Electronic Supplementary Material (ESI) for RSC Advances.

Evidence for a Multivalent Effect in Inhibition of Sulfatases Involved in Lysosomal Storage Disorders (LSDs)

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Supporting Information

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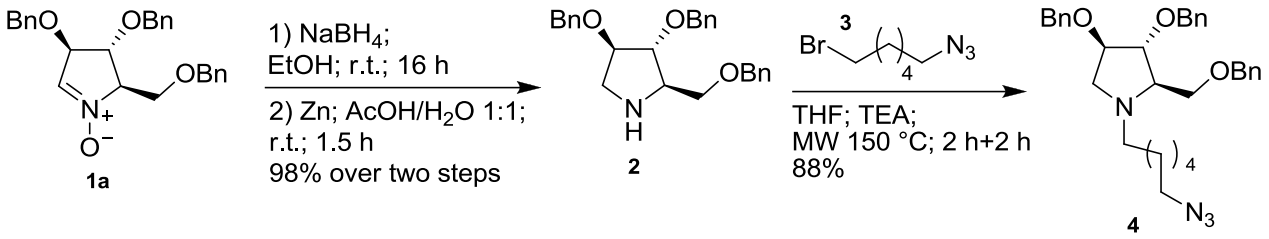
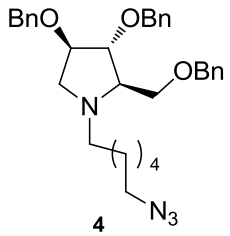
Synthesis of (2*R*,3*R*,4*R*)-3,4-bis(benzyloxy)-2-[(benzyloxy)methyl]-1-[6-azido)hexyl]-1*H*-pyrrolidine (4)



To a solution of **1a** (125 mg, 0.3 mmol) in 6 ml of ethanol, $NaBH_4$ (23 mg, 0.6 mmol) was added and the mixture was stirred at room temperature for 16 h, until a TLC analysis (AcOEt:EP 1:1) showed the disappearance of the starting material (R_f = 0.30) and the formation of a new product (R_f = 0.91). After the addition of 0.5 ml of MeOH and 1.5 ml of H_2O the mixture was raised at room temperature and stirred for another 3 h, then it was evaporated under reduced pressure and the crude obtained filtered through Celite® washing the solid several times with ethyl ether. The collected solution was evaporated under reduced pressure affording the desired pure hydroxylamine intermediate (124 mg, 0.3 mmol) that was dissolved in 4 ml of acetic acid. Zn powder (351 mg, 6 mmol) and 4 ml of H_2O were added. The reaction mixture was stirred at room temperature for 1.5 h until a TLC analysis (CH_2Cl_2:MeOH 30:1) showed the disappearance of the starting material (R_f = 0.81) and the formation of a new product (R_f = 0.21). After filtration through cotton, the mixture was concentrated at reduced pressure and then saturated aqueous solution of Na_2CO_3 was added at 0 °C until basic pH. After extraction with AcOEt (3 x 30 mL), the organic layers were dried on Na_2SO_4, concentrated under reduced pressure and the crude was purified by Flash Column Chromatography (CH_2Cl_2:MeOH 30:1) affording pure **2** (R_f = 0.21, 119 mg, 0.29 mmol, 98% over two steps) as a colorless oil [for an alternative synthesis see of compound **2** W.-C. Cheng, C.-Y. Wenga, W.-Y. Yun, S.-Y. Chang, Y.-C. Lin, F.-J. Tsai, F.-Y. Huang and Y.-R. Chen, *Bioorg. Med. Chem*, 2013, **21**, 5021].

A solution of **2** (175 mg, 0.43 mmol), 1-azido-6-bromohexane (**3**, 134 mg, 0.65 mmol) and TEA (120 µL, 0.86 mmol) in 4 ml of THF was stirred in microwave at 150°C. After 2 h a TLC analysis (CH_2Cl_2:MeOH 10:1) still showed the presence of the starting material (R_f = 0.51), then 0.5 equivalents of 1-azido-6-bromohexane (**3**) were added and the reaction mixture was stirred in microwave at 150 °C for another 2 h until complete formation of the desired product (R_f = 0.98, CH_2Cl_2:MeOH 10:1) was achieved. After evaporation under reduced pressure, the crude was purified by Flash Column Chromatography (EP:AcOEt 4:1) affording pure **4** (R_f = 0.31, 203 mg, 0.38 mmol, 88%) as a yellow oil. $[\alpha]_D^{30}$ = −25.1 (*c*

= 1.30, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.35-7.25 (m, 15H, H-Ar), 4.57-4.42 (m, 6H, H-Bn), 3.92 (d, *J* = 4.8 Hz, 1H, H-4), 3.88 (d, *J* = 3.9 Hz, 1H, H-3), 3.62-3.50 (m, 1H, Ha-6), 3.52 (dd, *J* = 9.8, 6.3 Hz, 1H, Hb-6), 3.24 (t, *J* = 7.1 Hz, 2H, H-12), 3.24-3.19 (m, 1H, Ha-5), 2.88-2.81 (m, 1H, Ha-7), 2.71 (bs, 1H, H-2), 2.55 (dd, *J* = 10.3, 4.9 Hz, 1H, Hb-5), 2.38-2.31 (m, 1H, Hb-7), 1.59 (quint, *J* = 7.1 Hz, 2H, H-11), 1.54-1.48 (m, 2H, H-8), 1.41-1.25 (m, 4H, H-9 and H-10); ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 138.5, 138.4, 138.3 (s, 3C, C-Ar), 128.3-127.6 (d, 15C, C-Ar), 85.5 (d, C-3), 81.7 (d, C-4), 73.2 (t, C-Bn) 71.3 (t, C-Bn), 71.2 (t, C-Bn), 71.0 (t, C-6), 69.4 (d, C-2), 57.3 (t, C-5), 55.5 (t, C-7), 51.4 (t, C-12), 28.8 (t, C-11), 28.0 (t, C-8), 27.0, 26.6 (t, 2C, C-9 and C-10); IR ($CDCl_3$): v = 3088, 3066, 3032, 3009, 2937, 2862, 1496, 1453, 1365, 1261, 1096 cm^{-1}; MS (ESI): m/z 529.42 ($[M+1]^+$; 100), 551.42 ($[M+Na]^+$; 38); elemental analysis calcd (%) for $C_{32}H_{40}N_4O_3$ (528.68): C 72.70, H 7.63, N 10.60; found: C 72.32, H 7.23, N 10.59.



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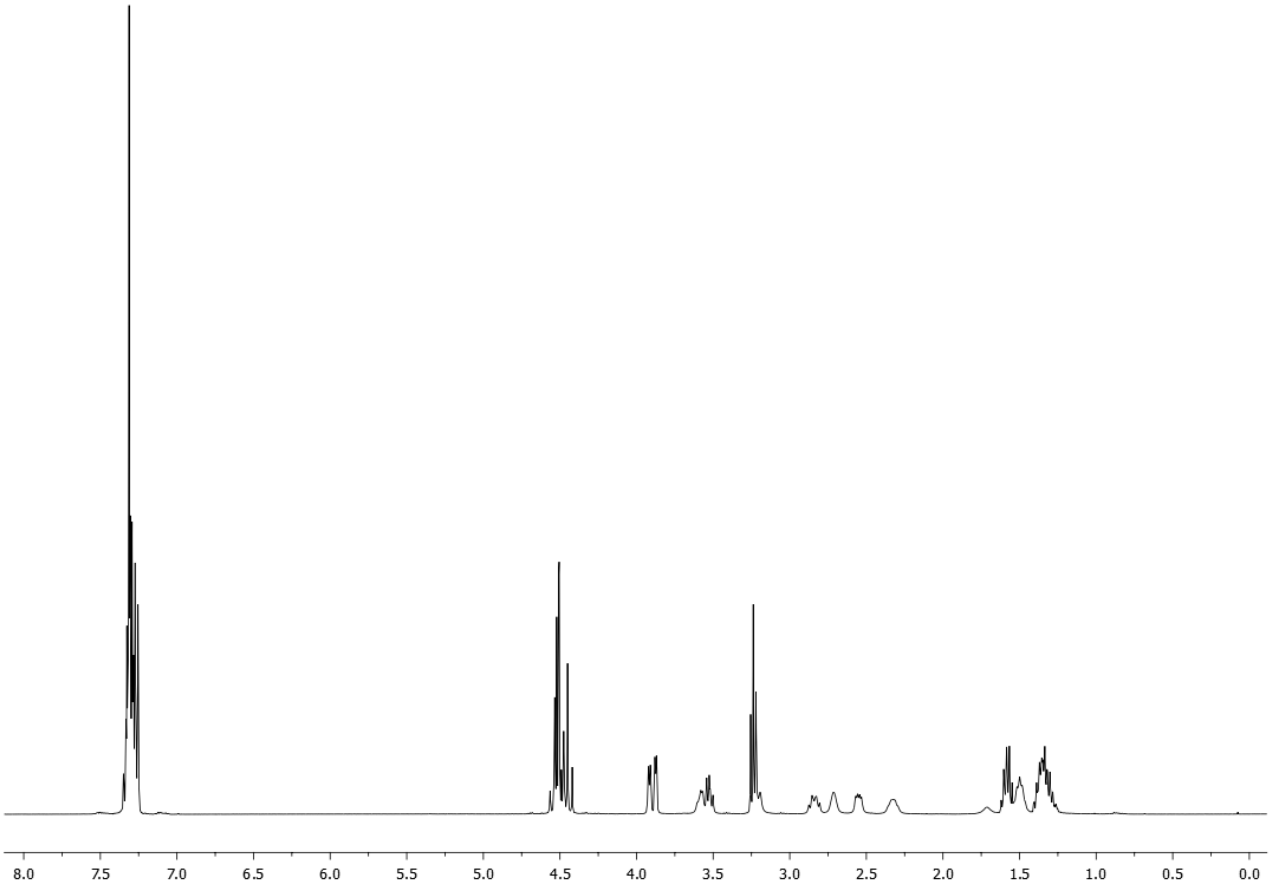
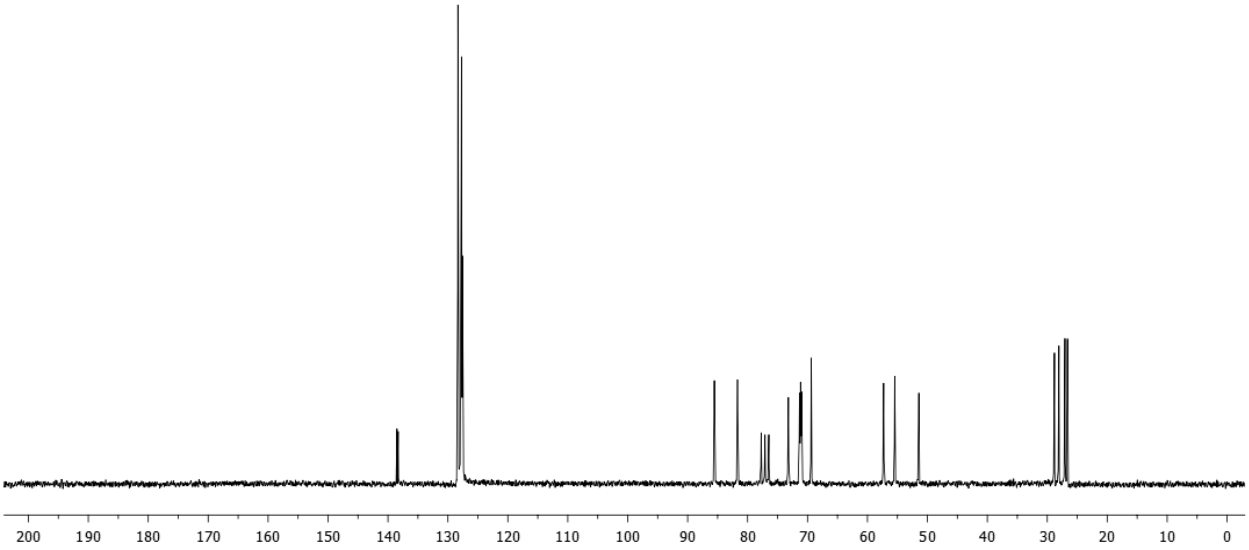
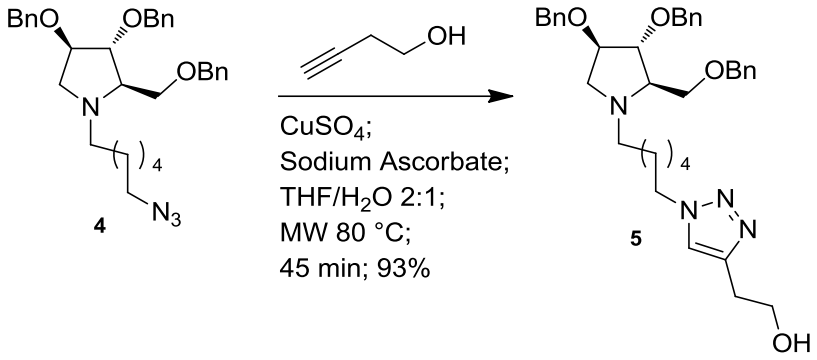


^{1}H-NMR spectrum of compound 4 (400 MHz, $CDCl_3$)

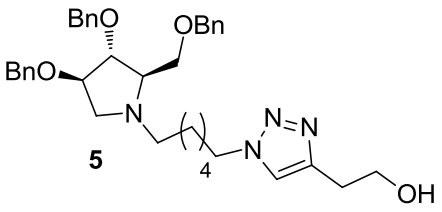


^{13}C-NMR spectrum of compound 4 (50 MHz, $CDCl_3$)

Synthesis of (2*R*, 3*R*, 4*R*)-1-hexyl[4-(hydroxyethyl)-1*H*-1,2,3-triazol-1-yl]-3,4-bis(benzyloxy)-2-[(benzyloxy)methyl]-pyrrolidine



To a solution of **4** (64 mg, 0.12 mmol) in 3 ml of 2:1 THF-H_2O $CuSO_4$ (30 mol%, 6 mg, 0.04 mmol), sodium ascorbate (60 mol%, 14 mg, 0.07 mmol) and 3-butyn-1-ol (9 mg, 0.13 mmol) were added. The reaction mixture was stirred in microwave at 80 °C for 45 min, until a TLC analysis (EP:AcOEt 3:1) showed the disappearance of the starting material (R_f = 0.49) and the formation of a new product (R_f = 0.00). After filtration through Celite®, the solvent was removed under reduced pressure and the crude was purified by Flash Column Chromatography (AcOEt) affording pure **5** (R_f = 0.23, 67 mg, 0.11 mmol, 93%) as a yellow oil. $[\alpha]_D^{26}$ = −20.3 (*c* = 0.94, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 7.34 (s, 1H, H-Triazole), 7.34-7.25 (m, 15H, H-Ar), 4.56-4.42 (m, 6H, H-Bn), 4.27 (t, *J* = 7.1 Hz, 2H, H-12), 3.93-3.90 (m, 1H, H-4), 3.91 (t, *J* = 6.0 Hz, 2H, *CH*$_2$OH), 3.88-3.87 (m, 1H, H-3), 3.58 (dd, *J* = 9.7, 5.3 Hz, 1H, Ha-6), 3.52 (dd, *J* = 9.7, 6.6 Hz, 1H, Hb-6), 3.22-3.19 (m, 1H, Ha-5), 2.93 (t, *J* = 6.0 Hz, 2H, *CH*$_2$CH$_2$OH), 2.86-2.79 (m, 1H, Ha-7), 2.74-2.72 (m, 1H, H-2), 2.55 (dd, *J* = 10.6, 5.1 Hz, 1H, Hb-5), 2.36-2.29 (m, 1H, Hb-7), 1.86 (quint, *J* = 7.0 Hz, 2H, H-11), 1.54-1.42 (m, 2H, H-8), 1.38-1.22 (m, 4H, H-9 and H-10); ^{13}C-NMR (50 MHz, $CDCl_3$): δ = 145.5 (s, C-Triazole), 138.4, 138.1, 138.0 (s, 3C, C-Ar), 128.3-127.5 (d, 15C, C-Ar), 121.4 (d, C-Triazole), 85.4 (d, C-3), 81.6 (d, C-4), 73.2 (t, C-Bn) 71.3 (t, C-Bn), 71.0 (t, 2C, C-Bn and C-6), 69.4 (d, C-2), 61.5 (t, *CH*$_2$OH), 57.2 (t, C-5), 55.4 (t, C-7), 50.1 (t, C-12), 30.2 (t, C-11), 28.8 (t, *CH*$_2$CH$_2$OH), 27.8 (t, C-8), 26.8, 26.3 (t, C-9 and C-10); IR ($CDCl_3$): v = 3620, 3466, 3088, 3066, 3031, 2936, 2862, 1496, 1453, 1310, 1207, 1098 cm^{-1}; MS (ESI): m/z 599.50 ($[M+1]^+$; 100), 621.42 ($[M+Na]^+$; 28); elemental analysis calcd (%) for $C_{36}H_{46}N_4O_4$ (598.77): C 72.21, H 7.74, N 9.36; found: C 72.06, H 7.52, N 9.27.



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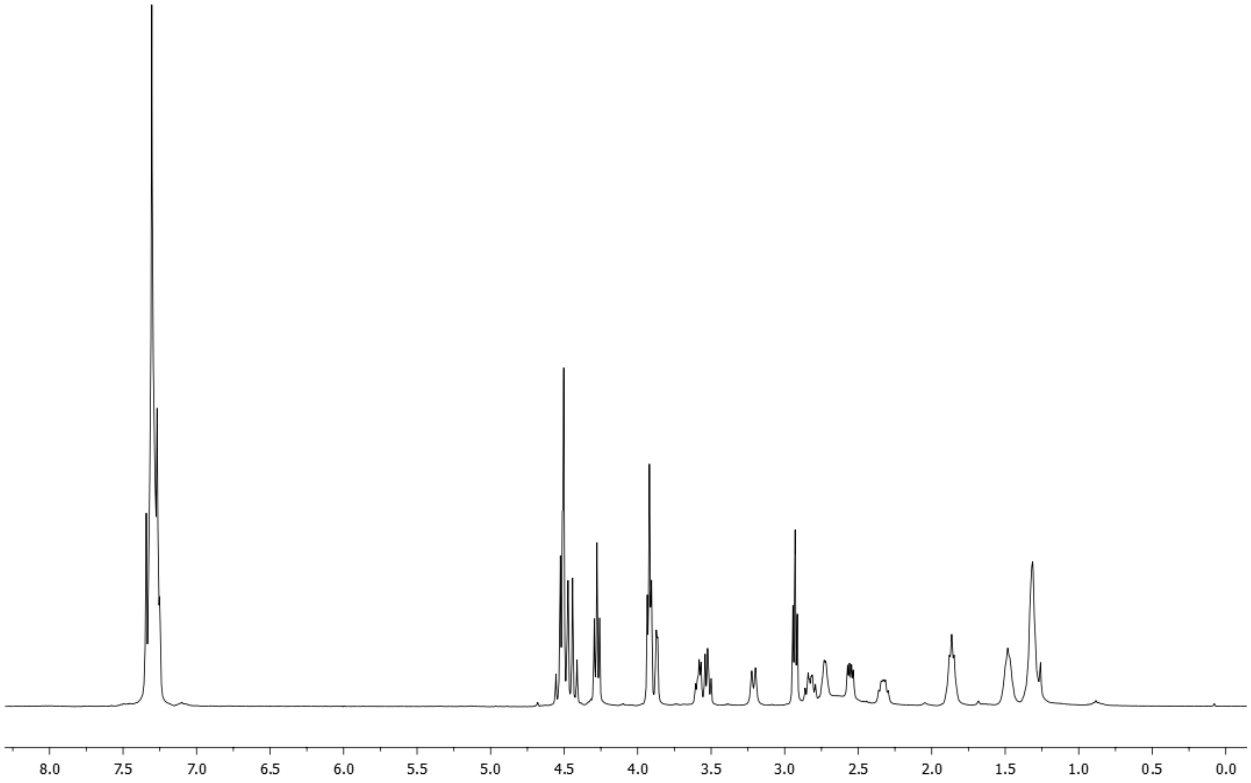
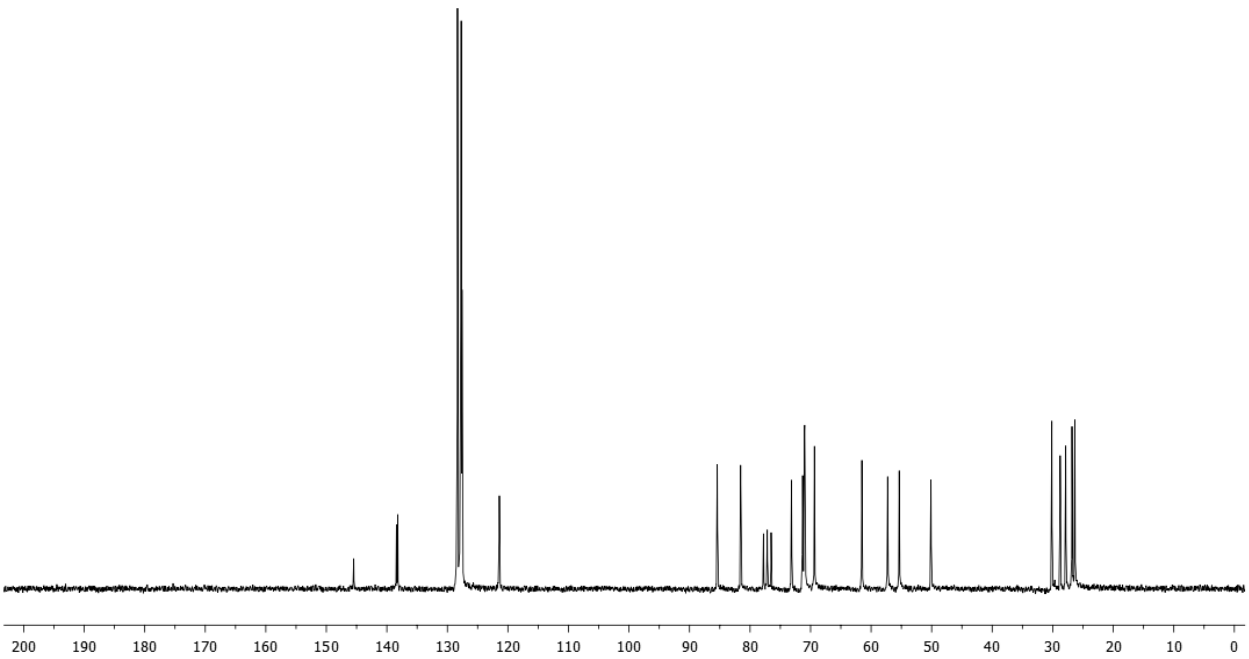
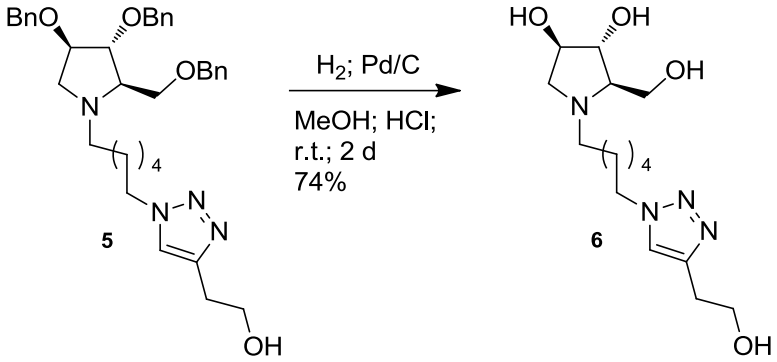


^{1}H-NMR spectrum of compound 5 (400 MHz, $CDCl_3$)

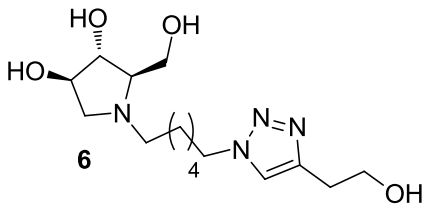


^{13}C-NMR spectrum of compound 5 (50 MHz, $CDCl_3$)

Synthesis of (2*R*, 3*R*, 4*R*)-1-hexyl[4-(hydroxyethyl)-1*H*-1,2,3-triazol-1-yl]-3,4-bis(hydroxy)-2-[(hydroxy)methyl]-pyrrolidine



To a solution of **5** (63 mg, 0.105 mmol) in 15 ml of methanol, 32 mg of 10% Pd/C and two drops of 37% HCl were added under nitrogen atmosphere, then the mixture was stirred under hydrogen atmosphere at room temperature for two days, until a NMR control showed the disappearance of the starting material. The mixture was then filtered through Celite® and the solvent was removed under reduced pressure affording a crude yellow oil. Free amine was obtained by passing the hydrochloride salt through a Dowex 50WX8 ion-exchange resin. Elution with 6% NH_4OH afforded the free base **6** (25 mg, 0.076 mmol, 74%) as a yellow oil. $[\alpha]_D^{23}$ = −15.7 (*c* = 0.21, MeOH); ^{1}H-NMR (400 MHz, D_2O): δ = 7.69 (s, 1H, H-Triazole), 4.26 (t, *J* = 6.8 Hz, 2H, H-12), 4.04-4.03 (m, 1H, H-4), 3.85-3.83 (m, 1H, H-3), 3.72 (t, *J* = 6.6 Hz, 2H, *CH*$_2$OH), 3.64 (d, *J* = 5.4 Hz, 2H, H-6), 3.04 (d, *J* = 11.2 Hz, 1H, Ha-5), 2.86-2.76 (m, 2H, Hb-5 and Ha-7), 2.80 (t, *J* = 6.4 Hz, 2H, *CH*$_2$CH$_2$OH), 2.68-2.62 (m, 1H, H-2), 2.45-2.39 (m, 1H, Hb-7), 1.76 (quint, *J* = 6.7 Hz, 2H, H-11), 1.46-1.32 (m, 2H, H-8), 1.24-1.08 (m, 4H, H-9 and H-10); ^{13}C-NMR (50 MHz, D_2O): δ = 145.2 (s, C-Triazole), 123.8 (d, C-Triazole), 78.3 (d, C-3), 75.0 (d, C-4), 72.8 (d, C-2), 60.6 (t, *CH*$_2$OH), 60.5 (t, C-6), 58.4 (t, C-5), 55.6 (t, C-7), 50.2 (t, C-12), 29.1 (t, C-11), 27.8 (t, *CH*$_2$CH$_2$OH), 25.9, 25.8, 25.2 (t, 3C, C-8, C-9 and C-10); MS (ESI): m/z 329.17 ($[M+1]^+$; 94), 351.08 ($[M+Na]^+$; 100); elemental analysis calcd (%) for $C_{15}H_{28}N_4O_4$ (328.41): C 54.86, H 8.59, N 17.06; found: C 54.79, H 8.57, N 17.09.



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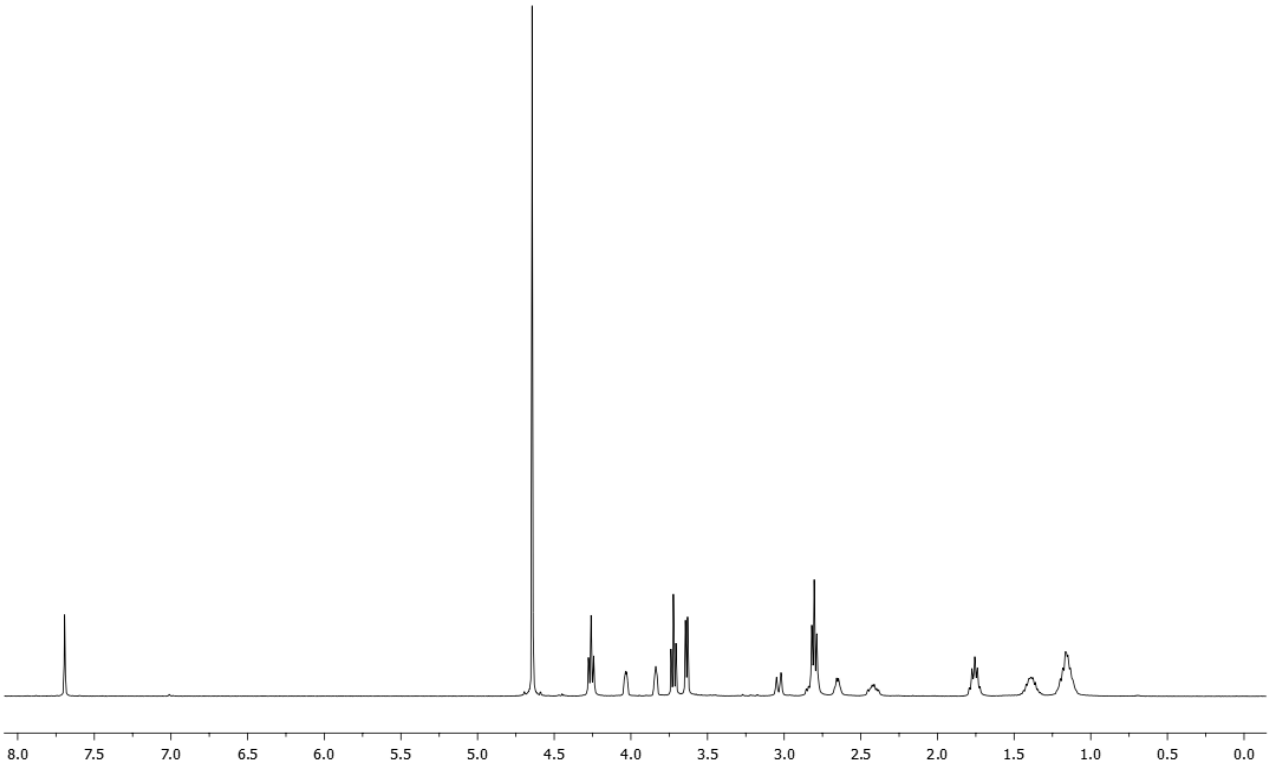
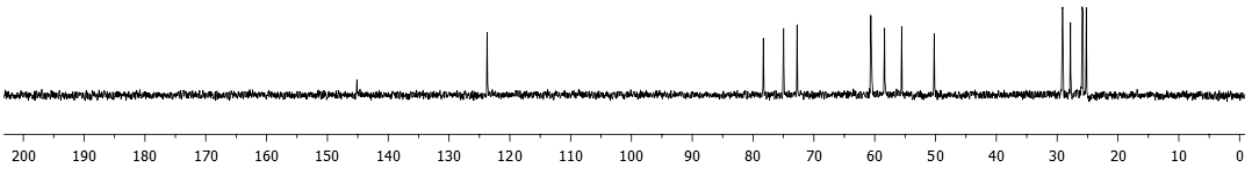
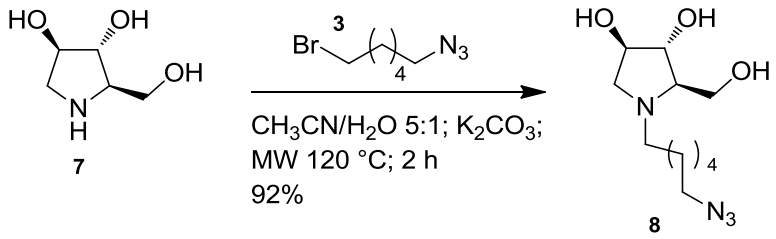


^{1}H-NMR spectrum of compound 6 (400 MHz, D_2O)

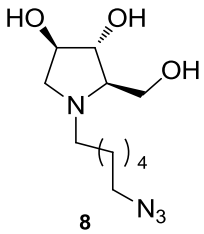


^{13}C-NMR spectrum of compound 6 (50 MHz, D_2O)

Synthesis of (2*R*,3*R*,4*R*)-1-[6-azido)hexyl]-3-hydroxymethylhexahydro-1*H*-pyrrolidine-1,2-diol



A solution of **7** (111 mg, 0.83 mmol), 1-azido-6-bromohexane (**3**, 258 mg, 1.25 mmol) and K_2CO_3 (173 mg, 1.25 mmol) in 6 ml of a mixture CH_3CN/H_2O 5:1 was stirred in microwave at 120°C for 2 h, until a TLC analysis (CH_2Cl_2:MeOH 2:1 + 1% v/v 6% NH_4OH) showed the disappearance of the starting material (R_f = 0.42) and the formation of a new product (R_f = 0.98). After filtration through Celite®, the solvent was removed under reduced pressure and the crude was purified by Flash Column Chromatography (CH_2Cl_2:MeOH 6:1 + 1% v/v 6% NH_4OH) affording pure **8** (R_f = 0.20, 197 mg, 0.76 mmol, 92%) as a yellow oil. $[\alpha]_D^{29}$ = −23.3 (*c* = 0.36, MeOH); ^{1}H-NMR (400 MHz, D_2O): δ = 3.99-3.97 (m, 1H, H-4), 3.81-3.79 (m, 1H, H-3), 3.63-3.55 (m, 2H, H-6), 3.20 (t, *J* = 6.9 Hz, 2H, H-12), 2.89 (d, *J* = 10.7 Hz, 1H, Ha-5), 2.71 (td, *J* = 11.2, 5.8 Hz, 1H, Ha-7), 2.63 (dd, *J* = 11.2, 5.9, 1H, Hb-5), 2.42 (q, *J* = 5.3 Hz, 1H, H-2), 2.26 (td, *J* = 11.2, 5.3 Hz, 1H, Hb-7), 1.53-1.46 (m, 2H, H-11), 1.44-1.34 (m, 2H, H-8), 1.32-1.17 (m, 4H, H-9 and H-10); ^{13}C-NMR (50 MHz, D_2O): δ = 79.4 (d, C-3), 75.6 (d, C-4), 72.1 (d, C-2), 61.4 (t, C-6), 58.5 (t, C-5), 55.4 (t, C-7), 51.4 (t, C-12), 28.0 (t, C-11), 26.7 (t, C-8), 26.4, 25.9 (t, 2C, C-9 and C-10); MS (ESI): m/z 281.25 ($[M+Na]^+$; 100); elemental analysis calcd (%) for $C_{11}H_{22}N_4O_3$ (258.32): C 51.15, H 8.58, N 21.69; found: C 51.08, H 8.35, N 21.54.



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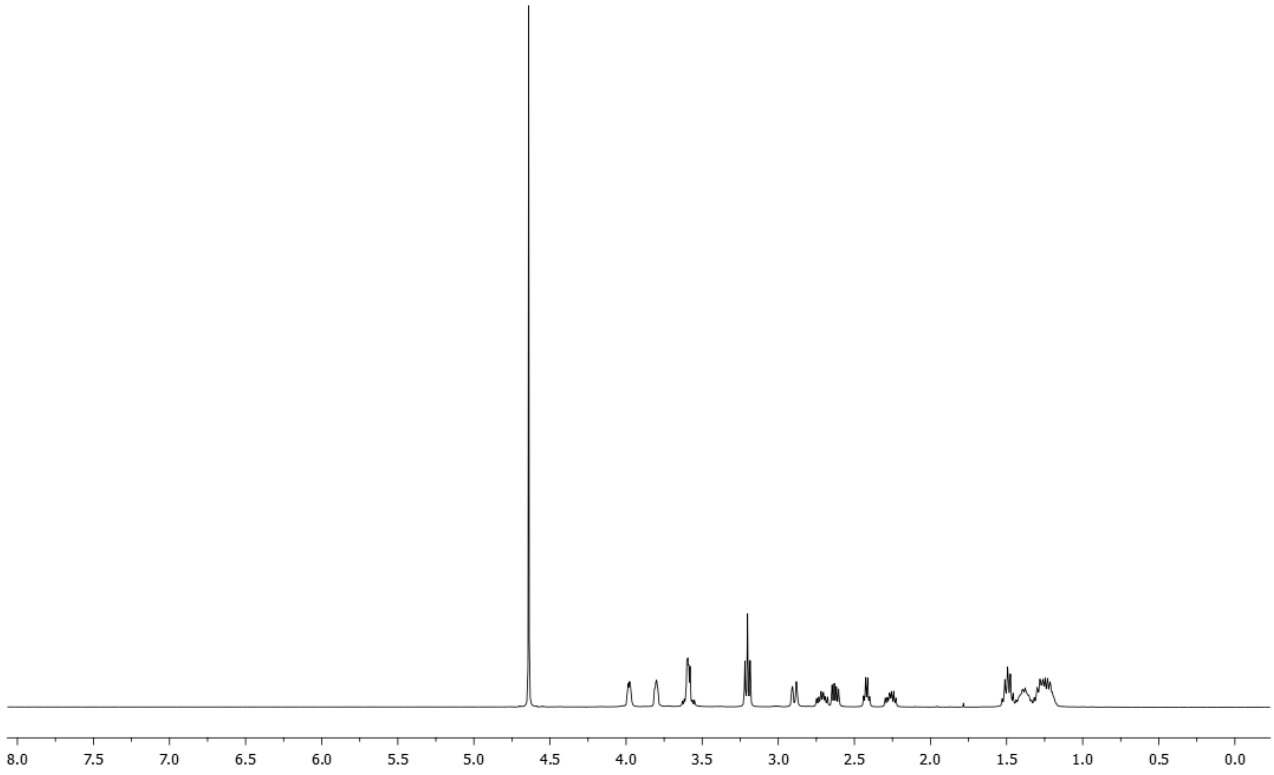
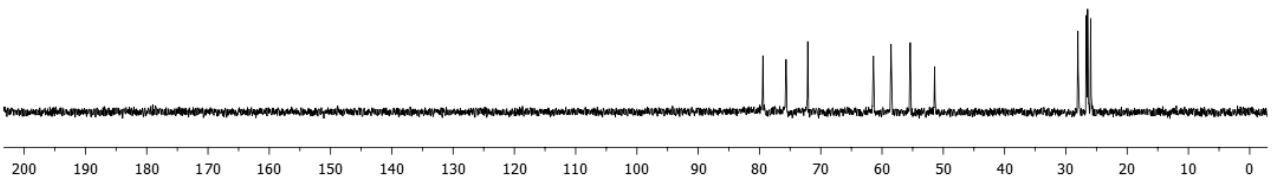
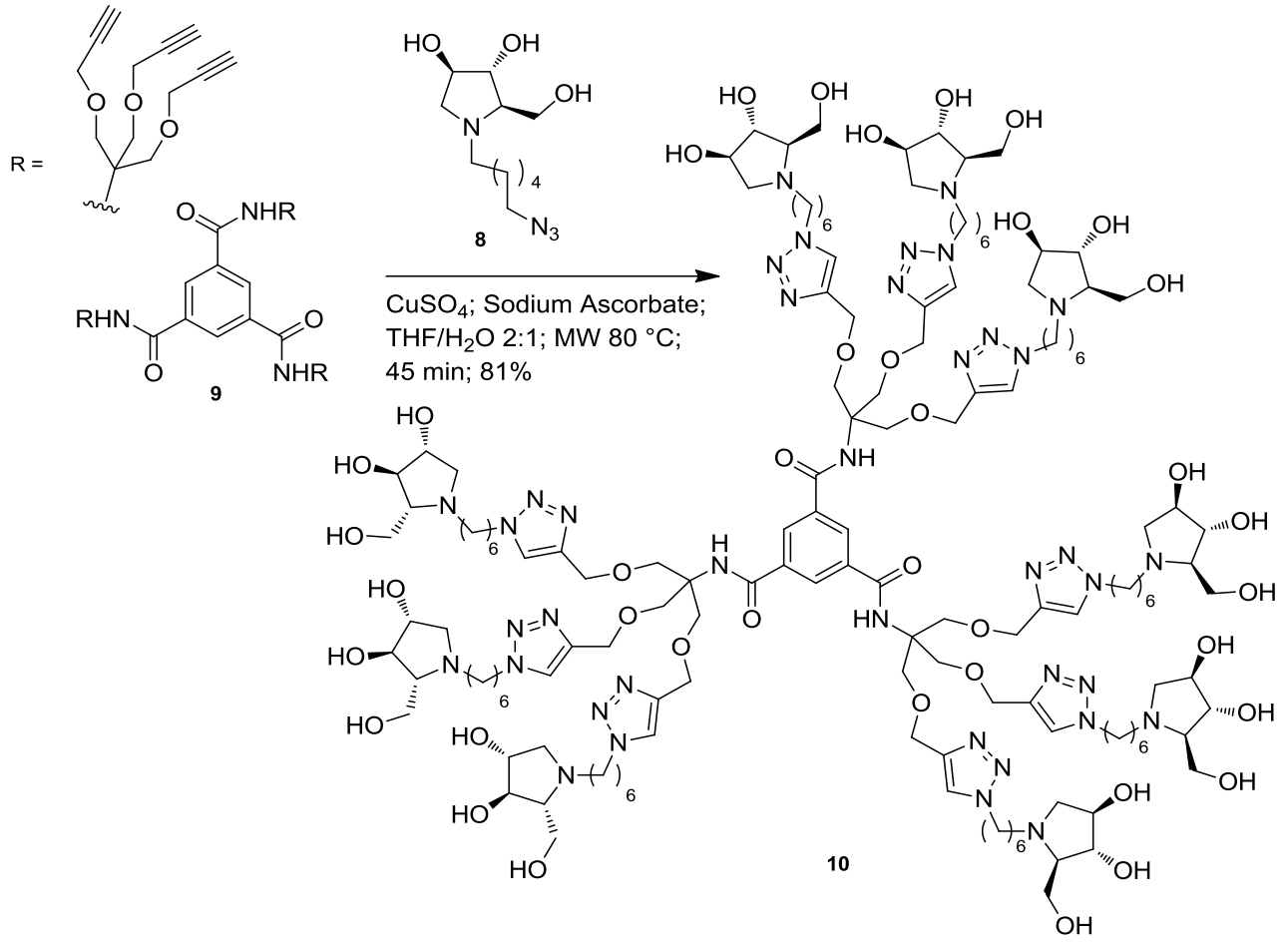


^{1}H-NMR spectrum of compound 8 (400 MHz, D_2O)



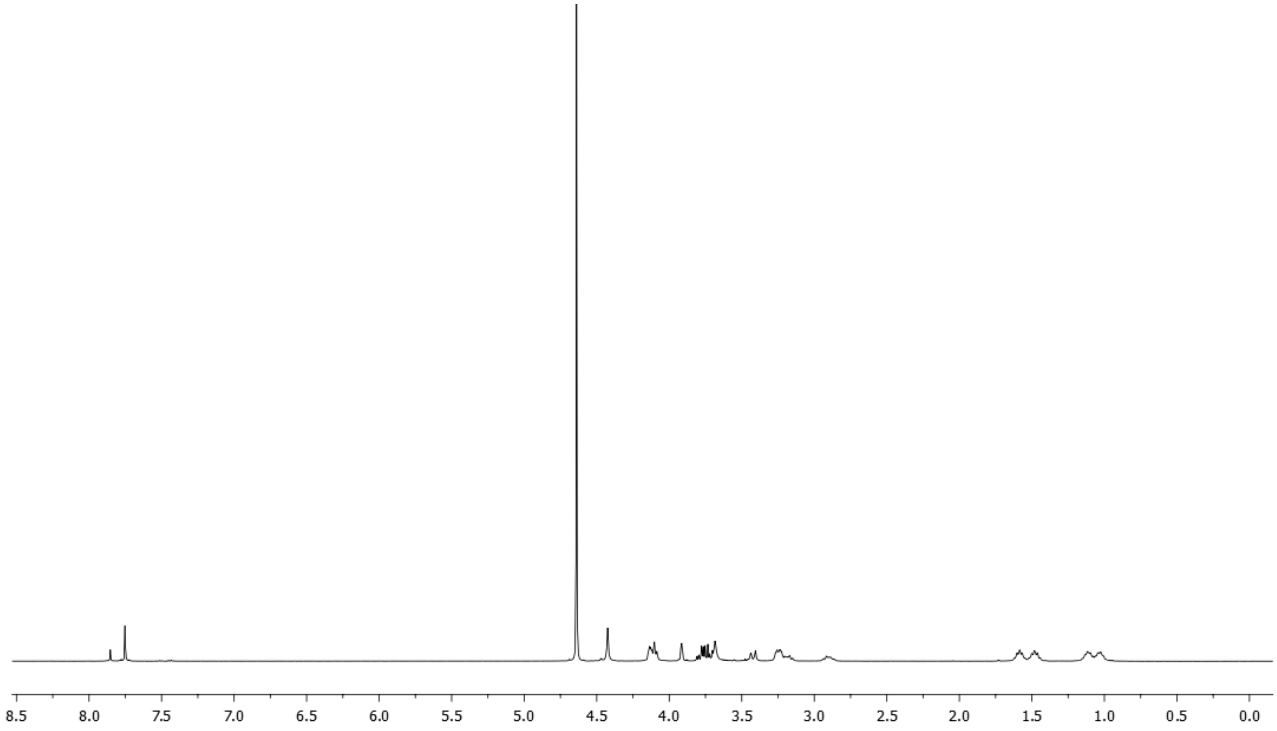
^{13}C-NMR spectrum of compound 8 (50 MHz, D_2O)

Synthesis of nonavalent pyrrolidine iminosugars 10

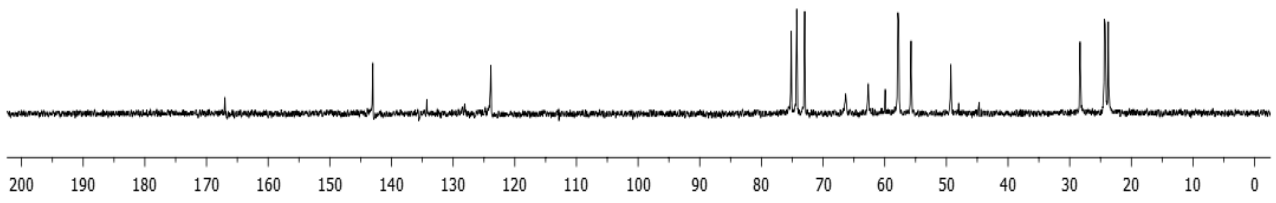


To a solution of **8** (169 mg, 0.65 mmol) in 7.5 ml of 2:1 THF/H_2O $CuSO_4$ (30 mol%, 3 mg, 0.02 mmol), sodium ascorbate (60 mol%, 8 mg, 0.04 mmol) and **9** (60 mg, 0.07 mmol) were added. The reaction mixture was stirred in microwave at 80 °C for 45 min, until a TLC analysis (EP:AcOEt 1:1) showed the disappearance of the starting material (R_f = 0.74) and the formation of a new product (R_f = 0.00). After filtration through Celite®, the solvent was removed under reduced pressure and the crude was purified by Flash Column Chromatography (MeOH:CH_2Cl_2:NH_4OH 2:1:0.5) and Size Exclusion Chromatography Sephadex LH-20 (eluting with H_2O) affording pure **10** (R_f = 0.88, 182 mg, 0.057 mmol, 81%) as a yellow oil. $[\alpha]_D^{28}$ = −4.2 (*c* = 0.9, H_2O); ^{1}H-NMR (400 MHz, D_2O): δ = 7.85 (s, 3H, H-Ar), 7.75 (s, 9H, H-Triazole), 4.43 (s, 18H, O*CH*$_2$Triazole), 4.14-4.13 (m, 9H, H-4), 4.10 (t, *J* = 7.1 Hz, 18H, H-12), 3.92-3.91 (m, 9H, H-3), 3.79 (dd, *J* = 12.4, 5.2 Hz, 9H, Ha-6), 3.73 (dd, *J* = 12.4, 7.6 Hz, 9H, Hb-6), 3.69 (s, 18H, C*CH*$_2$O), 3.44-3.41 (m, 9H, Ha-5), 3.27-3.15 (m, 27H, Hb-5, H-2 and Ha-7), 2.94-2.86 (m, 9H, Hb-7), 1.59 (quint, *J* = 7.2 Hz, 18H, H-11), 1.52-1.44 (m, 18H, H-8), 1.16-1.00 (m, 36H, H-9 and H-10); ^{13}C-NMR (50 MHz, D_2O): δ = 167.0 (s, 3C, C=O), 143.0 (s, 9C, C-Triazole), 134.2 (s, 3C, C-Ar), 128.5-

128.1 (d, 3C, C-Ar), 123.9 (d, 9C, C-Triazole), 75.2 (d, 9C, C-3), 74.3 (d, 9C, C-2), 73.0 (d, 9C, C-4), 66.3 (t, 9C, C*CH*$_2$O), 62.7 (t, 9C, O*CH*$_2$Triazole), 59.9 (s, 3C, *C*CH$_2$O), 57.9 (t, 9C, C-6), 57.7 (t, 9C, C-5), 55.7 (t, 9C, C-7), 49.3 (t, 9C, C-12), 28.3 (t, 9C, C-11), 24.4, 24.2 (t, 18C, C-9 and C-10), 23.7 (t, 9C, C-8); MS (ESI): m/z 1063.08 ([(M+3)/3]$^+$; 66); elemental analysis calcd (%) for $C_{147}H_{249}N_{39}O_{39}$ (3186.79): C 55.40, H 7.88, N 17.14; found: C 55.21, H 7.64, N 17.01.

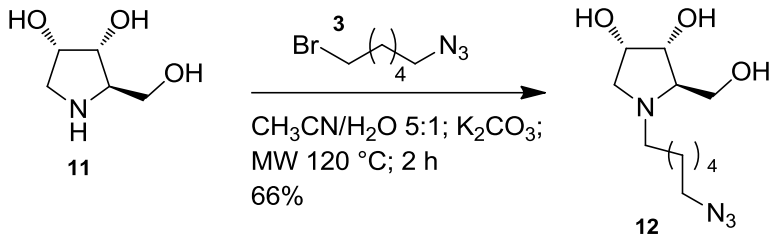


^{1}H-NMR spectrum of compound 10 (400 MHz, D_2O)

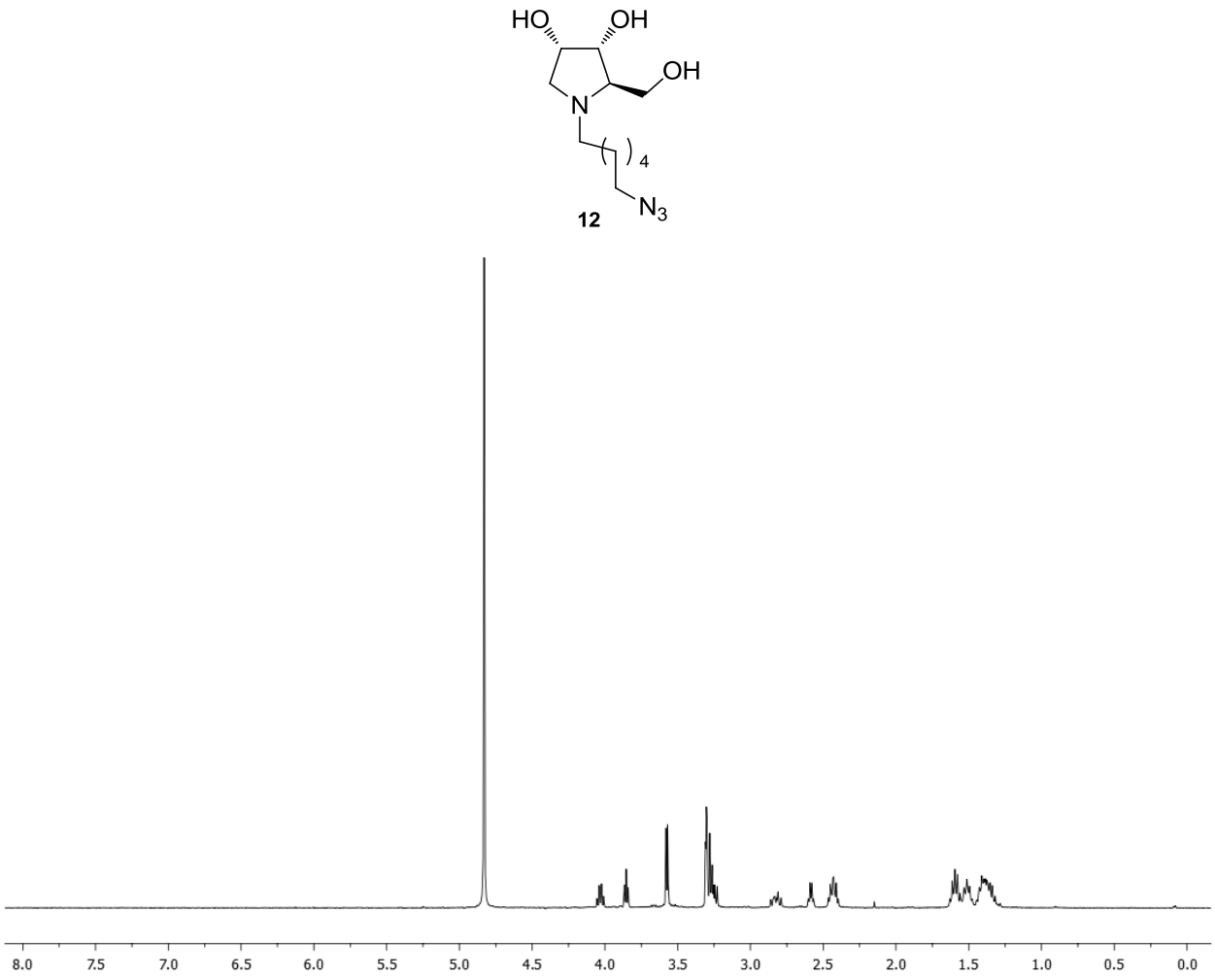


^{13}C-NMR spectrum of compound 10 (50 MHz, D_2O)

Synthesis of (2*R*,3*R*,4*S*)-1-[6-azido)hexyl]-3-hydroxymethylhexahydro-1*H*-pyrrolidine-1,2-diol

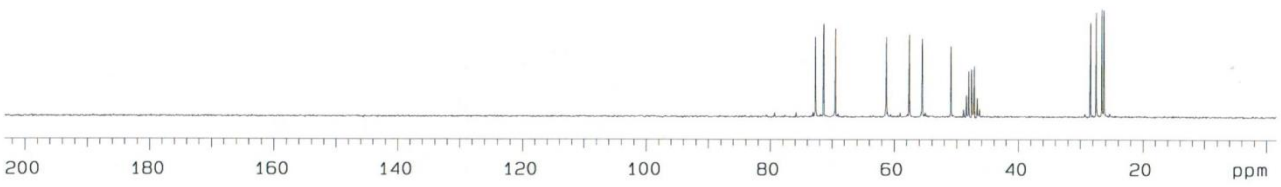


A solution of **11** (43 mg, 0.32 mmol), 1-azido-6-bromohexane (**3**, 98 mg, 0.48 mmol) and K_2CO_3 (66 mg, 0.48 mmol) in 3 ml of a mixture CH_3CN/H_2O 5:1 was stirred in microwave at 120°C for 2 h, until a TLC analysis (CH_2Cl_2:MeOH 2:1 + 1% v/v 6% NH_4OH) showed the disappearance of the starting material (R_f = 0.42) and the formation of a new product (R_f = 0.98). After filtration through Celite®, the solvent was removed under reduced pressure and the crude was purified by Flash Column Chromatography (CH_2Cl_2:MeOH 5:1 + 1% v/v 6% NH_4OH) affording pure **12** (R_f = 0.31, 53 mg, 0.21 mmol, 66%) as a yellow oil. $[\alpha]_D^{23}$ = −28.3 (*c* = 0.70, MeOH); ^{1}H-NMR (400 MHz, CD_3OD): δ = 4.06-4.00 (m, 1H, H-4), 3.85 (t, *J* = 5.1 Hz, 1H, H-3), 3.60-3.55 (m, 2H, H-6), 3.30-3.23 (m, 3H, H-12 and Ha-5), 2.83 (ddd, *J* = 11.9, 8.8, 7.3 Hz, 1H, Ha-7), 2.58 (q, *J* = 4.7 Hz, 1H, H-2), 2.46-2.40 (m, 2H, Hb-7 e Hb-5), 1.63-1.56 (m, 2H, H-11), 1.55-1.48 (m, 2H, H-8), 1.44-1.28 (m, 4H, H-9 e H-10); ^{13}C-NMR (50 MHz, CD_3OD): δ = 72.7 (d, C-3), 71.4 (d, C-2), 69.5 (d, C-4), 61.3 (t, C-6), 57.6 (t, C-5), 55.6 (t, C-7), 51.0 (t, C-12), 28.5 (t, C-11), 27.5 (t, C-8), 26.6, 26.3 (t, 2C, C-9 and C-10); MS (ESI): m/z: 259.17 ($[M+1]^+$; 100); elemental analysis calcd (%) for $C_{11}H_{22}N_4O_3$ (258.32): C 51.15, H 8.58, N 21.69; found: C 51.12, H 8.54, N 21.66.



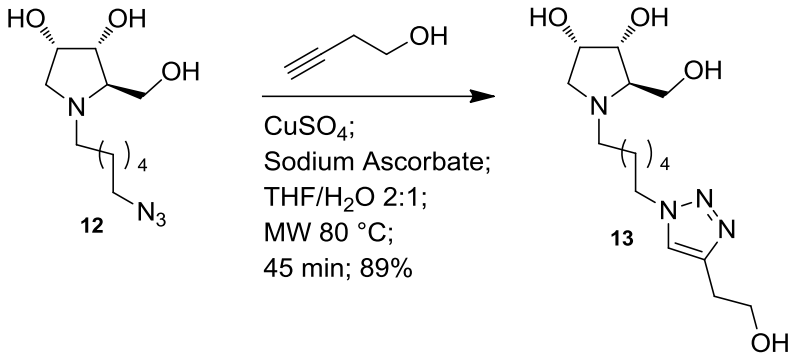
12

^{1}H-NMR spectrum of compound 12 (400 MHz, CD_3OD)

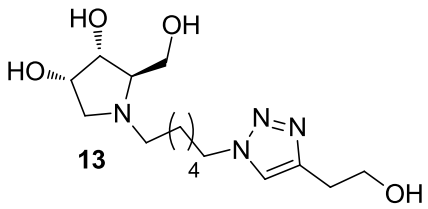


^{13}C-NMR spectrum of compound 12 (50 MHz, CD_3OD)

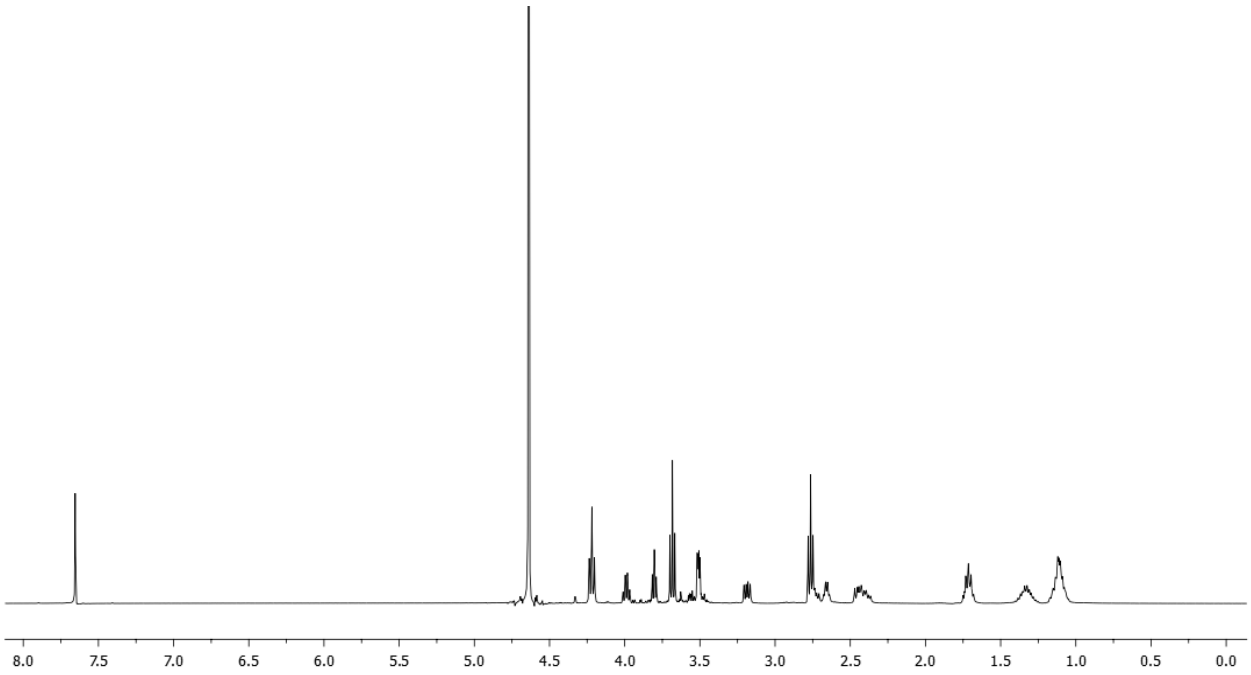
Synthesis of (2*R*, 3*R*, 4*S*)-1-hexyl[4-(hydroxyethyl)-1*H*-1,2,3-triazol-1-yl]-3,4-bis(hydroxy)-2-[(hydroxy)methyl]-pyrrolidine



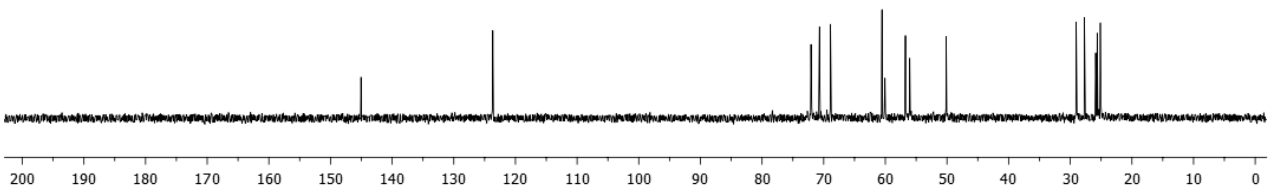
To a solution of **12** (64 mg, 0.22 mmol) in 6 ml of 2:1 THF/H_2O $CuSO_4$ (30 mol%, 11 mg, 0.07 mmol), sodium ascorbate (60 mol%, 26 mg, 0.13 mmol) and 3-butyn-1-ol (20 µL, 0.26 mmol) were added. The reaction mixture was stirred in microwave at 80 °C for 45 min, until a TLC analysis (CH_2Cl_2:MeOH 6:1 + 1% v/v 6% NH_4OH) showed the disappearance of the starting material (R_f = 0.22) and the formation of a new product (R_f = 0.00). After filtration through Celite®, the solvent was removed under reduced pressure and the crude was purified by Flash Column Chromatography (CH_2Cl_2:MeOH 6:1 + 1% v/v 6% NH_4OH) affording pure **13** (R_f = 0.13, 64 mg, 0.19 mmol, 89%) as a yellow oil. $[\alpha]_D^{22}$ = −10.0 (*c* = 0.67, MeOH); ^{1}H-NMR (400 MHz, D_2O): 7.65 (s, 1H, H-Triazole), 4.22 (t, *J* = 6.9 Hz, 2H, H-12), 3.99 (q, *J* = 5.8 Hz, 1H, H-4), 3.80 (t, *J* = 5.4 Hz, 1H, H-3), 3.68 (t, *J* = 6.4 Hz, 2H, *CH*$_2$OH), 3.58-3.45 (m, 2H, H-6), 3.19 (dd, *J* = 10.7, 5.9 Hz, 1H, Ha-5), 2.76 (t, *J* = 6.4 Hz, 2H, *CH*$_2$CH$_2$OH), 2.76-2.71 (m, 1H, Ha-7), 2.66 (q, *J* = 5.2 Hz, 1H, H-2), 2.45 (dd, *J* = 10.7, 6.8 Hz, 1H, Hb-5), 2.39 (td, *J* = 11.7, 5.4 Hz, 1H, Hb-7), 1.75-1.68 (m, 2H, H-11), 1.39-1.27 (m, 2H, H-8), 1.17-1.05 (m, 4H, H-9 and H-10); ^{13}C-NMR (100 MHz, D_2O): δ = 145.2 (s, C-Triazole), 123.8 (d, C-Triazole), 72.1 (d, C-3), 70.8 (d, C-2), 69.0 (d, C-4), 60.7, 59.9 (t, C-6 and *CH*$_2$OH), 56.9 (t, C-5), 56.2 (t, C-7), 50.2 (t, C-12), 29.1 (t, C-11), 27.8 (t, *CH*$_2$CH$_2$OH), 25.8, 25.7, 25.2 (t, 3C, C-8, C-9 and C-10); MS (ESI): m/z 329.40 ($[M+1]^+$; 32), 351.36 ($[M+Na]^+$; 100); elemental analysis calcd (%) for $C_{15}H_{28}N_4O_4$ (328.41): C 54.86, H 8.59, N 17.06; found: C 54.81, H 8.56, N 17.04.



13

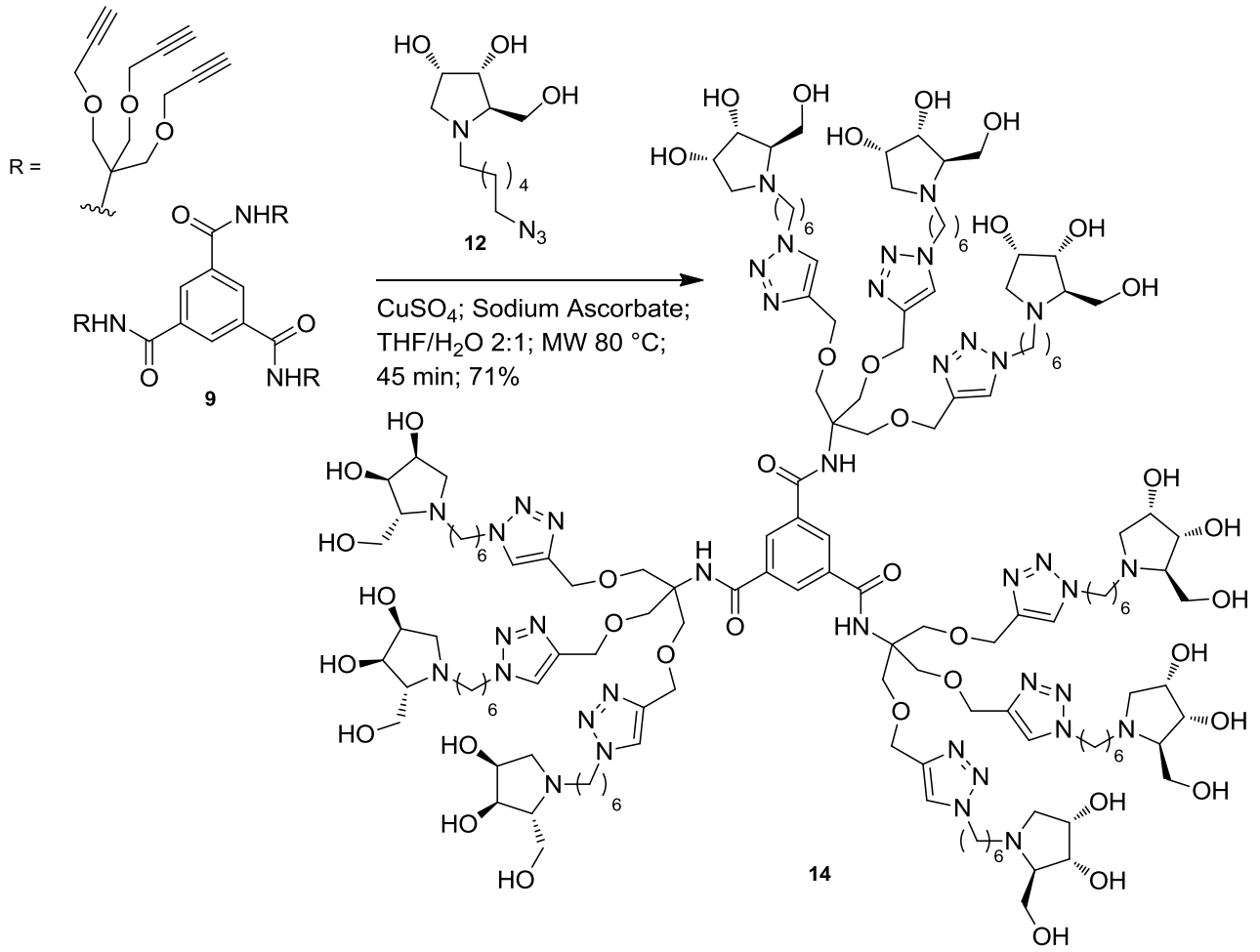


^{1}H-NMR spectrum of compound 13 (400 MHz, D_2O)



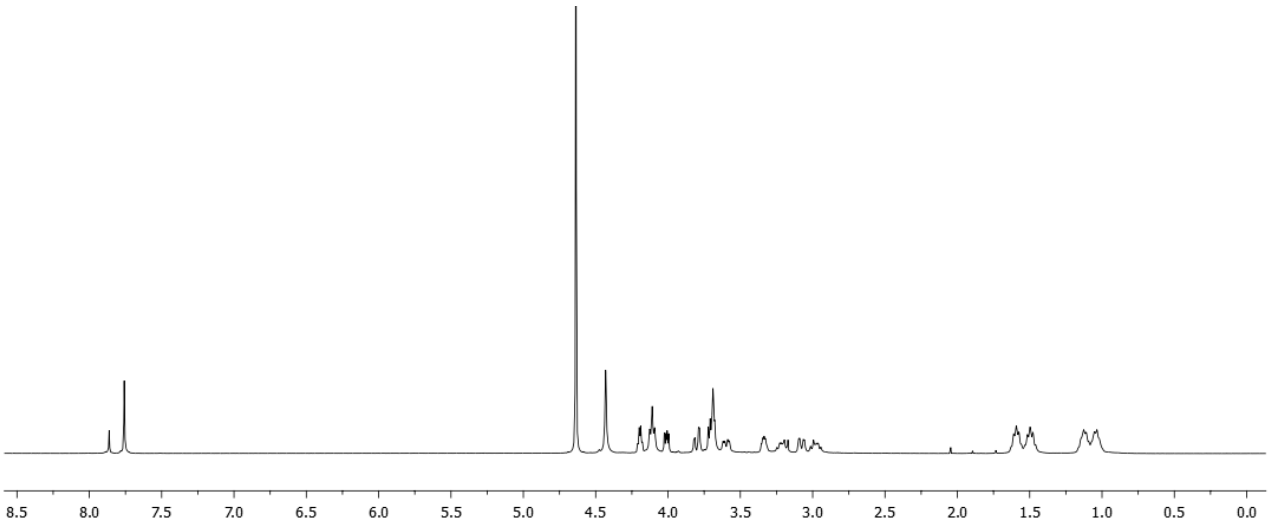
^{13}C-NMR spectrum of compound 13 (100 MHz, D_2O)

Synthesis of nonavalent pyrrolidine iminosugars 14

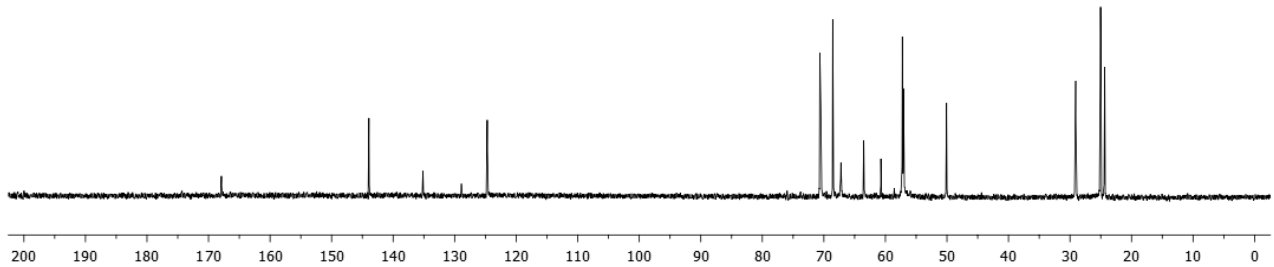


To a solution of **12** (41 mg, 0.158 mmol) in 3 ml of 2:1 THF/H_2O $CuSO_4$ (30 mol%, 1 mg, 0.005 mmol), sodium ascorbate (60 mol%, 2 mg, 0.010 mmol) and **9** (15 mg, 0.017 mmol) were added. The reaction mixture was stirred in microwave at 80 °C for 45 min, until a TLC analysis (EP/AcOEt 2:1) showed the disappearance of the starting material (R_f = 0.26) and the formation of a new product (R_f = 0.00). After filtration through Celite®, the solvent was removed under reduced pressure and the crude was purified by Flash Column Chromatography (MeOH:CH_2Cl_2:NH_4OH 2:1:0.75) and Size Exclusion Chromatography Sephadex LH-20 (eluting with H_2O) affording pure **14** (R_f = 0.26, 38 mg, 0.012 mmol, 71%) as a yellow oil. $[\alpha]_D^{23}$ = −2.2 (*c* = 0.75, H_2O); ^{1}H-NMR (400 MHz, D_2O): δ = 7.86 (s, 3H, H-Ar), 7.76 (s, 9H, H-Triazole), 4.43 (s, 18H, O*C*H_2Triazole), 4.19 (pseudo q, *J* = 4.1 Hz, 9H, H-4), 4.11 (t, *J* = 6.9 Hz, 18H, H-12), 4.01 (dd, *J* = 7.3, 4.4 Hz, 9H, H-3), 3.80 (dd, *J* = 13.1, 3.4 Hz, 9H, Ha-6), 3.70 (dd, *J* = 13.1, 4.9 Hz, 9H, Hb-6), 3.69 (s, 18H, C*C*H_2O), 3.60 (dd, *J* = 12.7, 4.4 Hz, 9H, Ha-5), 3.36-3.33 (m, 9H, H-2), 3.21 (dt, *J* = 12.2, 8.3 Hz, 9H, Ha-7), 3.08 (dd, *J* = 12.7, 2.9 Hz, 9H, Hb-5), 2.98 (dt, *J* = 12.2, 8.1 Hz, 9H, Hb-7), 1.64-1.56 (m,

18H, H-11), 1.56-1.44 (m, 18H, H-8), 1.18-0.98 (m, 36H, H-9 and H-10); ^{13}C-NMR (100 MHz, D_2O): δ = 167.9 (s, 3C, C=O), 143.9 (s, 9C, C-Triazole), 135.2 (s, 3C, C-Ar), 128.9 (d, 3C, C-Ar), 124.7 (d, 9C, C-Triazole), 70.7 (d, 9C, C-3), 70.5 (d, 9C, C-2), 68.6 (d, 9C, C-4), 67.2 (t, 9C, C*CH*$_2$O), 63.5 (t, 9C, O*CH*$_2$Triazole), 60.7 (s, 3C, *C*CH$_2$O), 57.2, 57.1, 57.0 (t, 27C, C-6, C-5 and C-7), 50.1 (t, 9C, C-12), 29.1 (t, 9C, C-11), 25.0, 24.9 (t, 18C, C-9 and C-10), 24.4 (t, 9C, C-8); MS (ESI): m/z 1070.58 ([(M+3)/3]$^+$; 81); elemental analysis calcd (%) for $C_{147}H_{249}N_{39}O_{39}$ (3186.79): C 55.40, H 7.88, N 17.14; found: C 55.29, H 7.74, N 17.02.



^{1}H-NMR spectrum of compound 14 (400 MHz, D_2O)



^{13}C-NMR spectrum of compound 14 (100 MHz, D_2O)

Biochemical tests on human GALNS and IDS

For nonavalent compounds **10** and **14** and their monovalent counterparts **6** and **13** the percentage of inhibition, at 1 mM concentration, 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.[1]

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 (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.[2]

IC_{50} determination[3]

The IC_{50} values against the GALNS and IDS inhibitors were determined by measuring the initial hydrolysis rate under fixed 4-methylumbelliferyl-ß-galactoside-6-sulphate·Na concentration (6.66 mM). and 4-methylumbelliferyl-α-L-Iduronide-2-sulphate·2Na

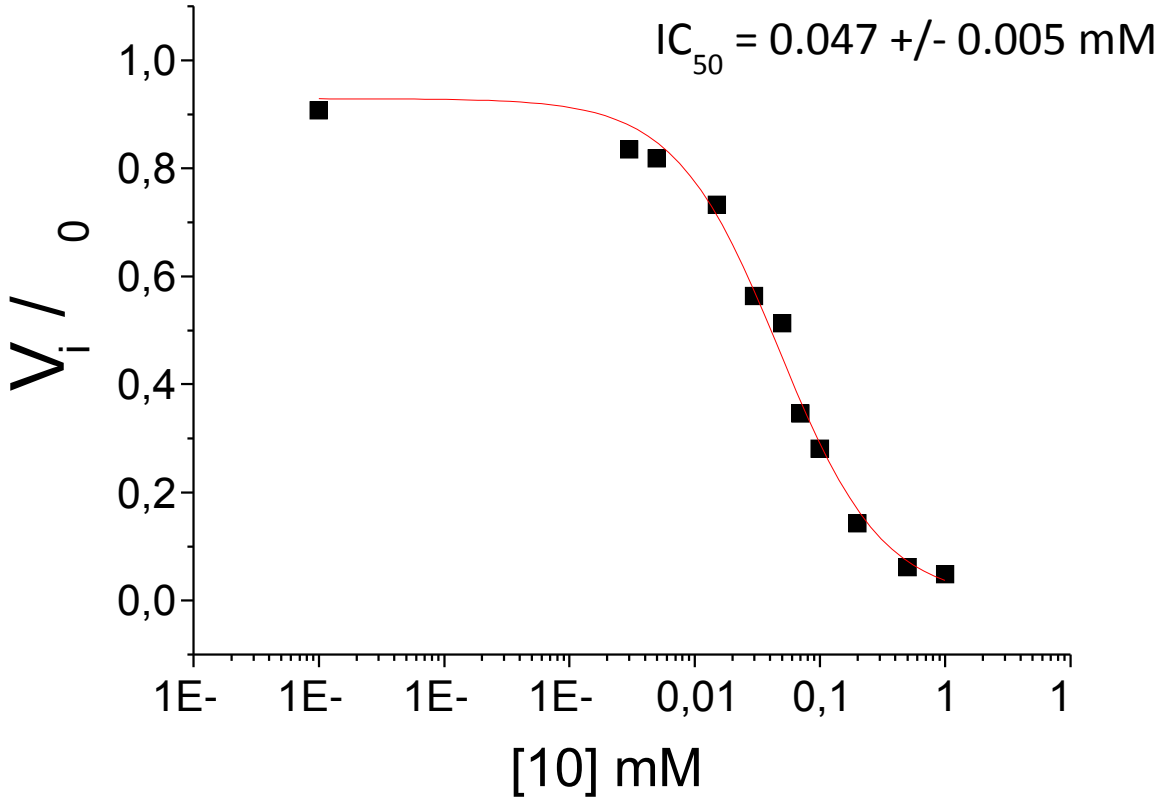
[1] O. P. Van Diggelen, H. Zhao, W. J. Kleijer, H. C. Janse, B. J. H. M. Poorthuis, J. Van Pelt, J. P. Kamerling and H. Galjaard, *Clinica Chimica Acta*, 1990, **187**, 131.
[2] Y. V. Voznyi, J. L. Keulemans and O. P. van Diggelen, *J. Inher. Metabol. Dis.*, **2001**, 24, 675.
[3] R. Ottanà, R. Maccari, J. Mortier, A. Caselli, S. Amuso, G. Camici, A. Rotondo, G. Wolber and P. Paoli, *Eur. J. Med. Chem.*, 2014, **71**, 112.

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 Vi/Vo, represents the ratio between the activity measured in the presence of the inhibitor (V_i) 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.

GALNS



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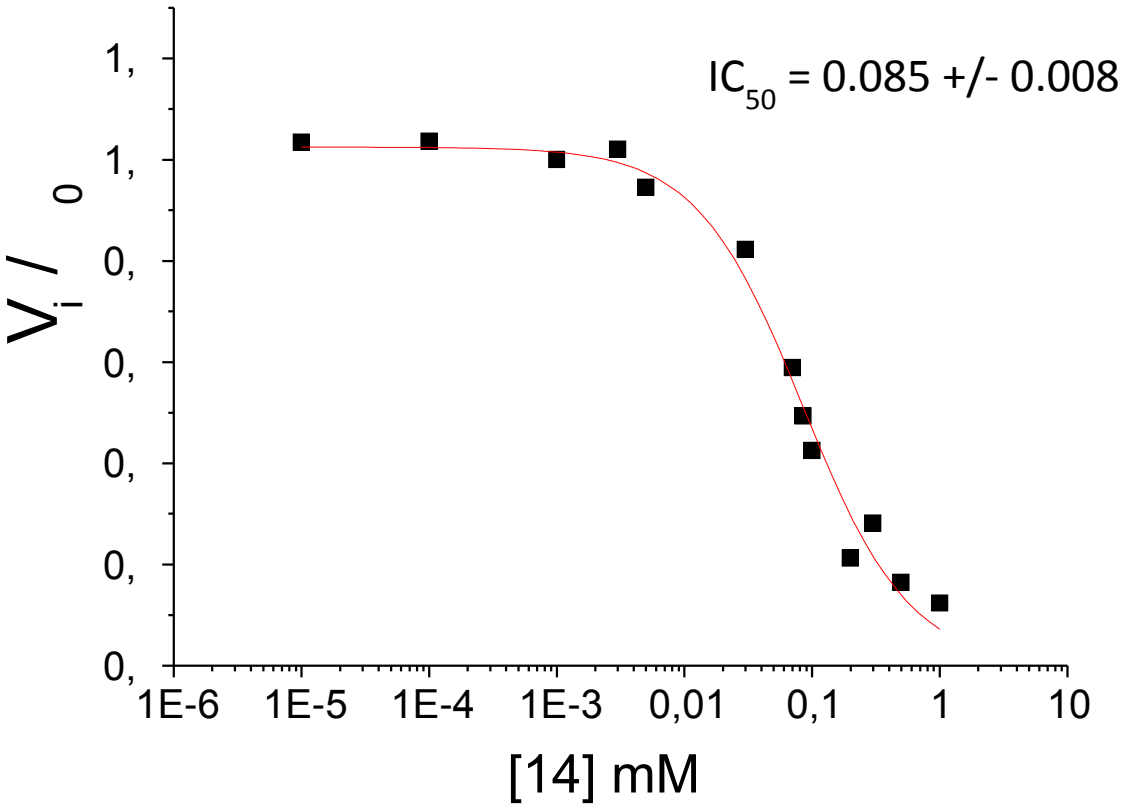

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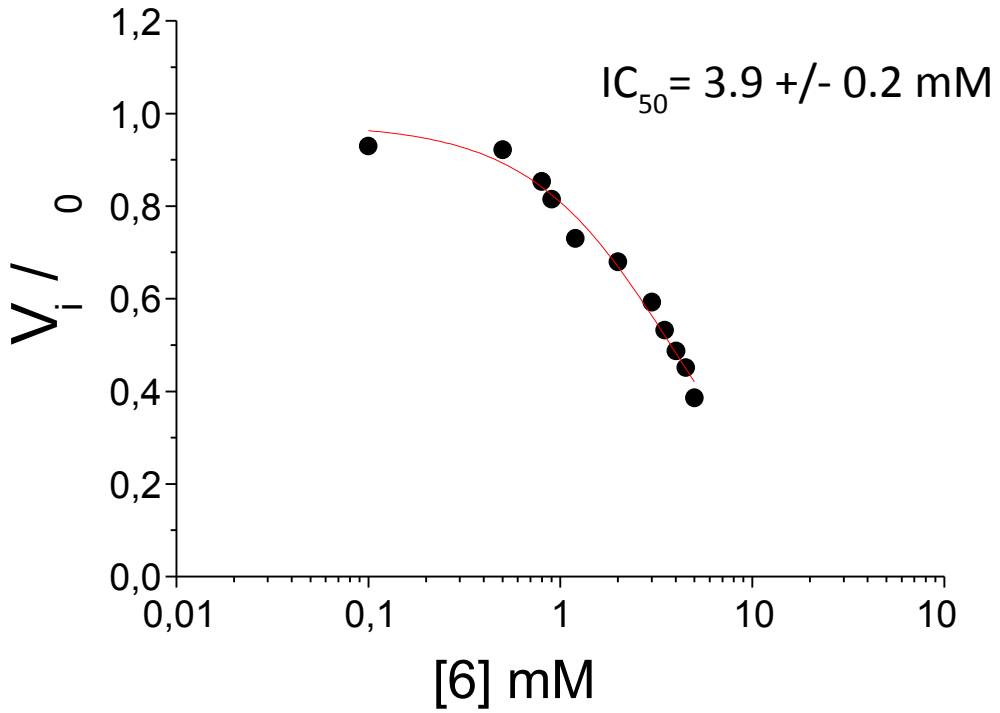

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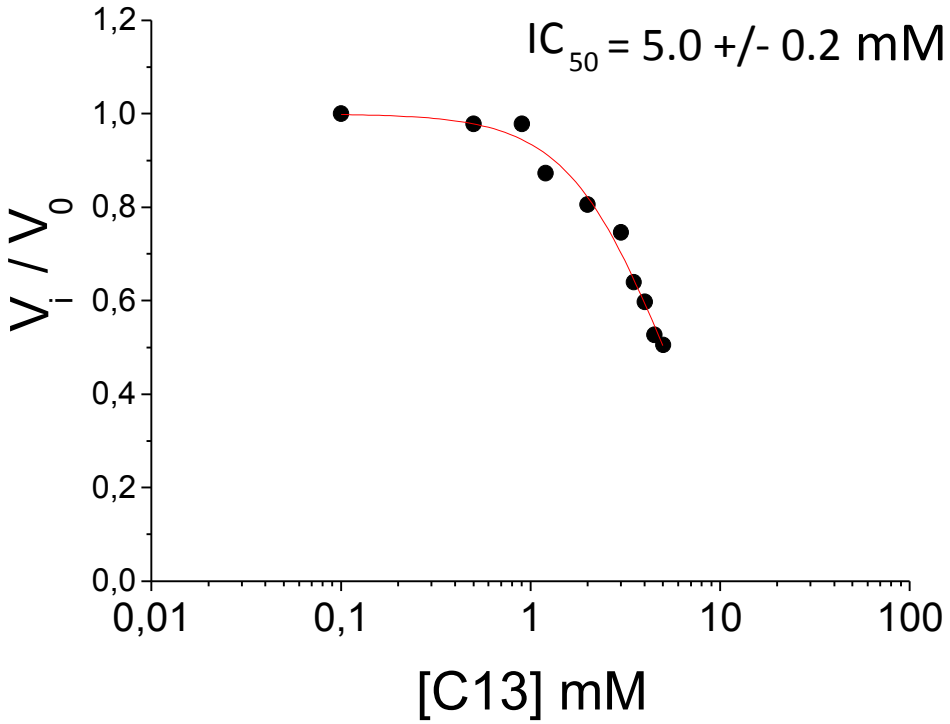
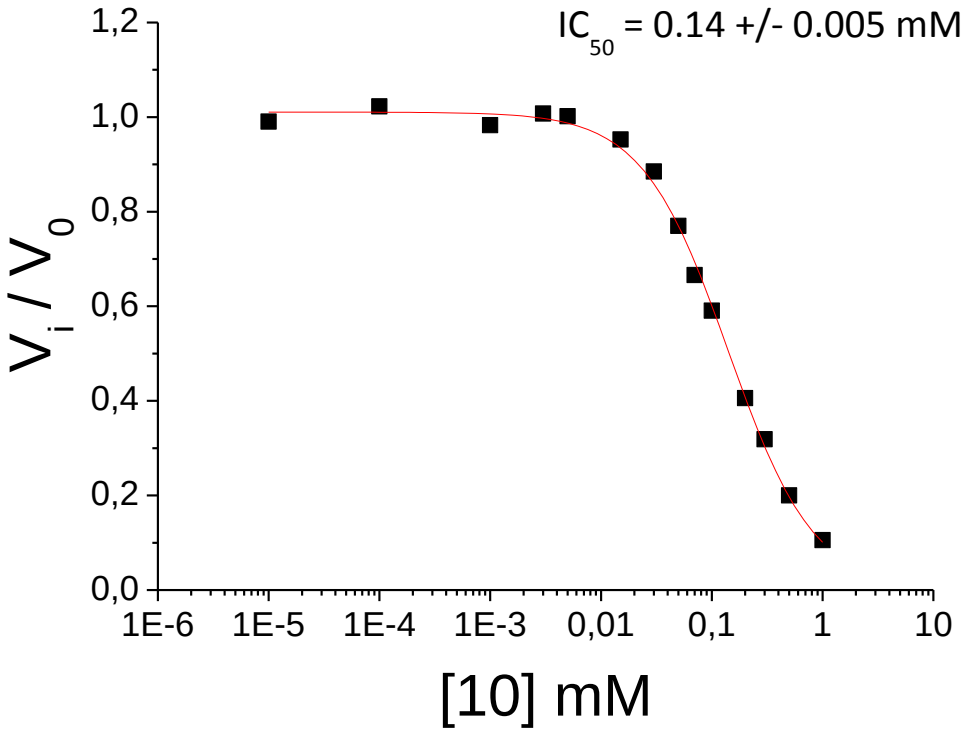


IDS


IC50 = 0.14 +/- 0.005 mM
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0,6
0,4
0,2
0,0
Vi / V0
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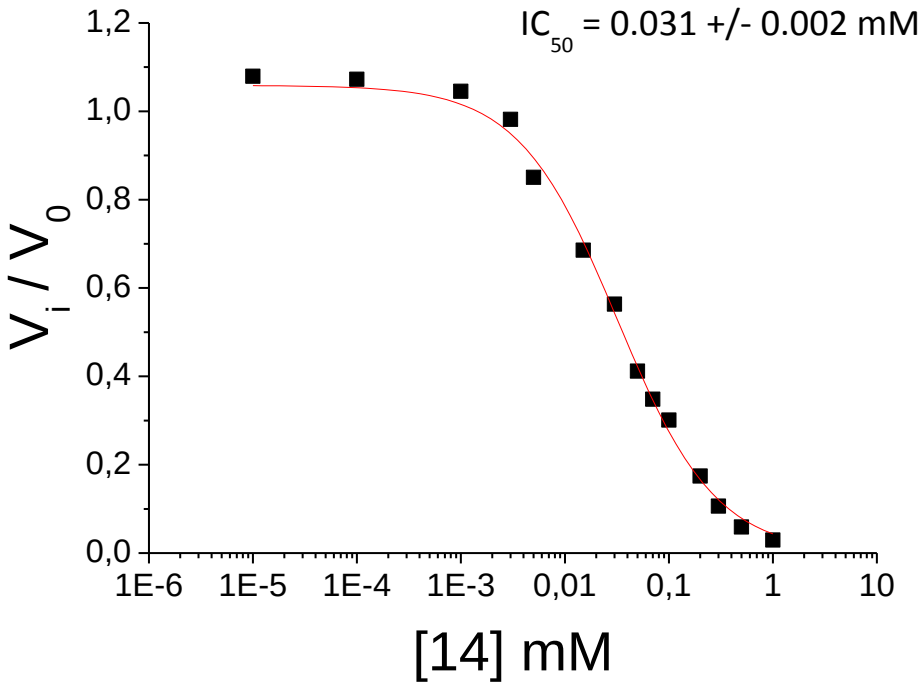
1E-5
1E-4
1E-3
0,01
0,1
1
10
[10] mM

IDS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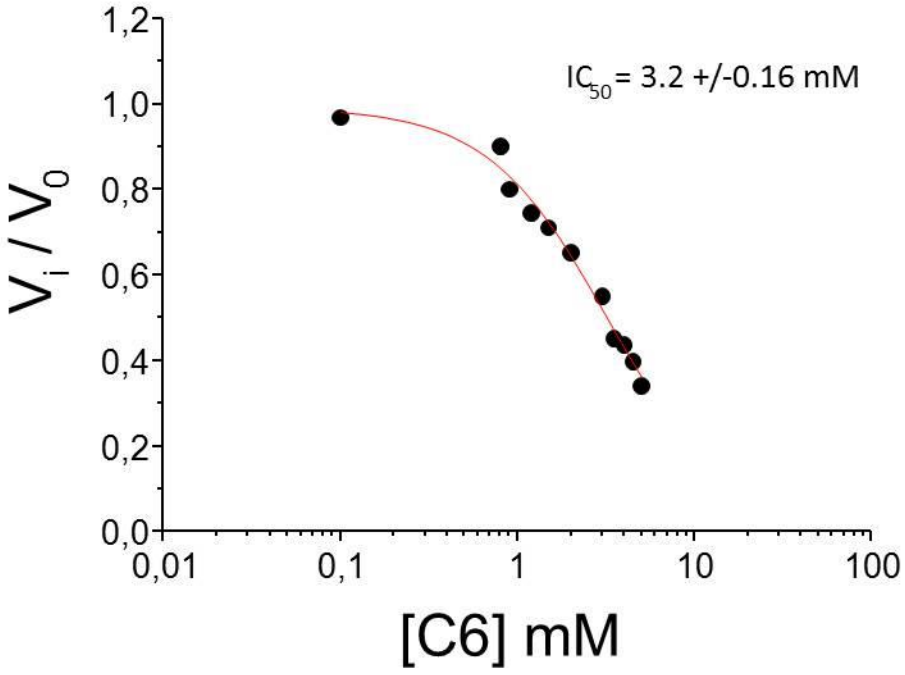
0,01
0,1
1
10
[14] mM

IDS



IDS