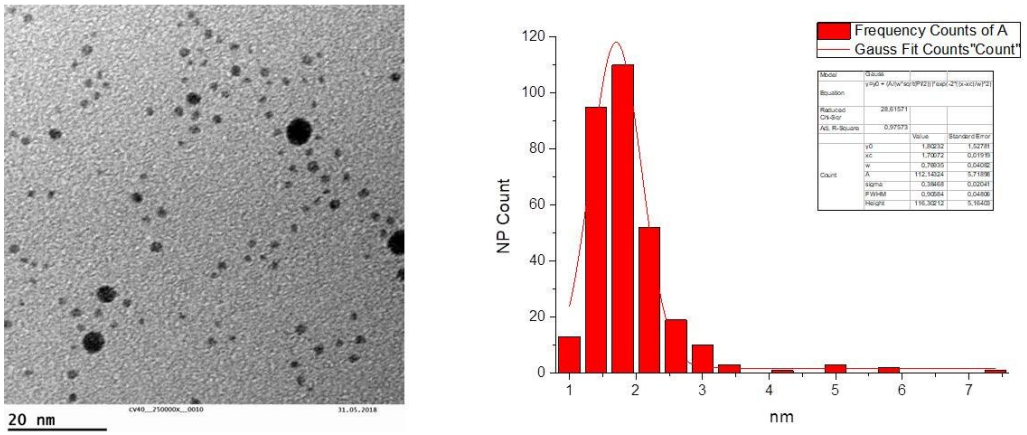


^{1}H NMR of sugar/iminosugar ligands mixture before (left) and after (right) formation of Au GNPs **12a** (400 MHz, CD_3OD).

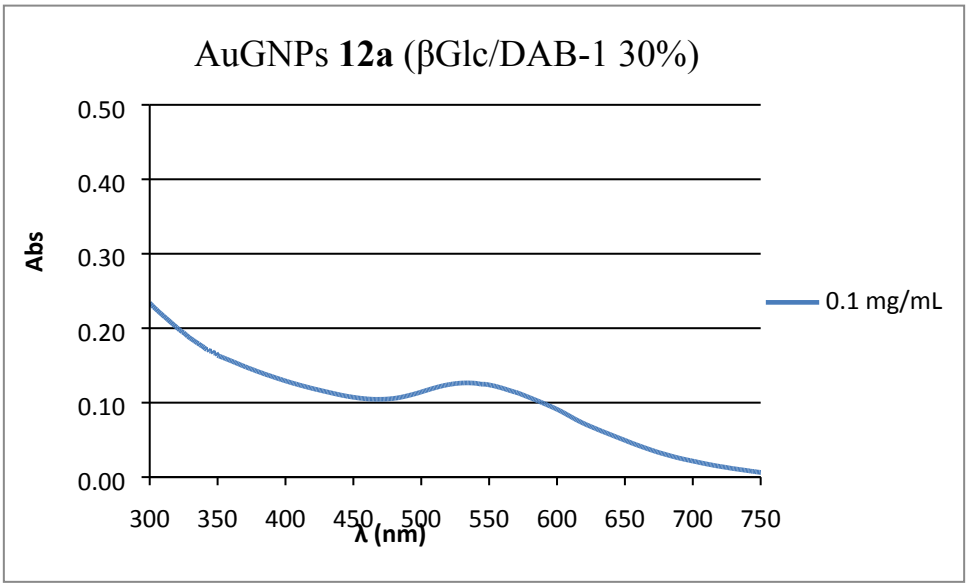


^{1}H NMR and ^{1}H qNMR with TSP-d$_4$ of Au GNPs **12a** (400 MHz, D_2O).

[3] In the quantitative NMR (qNMR) the multiplet corresponding to Hb-5 proton signal (δ = 2.62-2.53 ppm, 1H) of DAB-1 conjugate, was selected for integration as it falls in a spectral region free of other signals.

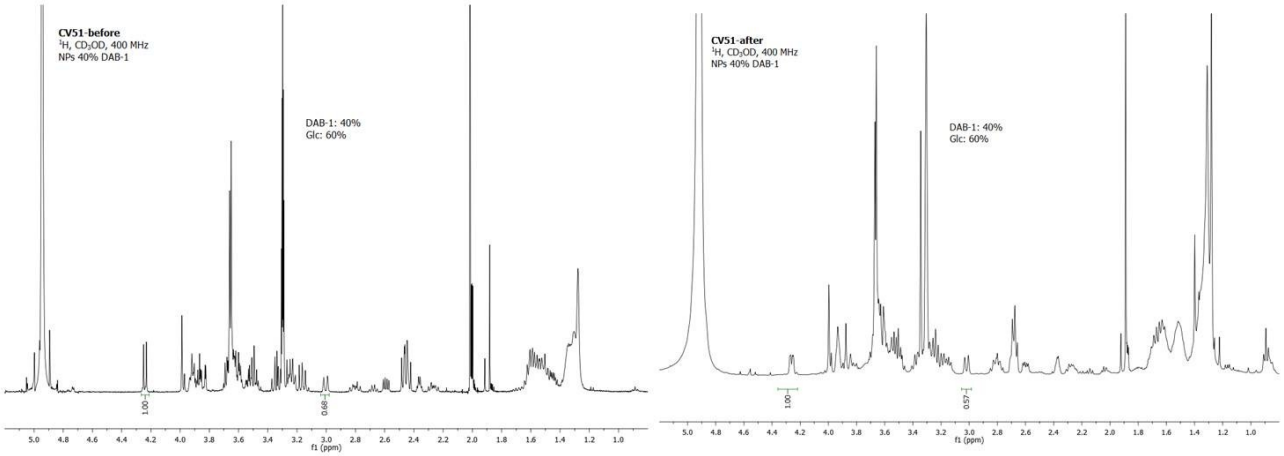


TEM micrograph in H_2O and size-distribution histogram obtained by measuring 300 nanoparticles (average diameter 1.7 ± 0.4 nm; less than 5% of nanoparticles show a gold diameter bigger than5 nm).

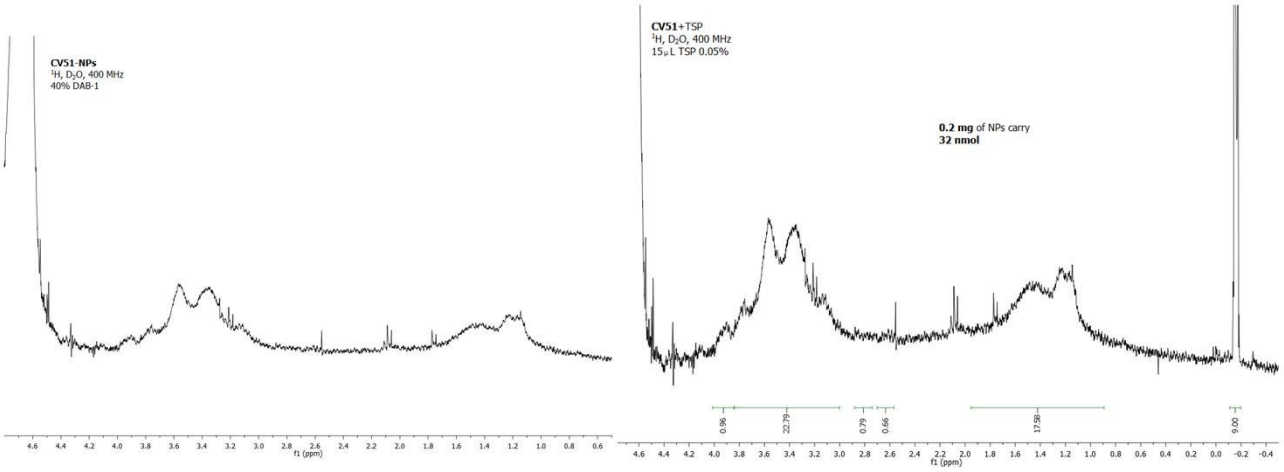


UV/vis spectrum of H_2O solution of Au GNPs **12a** recorded at concentration of 0.1 mg/mL.

Preparation of 40% DAB-1-βGlc NPs (12b): A 1: 1.5 mixture of thiol-ending **10** (7.80 mg, 12.9 µmol) and βGlcC$_5$S **11** (5.4 mg, 19.3 µmol) in CD_3OD (2.7 mL) was used to obtain 0.4 mg of Au GNPs **12b**. TEM (average diameter): 1.9 ±0.3 nm. Quantitative ^{1}H NMR (400 MHz, D_2O containing 0.05 wt. % of 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid, sodium salt as an internal standard): 0.20 mg of **12b** were dissolved in 150 µL of D_2O and 15 µL of D_2O containing 0.05 wt.% TSP were added and 32 nmoles of DAB-1 conjugate were found.[4] Significant peaks: δ = 3.98 (br s, $NHCOCH_2$- from DAB-1 conjugate), 3.81-2.98 (m), 2.87-2.75 (m, 1H, from DAB-1 conjugate), 2.67-2.57 (m, 1H, from DAB-1 conjugate), 1.90-1.00 ppm (m).

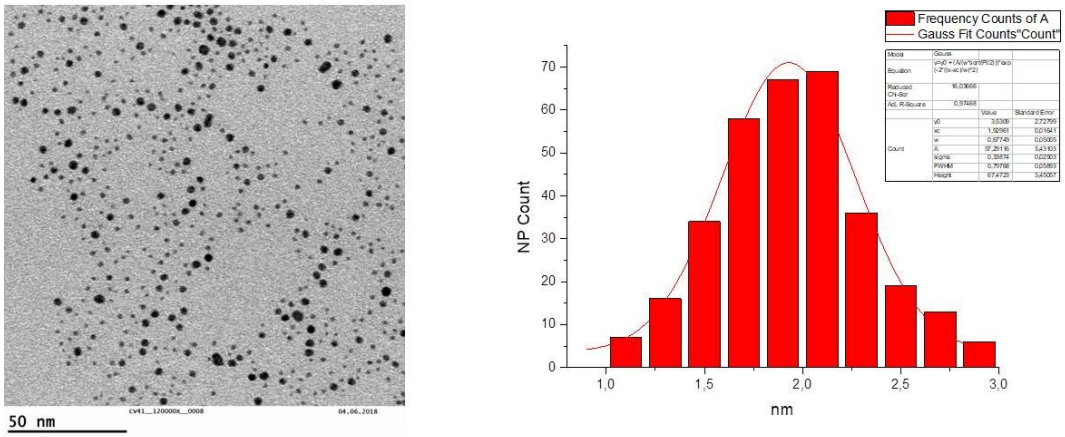


^{1}H NMR of sugar/iminosugar ligands mixture before (left) and after (right) formation of Au GNPs **12b** (400 MHz, CD_3OD).

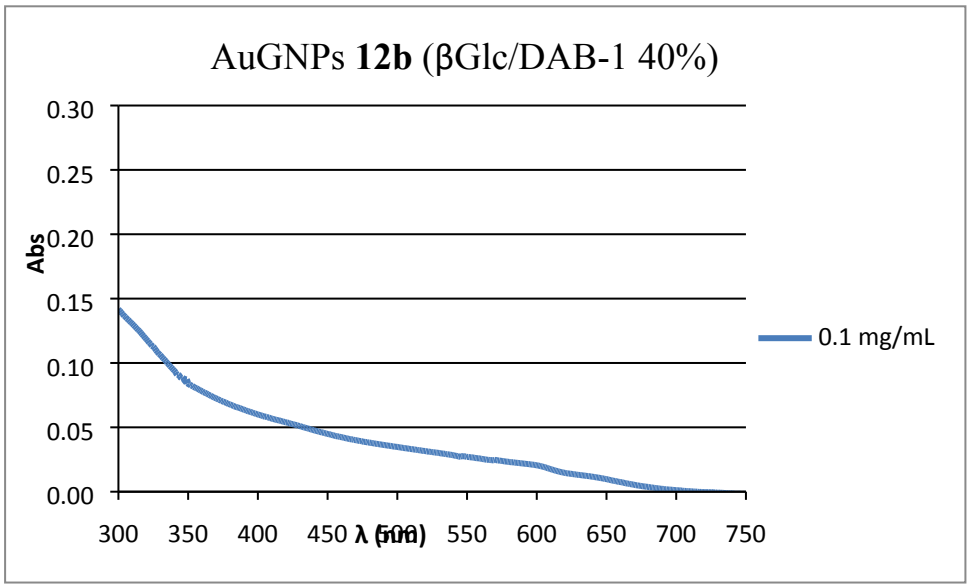


^{1}H NMR and ^{1}H qNMR with TSP-d_4 of Au GNPs **12b** (400 MHz, D_2O).

[4] In the quantitative NMR (qNMR) the multiplet corresponding to Hb-5 proton signal (δ = 2.67-2.57 ppm, 1H) of DAB-1 conjugate, was selected for integration as it falls in a spectral region free of other signals.

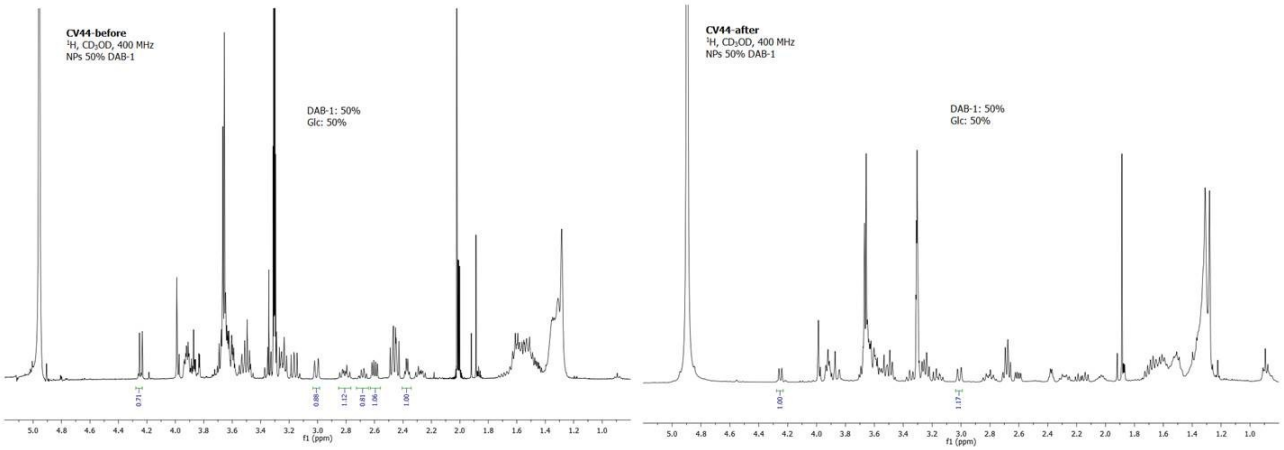


TEM micrograph in H_2O and size-distribution histogram obtained by measuring 300 nanoparticles (average diameter double distribution: 1.9 ±0.3 nm).

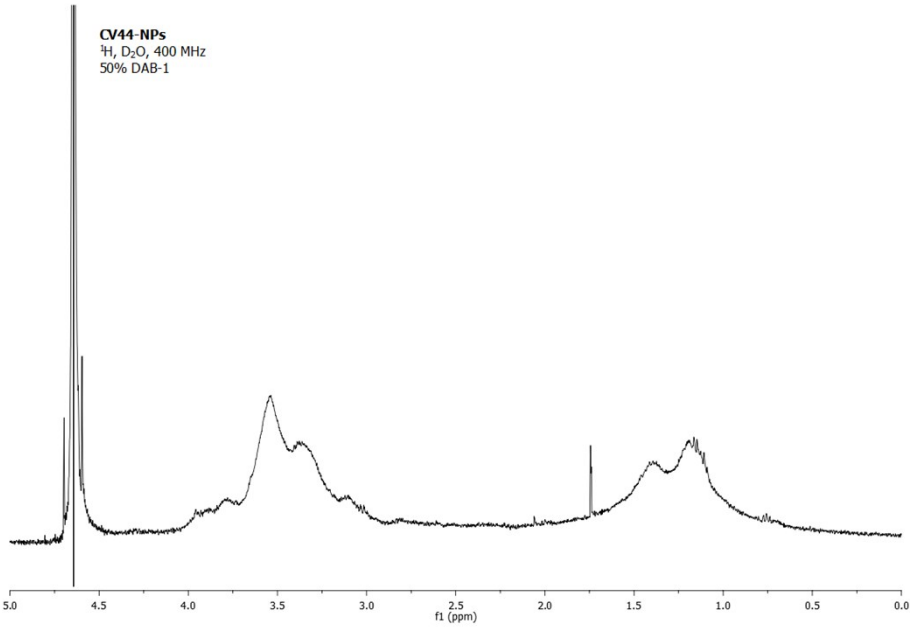


UV/vis spectrum of H_2O solution of Au GNPs **12b** recorded at concentration of 0.1 mg/mL.

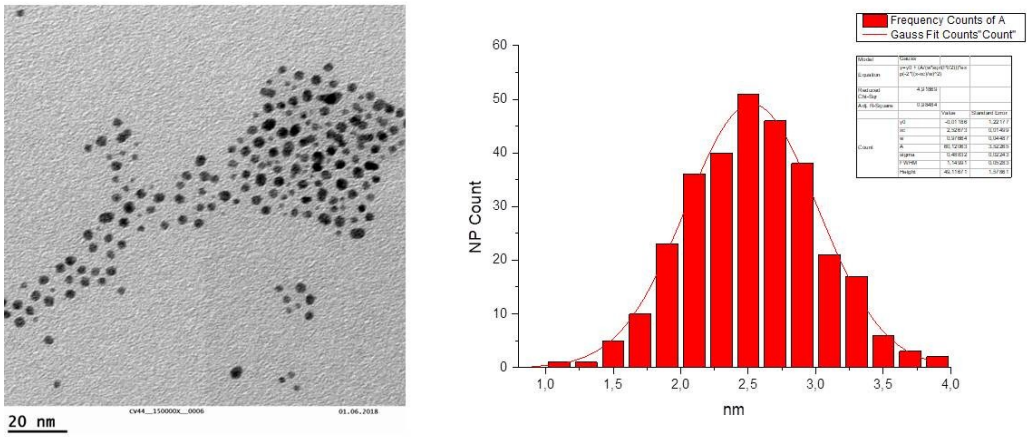
Preparation of 50% DAB-1-βGlc NPs (12c): A 1: 1 mixture of thiol-ending **10** (7.80 mg, 12.9 µmol) and βGlcC_5S **11** (3.6 mg, 12.9 µmol) in CD_3OD (2.1 mL) was used to obtain 0.45 mg of Au GNPs **12c.** These nanoparticles were difficult to isolate after washing with MeOH and they were not stable when dispersed in water; indeed flocculation was observed after a few hours. For this reason a qNMR was impossible to be recorded (15-18 h of acquisition time are usually necessary for these experiments).TEM (average diameter): 2.5 ±0.5 nm.



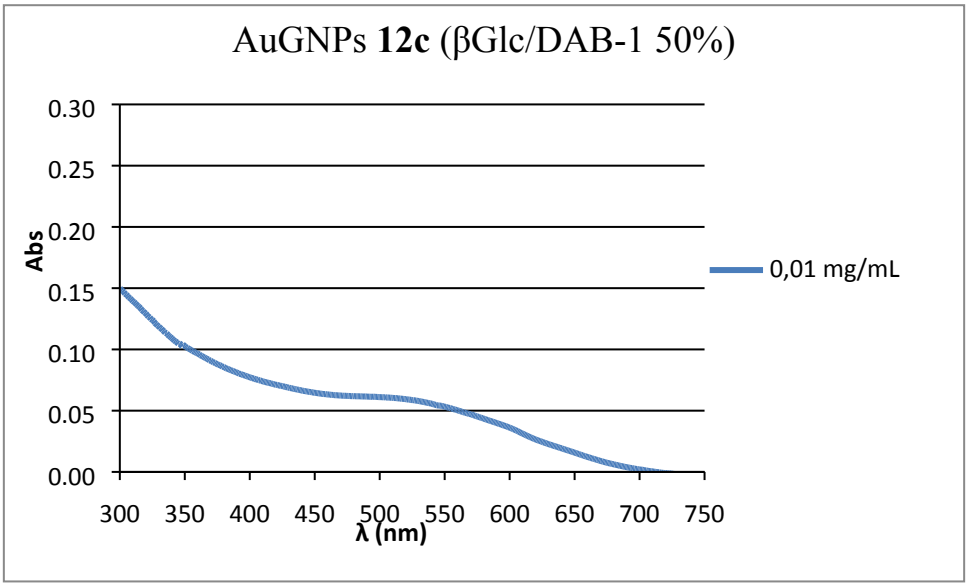
^{1}H NMR of sugar/iminosugar ligands mixture before (left) and after (right) formation of Au GNPs **12c** (400 MHz, CD_3OD).



^{1}H NMR of Au GNPs **12c** (400 MHz, D_2O).

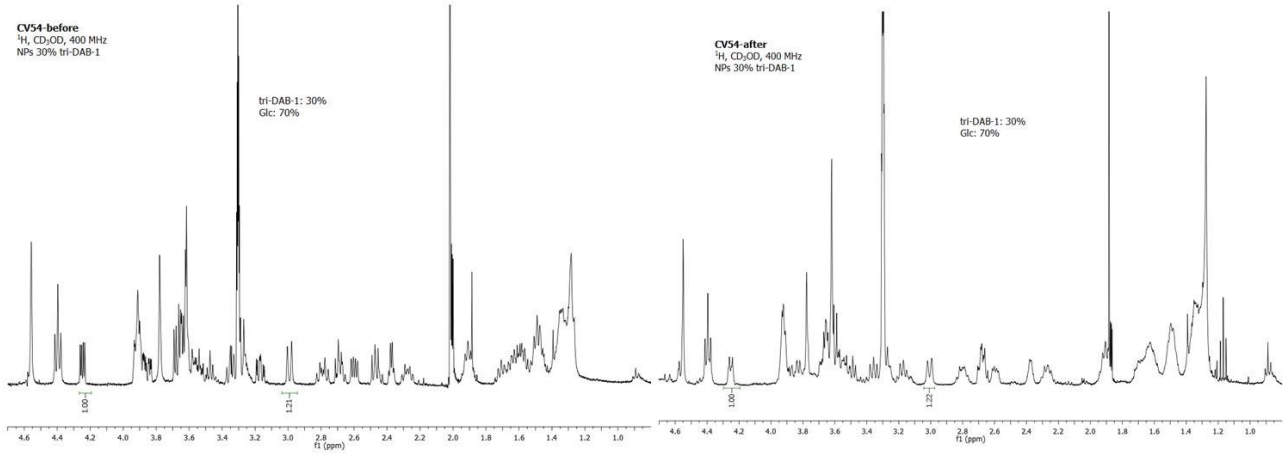


TEM micrograph in H_2O and size-distribution histogram obtained by measuring 300 nanoparticles (average diameter: 2.5 ±0.5 nm.

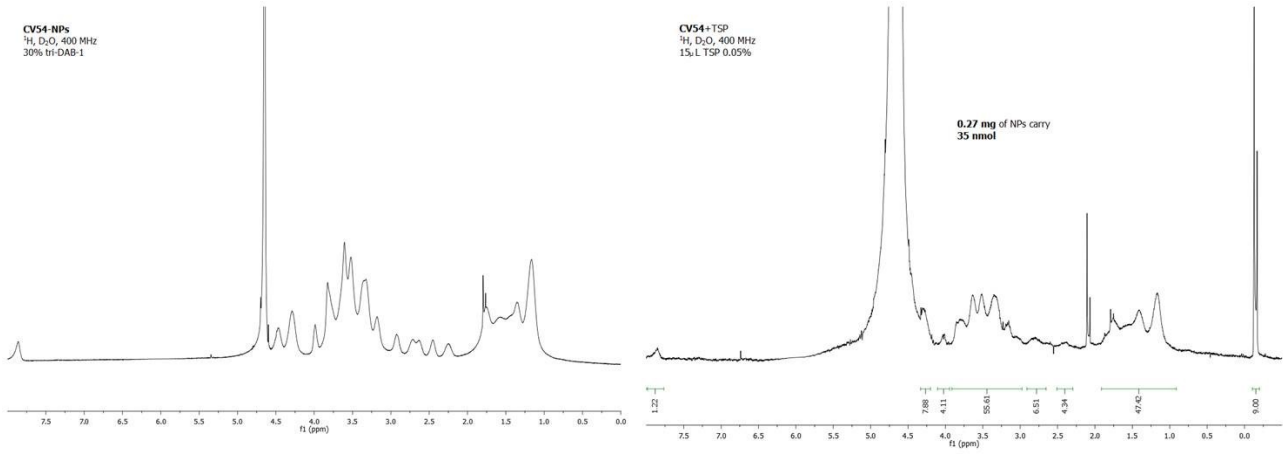


UV/vis spectrum of H_2O solution of Au GNPs **12c** recorded at concentration of 0.1 mg/mL.

Preparation of 30% tri-DAB-1-βGlc NPs (16): A 1: 4 mixture of thiol-ending **15** (10.0 mg, 5.5 µmol) and βGlcC$_5$S **11** (6.3 mg, 22.1 µmol) in CD_3OD (2.3 mL) was used to obtain 2.2 mg of AuGNPs **16**. TEM (average diameter): 1.7 ±0.5 nm. The ^{1}HNMR spectrum of the "before mixture" showed a 1: 2.3 ratio between **15** and **11**, corresponding to 30% tri-DAB-1-βGlc NPs, also confirmed by the ^{1}H NMR spectrum of the "after mixture". Quantitative ^{1}H NMR (400 MHz, D_2O containing 0.05 wt. % of 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid, sodium salt as an internal standard): 0.27 mg of **16** were dissolved in 150 µL of D_2O and 15 µL of D_2O containing 0.05 wt.% TSP were added and 35 nmoles of DAB-1 conjugate were found.[5] Significant peaks: δ = 7.85 (br s, 3H, triazole from DAB-1 conjugate), 4.29 (br s, from βGlcC5S), 4.03 (br s, $NHCOCH_2$-, 6H, from DAB-1 conjugate), 3.93-3.00 (m), 2.90-2.65 (m, 9H, from DAB-1 conjugate), 2.48-2.30 (m, 6H, from DAB-1 conjugate), 1.92-0.93 ppm (m).

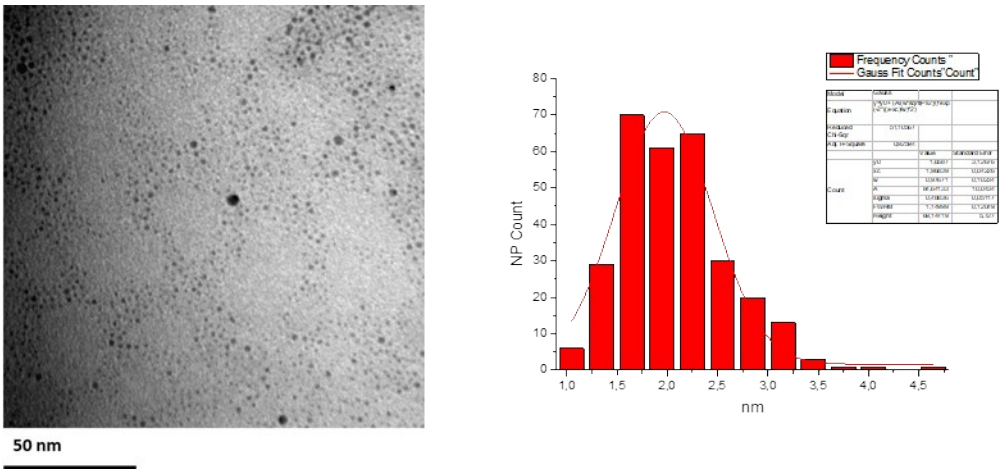


^{1}H NMR of sugar/iminosugar ligands mixture before (left) and after (right) formation of Au GNPs **16** (400 MHz, CD_3OD).

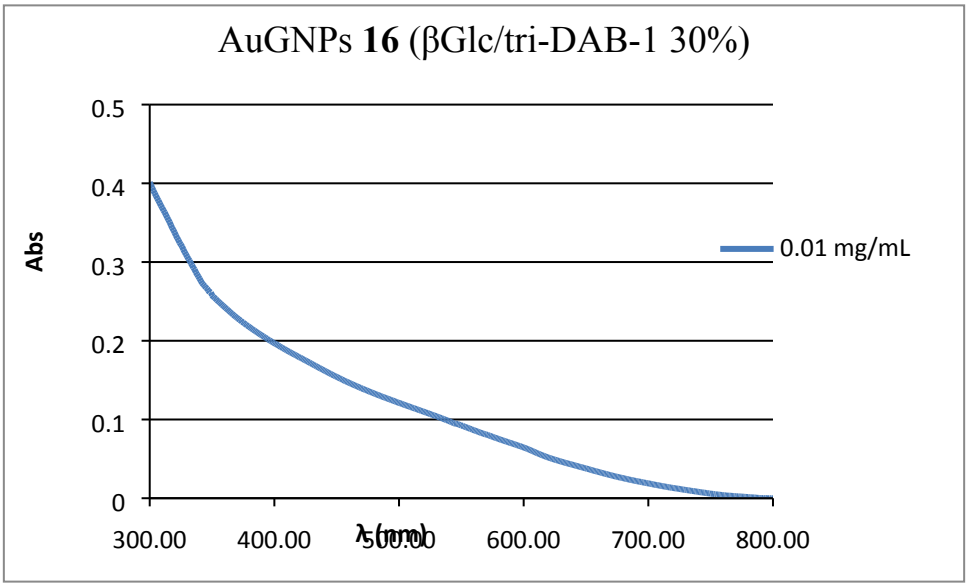


^{1}H NMR and ^{1}H qNMR with TSP-d_4 of Au GNPs **16** (400 MHz, D_2O).

[5] In the quantitative NMR (qNMR) the multiplet corresponding to H-5 and Ha-1' proton signals (δ = 2.90-2.65 ppm, 9H) of tri-DAB-1 conjugate **16** was selected for integration as it falls in a spectral region free of other signals. This is in agreement with the multiplet corresponding to H-2 and Hb-1' (δ = 2.48-2.30 ppm, 6H). Conversely, even if the signal corresponding to the triazole (br s at δ = 7.85 ppm) falls in a spectral region free of other signals, it is not suitable as reference signal, because it usually integrates much less than other protons.



TEM micrograph in H_2O and size-distribution histogram obtained by measuring 300 nanoparticles (average diameter 2.0 ± 0.5 nm).



UV/vis spectrum of H_2O solution of Au GNPs **16** recorded at concentration of 0.1 mg/mL.

Determination of GALNS and IDS activity in leukocytes homogenate from healthy donors.

For compounds **3**, **12a-b**, **16**, **17** and **18** the percentage of inhibition (at 1 mM concentration for **3** and **18** and at 0.2 mg/mL for AuGNPs **12a-b**, **16** and **17**) towards GALNS and IDS, using leukocyte extracts from healthy donors, was evaluated.

Leukocyte pellets were disrupted by sonication in water and the micro BCA protein assay kit (Sigma-Aldrich) was used to set up the protein amount for the enzymatic assay, according to the manufacturer's instructions.

GALNS enzymatic test:

Enzyme activity was measured by setting the reaction in 0.2 ml tubes and performing the experiments in triplicates as follows.

Step 1: Iminosugars solution (3 µl), leukocytes homogenate (7 µl) and 20 µl of 4-methylumbelliferyl-ß-galactoside-6-sulphate·Na (Moscerdam Substrates) substrate solution in Na-Acetate/acetic acid buffer (0.1 M/0.1 M, pH 4.3) containing 0.1 M NaCl , 0.02% (w/v) NaN_3 and 5 mM Pb-acetate were incubated for 17 h at 37 °C.

Step 2: After step 1 the tubes were placed on an ice cooler and the reaction was stopped by addition of 5 µl of Na-phosphate buffer (0.9 M, pH 4.3) containing 0.02 % of NaN_3 and by efficient mixing with vortex. Then, 10 µl of β-Gal-A-10U were added to each sample and the suspension mixed again in vortex apparatus, then samples were incubated for 2 h at 37°C.

At the end of this period the tubes were placed on an ice cooler and the samples were transferred in a cooled flat–bottomed 96 well plate and the reaction was immediately stopped with 200 µl of $NaHCO_3$/Na_2CO_3 buffer (0.5M/0.5M pH 10.7) containing 0.025% (w/v) of Triton X-100. Fluorescence was measured in a SpectraMax M2 microplate reader (Molecular-Devices) using a 365 nm excitation wavelength and a 435 nm emission wavelength.

Percentage of GALNS inhibition was given with respect to the control (without iminosugar). Experiments were performed in triplicate, and the mean ± S. D. was calculated.[6]

IDS enzymatic test:

Enzyme activity was measured by setting the reaction in 0.2 ml tubes and performing the experiments in triplicates as follows.

Step 1: Iminosugars solution (3 µl), leukocytes homogenate (7 µl) and 20 µl of 4-methylumbelliferyl-α-L-Iduronide-2-sulphate·2Na (Moscerdam Substrates) substrate solution were incubated for 4 h at 37 °C.

Step 2: After step 1 the tubes were placed on an ice cooler and the reaction stopped by addition of 20 µl of Na-Phosphate/Citrate buffer (0.2M/ 0.1M pH 4.5) and by efficient mixing with vortex. Then, 10 µl of LEBT

[6]O. P. Van Diggelen, H. Zhao, W. J. Kleijer, H. C. Janse, B. J. H. M. Poorthuis, J. Van Pelt, J. P. Kamerling and H. Galjaard, *Clinica Chimica Acta*, **1990**, *187*, 131.

(Lysosomal Enzymes purified from Bovine Testis) were added to each sample and the incubation was continued for 24 hours at 37°C.

At the end of this period tubes were placed on an ice cooler and the samples were transferred in a cooled flat–bottomed 96 well plate and the reaction was immediately stopped by addition of 200 µl of $NaHCO_3/Na_2CO_3$ buffer (0.5M/0.5M pH 10.7) containing 0.025% (w/v) of Triton X-100.

Fluorescence was then measured in a SpectraMax M2 microplate reader (Molecular-Devices) using a 365 nm excitation wavelength and a 435 nm emission wavelength.

Percentage of IDS inhibition was given with respect to the control (without iminosugar). Experiments were performed in triplicate, and the mean ± S. D. was calculated.[7]

IC_{50} determination[8]

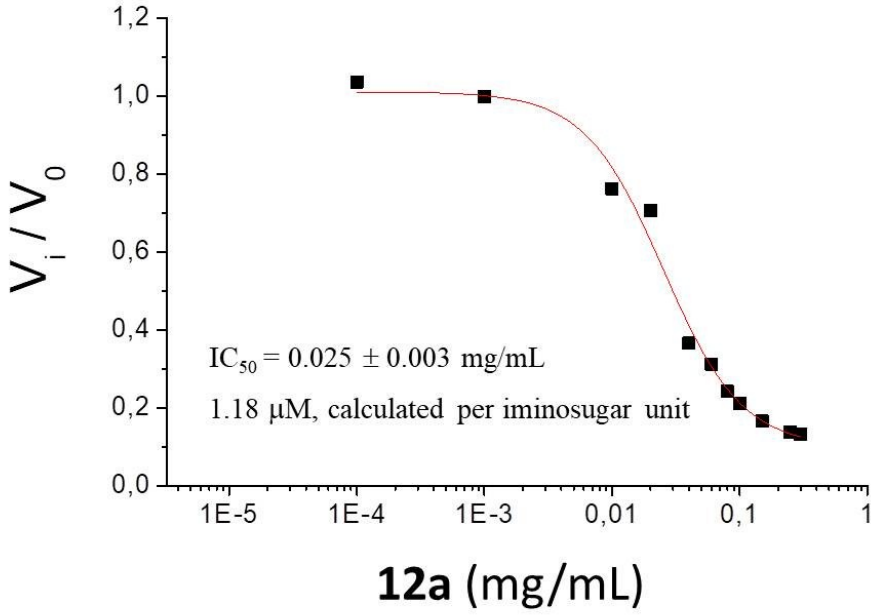
The IC_{50} values against the GALNS and IDS inhibitors were determined by measuring the initial hydrolysis rate under fixed 4-methylumbelliferyl-β-galactoside-6-sulphate·Na concentration (6.66 mM) and 4-methylumbelliferyl-α-L-Iduronide-2-sulphate·2Na concentration (0.833 mM) respectively. Data obtained were fitted to the following equation using the Origin Microcal program:

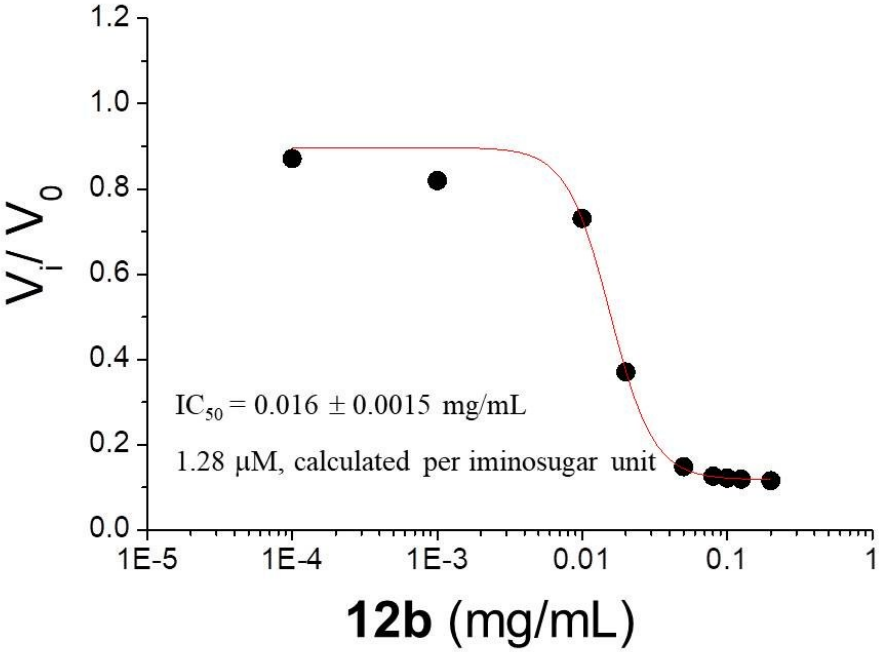
$$\frac{Vi}{Vo} = \frac{Max - Min}{1 + \left(\frac{x}{IC_{50}}\right)^{slope}} + Min$$

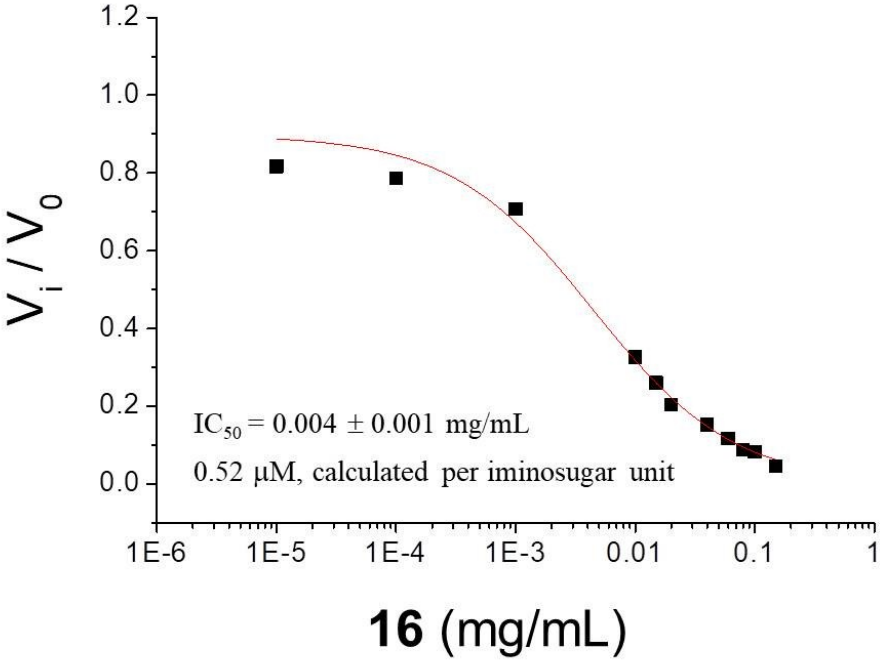
where V_i/V_0, represents the ratio between the activity measured in the presence of the inhibitor (Vi) and the activity of the control without the inhibitor (V_0), "x" the inhibitor concentration, Max and Min, the maximal and minimal enzymatic activity observed, respectively.

[7] Y. V. Voznyi, J. L. Keulemans and O. P. van Diggelen, *J. Inher. Metabol. Dis.*, **2001**, *24*, 675.

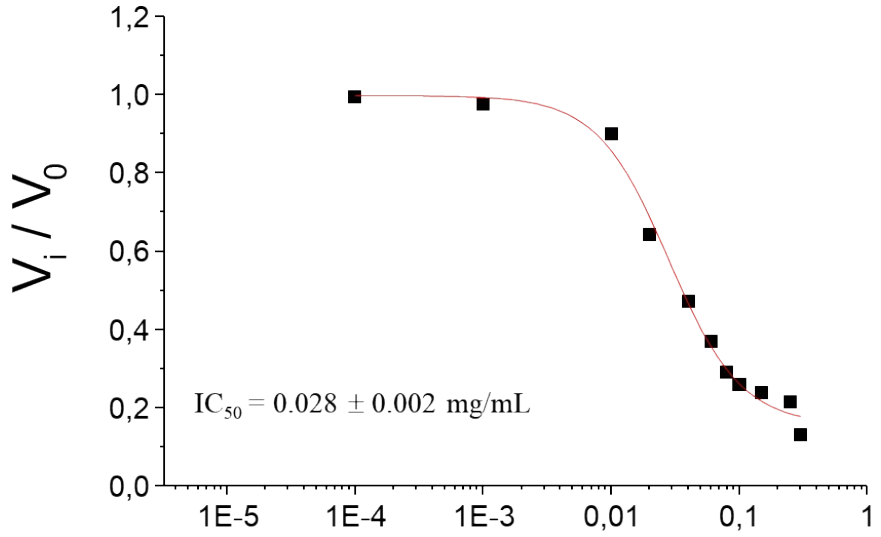
[8] R. Ottanà, R. Maccari, J. Mortier, A. Caselli, S. Amuso, G. Camici, A. Rotondo, G. Wolber and P. Paoli, *Eur. J. Med. Chem*., **2014**, *71*, 112.


1,2
1,0
0,8
0,6
0,4
0,2
0,0
V_i / V_0
IC_{50} = 0.025 ± 0.003 mg/mL
1.18 μM, calculated per iminosugar unit
1E-5
1E-4
1E-3
0,01
0,1
1
12a (mg/mL)


1.2
1.0
0.8
0.6
0.4
0.2
0.0
V_i / V_0
IC_{50} = 0.016 ± 0.0015 mg/mL
1.28 μM, calculated per iminosugar unit
1E-5
1E-4
1E-3
0.01
0.1
1
12b (mg/mL)



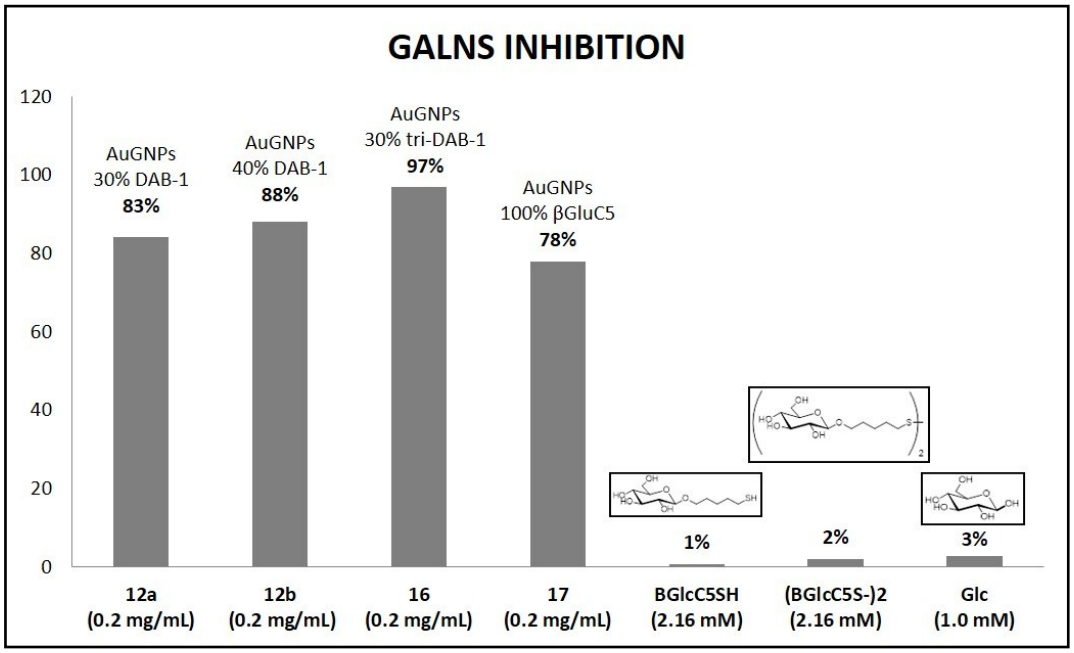
16 (mg/mL)



17 (mg/mL)

Inhibition of GALNS by Au GNPs and βGlucose monovalent derivatives

To verify the essential contribution of the multivalent presentation of β-GluC5S in GALNS inhibition, the free ligand was also tested towards this enzyme, following the previously reported protocol. In particular, due to the spontaneous oxidation of the thiol ligand to the corresponding disulphide, we decided to test both the thiol form (obtained by deprotecting peracetylated **11** in deoxygenated atmosphere and subsequently kept under N_2 atmosphere) and the disulphide (obtained by oxidation of thiol **11** in O_2 atmosphere). For seek of completeness we also tested the simple sugar β-Glucose, as purchased from Sigma Aldrich. None of the three forms of the sugar showed GALNS inhibition. In the chart below the percentage of inhibition obtained with AuGNPs **12a**, **12b**, **16** and **17** are also reported for comparison.



Percentage of inhibition obtained by screening AuGNPs **12a-b**, **16** and **17** at 0.2 mg/mL, β-GlcC5S ligands (thiol and disulphide) at 2.16 mM and β-Glucose at 1.0 mM towards GALNS.