Electronic Supplementary Information for

Synthesis, Physico-Chemical and Biological Properties of Amphiphilic Amino Acid Conjugates of Nitroxides

Grégory Durand,[a,] Fanny Choteau,[a] Robert A. Prosak,[b] Antal Rockenbauer,[c] Frederick A. Villamena[b,d]* and Bernard Pucci[a]*

[a]Laboratoire de Chimie BioOrganique et des Systèmes Moléculaires Vectoriels, Faculté des Sciences, Université d'Avignon et des Pays de Vaucluse, 33 Rue Louis Pasteur, 84000 Avignon, France

[b]Department of Pharmacology, [d]Center for Biomedical EPR Spectroscopy and Imaging, The Davis Heart and Lung Research Institute, College of Medicine, The Ohio State University, Columbus, OH 43210

[c]Chemical Research Center, Institute of Structural Chemistry, H-1025 Budapest, Pusztaszeri 59, Hungary

gregory.durand@univ-avignon.fr frederick.villamena@osumc.edu

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Material, general procedure and instrumentation for the synthesis

Synthesis. All reagents were used as purchased from Sigma-Aldrich or Acros except for amino acids which were purchased from Iris Biotech. and 1*H*,1*H*,2*H*,2*H*-perfluorooctyl iodide which was from Elf Atochem and were used without further purification. All solvents were distilled and dried according to standard procedures. TLC analyses were performed on sheets precoated with silica gel 60F254 (Merck). Compound detection was achieved either by exposure to ultraviolet light (254 nm), by spraying with a 5% sulphuric acid solution in ethanol or 2% ninhydrin solution in ethanol and then heating at ~150°C. Flash chromatography purifications were carried out on Merck silica gel Gerduran Si 60 (40-63 µm). Size exclusion chromatography purifications were carried out on Sephadex LH20 resin (Amersham Biosciences). UV spectra were recorded on a Varian CARY 100. Melting points were measured on an Electrothermal IA9100 apparatus and have not been corrected. Optical rotations were measured at 25°C on a Perkin Elemer MC 241 polarimeter. The ^{1}H, ^{13}C, ^{19}F and DEPT NMR sequences were recorded on a Brüker AC-250 spectrometer and were performed at 250, 62.86 and 235 MHz for ^{1}H, ^{13}C and ^{19}F experiments, respectively. Chemical shifts are given in ppm relative to the solvent residual peak as a heteronuclear reference for ^{1}H and ^{13}C. Abbreviations used for signal patterns are: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublet. Mass spectra were recorded on a Qstar Elite (Applied Biosystems SCIEX) for MS ESI+ experiments and on a 3200 Qtrap (Applied Biosystems SCIEX) for HR-MS ESI+ experiments.

Experimental procedures for 10, 11, 13 and 14

***N*-(2,3,4,6,2',3',4',6'-O-acetyl-lactobionyl)-*β*-(4-amidoTEMPO)-L-aspartyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (10).** At 0°C, compound **9**[23] (0.3 g, 2.48 10^{-4} mol) was dissolved in a TFA/CH_2Cl_2 4:6 (v/v) mixture. After 2 h of being stirred, the solution was concentrated under vacuum to give the corresponding acid derivative as a white powder (0.252 g, 2.18 10^{-4} mol, 88%). The acid derivative (0.240 g, 2.08 10^{-4} mol), EDC (0.075 g, 3.91 10^{-4} mol), 4-AT (0.024 g, 4.24 10^{-4} mol) and a catalytic amount of HOBt were dissolved in dry CH_2Cl_2 with DIEA (pH = 8-9) under argon. The mixture was stirred for 28 h at room temperature and then the solvent was evaporated under vacuum. The crude mixture was purified by flash chromatography (EtOAc/cyclohexane 7:3 v/v) followed by size exclusion chromatography (CH_2Cl_2/MeOH 1:1 v/v) to give compound **10** (0.162 g, 1.24 10^{-4} mol, 60%) as an orange powder. R_f 0.56 (EtOAc). mp 66-67°C (decomposition). $\lambda_{max}(CH_2Cl_2)$/nm 229. MS (ESI+, m/z) 1308.4 [$(M+H)^+$], 1325.4 [$(M+NH_4)^+$], 1330.4 [$(M+Na)^+$], 1346.4 [$(M+K)^+$].

***N*-Lactobionyl-*β*-(4-amidoTEMPO)-L-aspartyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (11).** Compound **10** (0.150 g, 1.15 10^{-4} mol) was dissolved under argon in MeOH and a catalytic amount of sodium methoxide was added. The mixture was stirred for 4 h and HCl 1N was added dropwise to neutralize the solution. The solvent was evaporated under vacuum and the crude mixture was purified by size exclusion chromatography (MeOH) to give compound **11** (0.073 g, 9.89 10^{-5} mol, 86%) as an orange powder. R_f 0.55

(EtOAc/MeOH/H_2O 7:2:1 v/v/v). mp 78-79°C (decomposition). $[\alpha]^{D}_{25}$ +18.2 (*c* 0.l in MeOH). λ_{max}(MeOH)/nm 229. HR-MS (ESI+, m/z) calcd for $C_{33}H_{49}N_4O_{14}F_{13}$ $[(M+H)^+]$: 972.3032, found 972.3030.

***N*-(2,3,4,6,2',3',4',6'-O-acetyl-lactobionyl)-*N*$^{\varepsilon}$-(carboxyproxyl)-L-Lysinyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (13).** At 0°C, compound **12**[23] (0.3 g, 2.30 10^{-4} mol) was dissolved in ethanol/acetic acid 99:1 (v/v) and 0.018 g of 10% Pd/C was portion wise added under stirring. The reaction mixture was submitted to a hydrogen atmosphere for 12 h (8 bars). After filtration of the catalyst through a pad of Celite, the solvent was evaporated under vacuum to give the corresponding amino derivative (0.263 g, 2.26 10^{-4} mol). The resulting amino (0.250 g, 2.14 10^{-4} mol), EDC (0.049 g, 2.57 10^{-3} mol), 3-CP (0.048 g, 2.57 10^{-3} mol) and a catalytic amount of HOBT were dissolved in dry CH_2Cl_2 with DIEA (pH = 8-9) under argon. The mixture was stirred for 18 h at room temperature and the solvent was evaporated under vacuum. The crude mixture was purified by flash chromatography (EtOAc) and by size exclusion chromatography (CH_2Cl_2/MeOH 1:1 v/v) to give compound **13** (0.082 g, 6.14 10^{-5} mol, 28%) as a yellow powder. R_f 0.58 (EtOAc). mp 82.6-83.8°C (decomposition). $[\alpha]^{D}_{25}$ +3.6 (*c* l in CH_2Cl_2). λ_{max}(CH_2Cl_2)/nm 203. MS (ESI+, m/z) 1336.5 $[(M+H)^+]$, 1353.6 $[(M+NH_4)^+]$, 1358.5 $[(M+Na)^+]$, 1374.5 $[(M+K)^+]$, 1334.5 $[(M-H)^-]$

***N*-lactobionyl-*N*$^{\varepsilon}$-(carboxyproxyl)-L-Lysinyl-1*H*,1*H*,2*H*,2*H*-perfluorooctylamide (14).** Compound **13** (0.070g, 5.24 10^{-5} mol) was dissolved under argon in MeOH and a catalytic amount of sodium methoxide was added. The mixture was stirred for 4 h and HCl 1N was added dropwise to neutralize the solution. The solvent was evaporated under vacuum and the crude mixture was purified by size exclusion chromatography (MeOH), to give the compound **14** (0.046g, 4.6 10^{-5} mol, 88%) as a yellow powder. R_f 0.51 (EtOAc/MeOH/H_2O 7:2:1 v/v/v). mp 108.5-109.5°C (decomposition). $[\alpha]^{D}_{25}$ +11.3 (*c* 0.1 in MeOH). λ_{max}(MeOH)/nm 203. HR-MS (ESI+, m/z) calcd for $C_{35}H_{53}N_4O_{14}F_{13}$•$[(M+H)^+]$: 1000.3345, found 1000.3353; calcd for $C_{35}H_{56}N_5O_{14}F_{13}$•$[(M+NH_4)^+]$: 1017.3611, found 1017.3621.

Material and general procedure for cell culturing

All reagents or materials were purchased and used without further purification. Bovine aortic endothelial cells (BAEC) were purchased from Cell Systems (Kirkland, WA). Cells were cultured in 75 cm^2 tissue culture flasks using Dulbecco's modified eagle medium with 4.5 g/L D-glucose and supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 2.5 mg/L endothelial cell growth supplement, and 1% non-essential amino acids (Gibco) in the absence of antibiotics at 37°C in a humidified atmosphere of 5% CO_2 and 20% O_2. The medium was changed every 2-3 days and cells were sub-cultured once they reached 90–95% confluence.

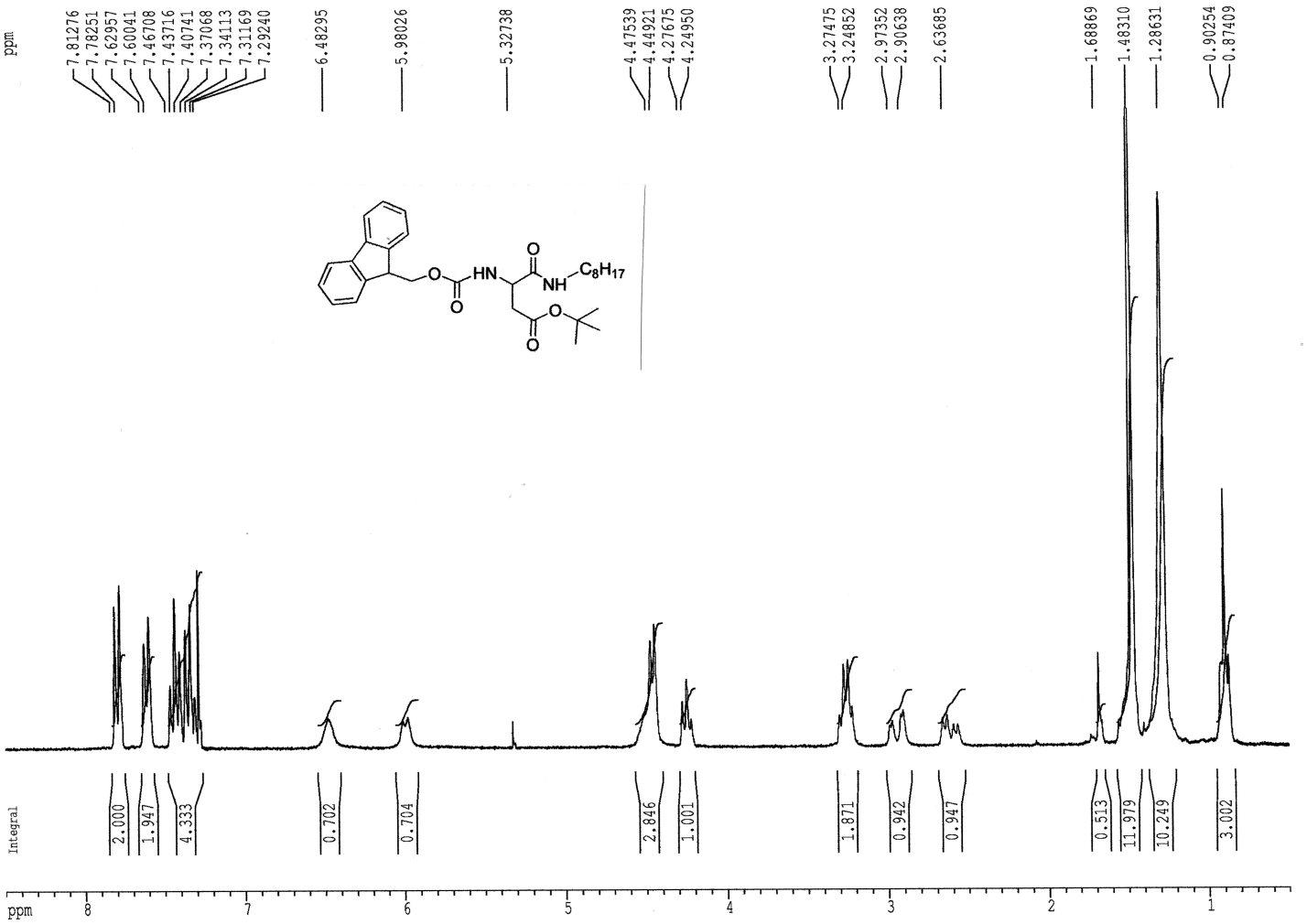

Figure S1. ^{1}H NMR Spectrum of **1** in $CDCl_3$

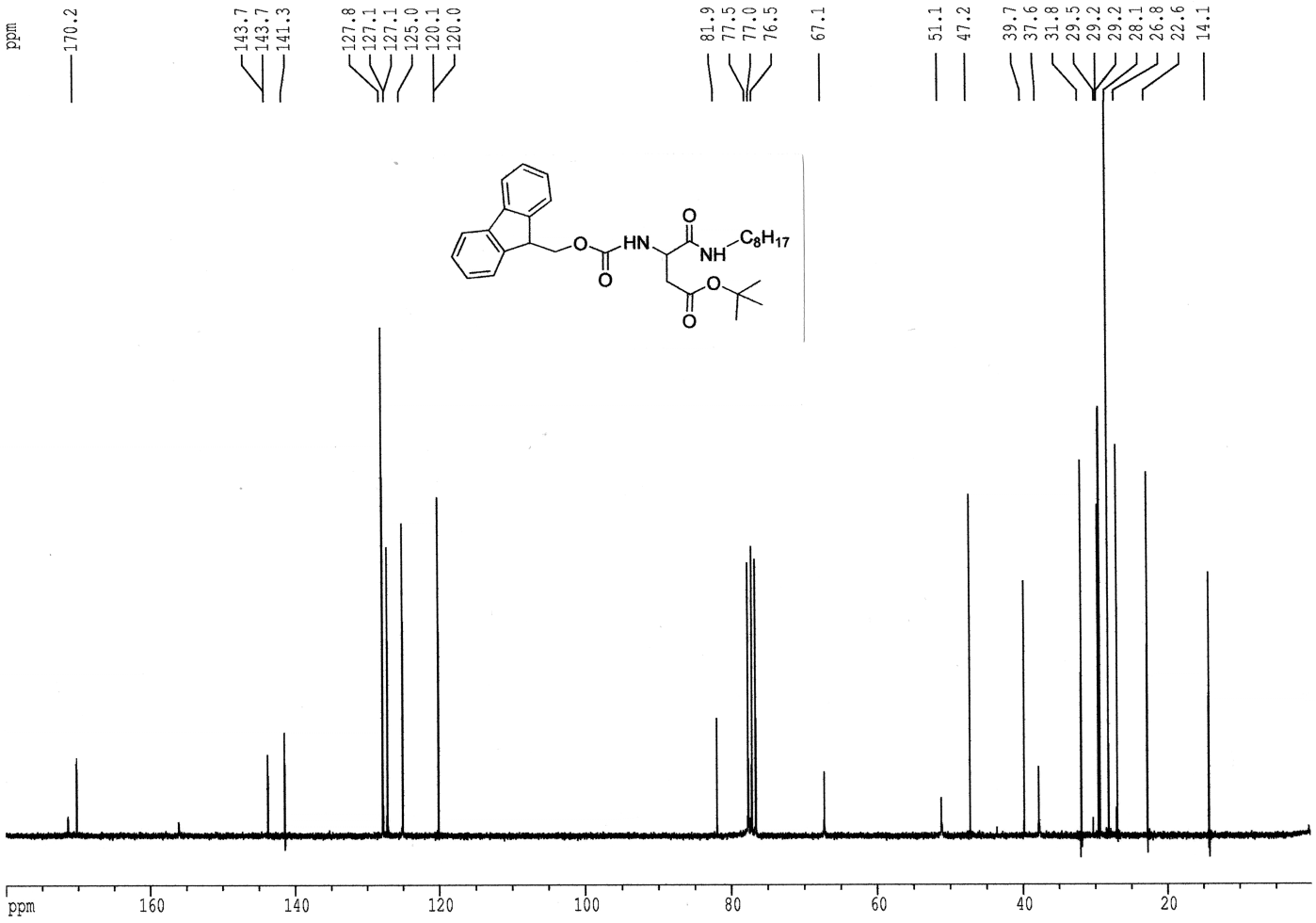

Figure S2. ^{13}C NMR spectrum of **1** in $CDCl_3$

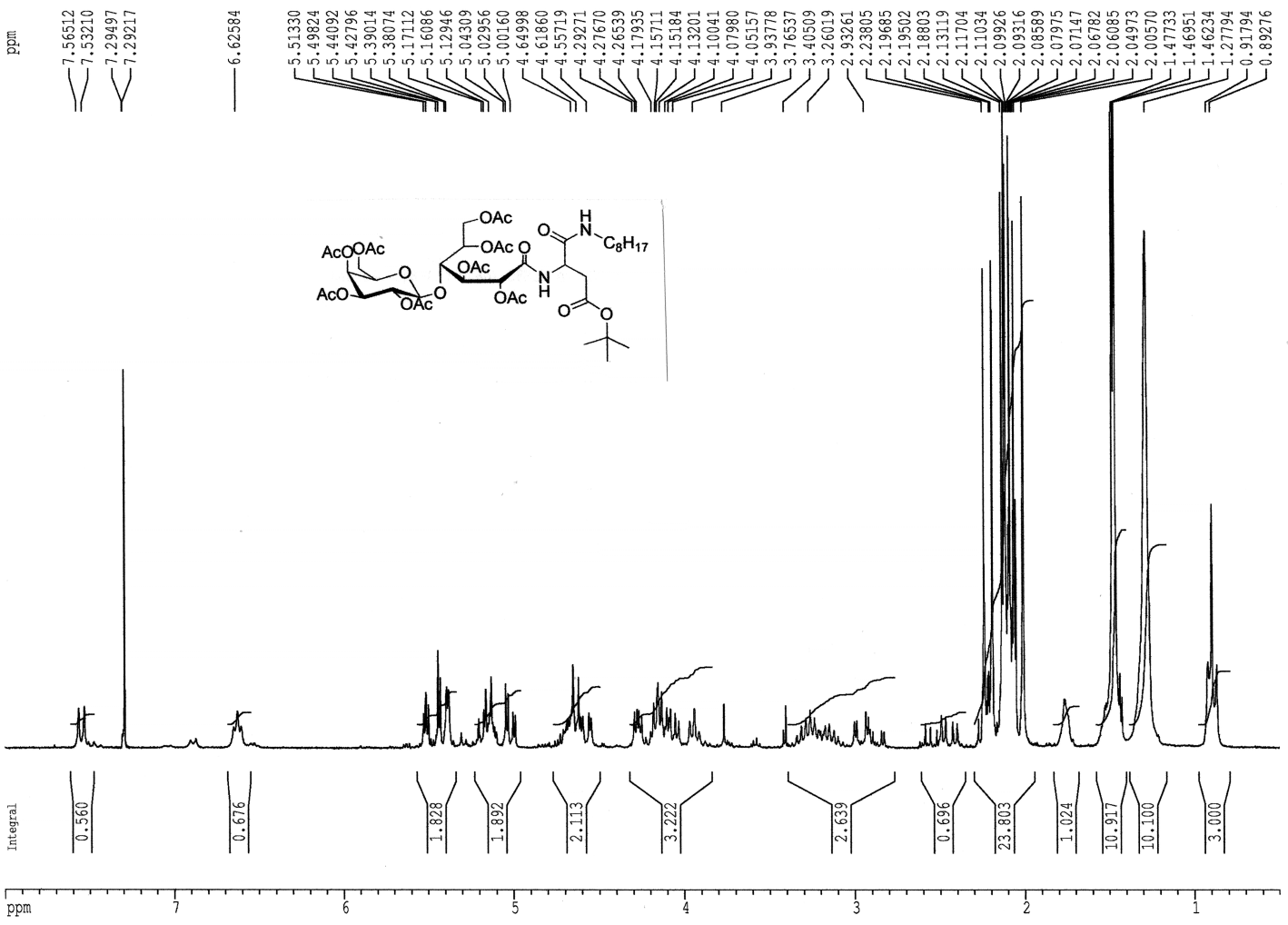

Figure S3. ^{1}H NMR spectrum of LAH (**2**) in $CDCl_3$

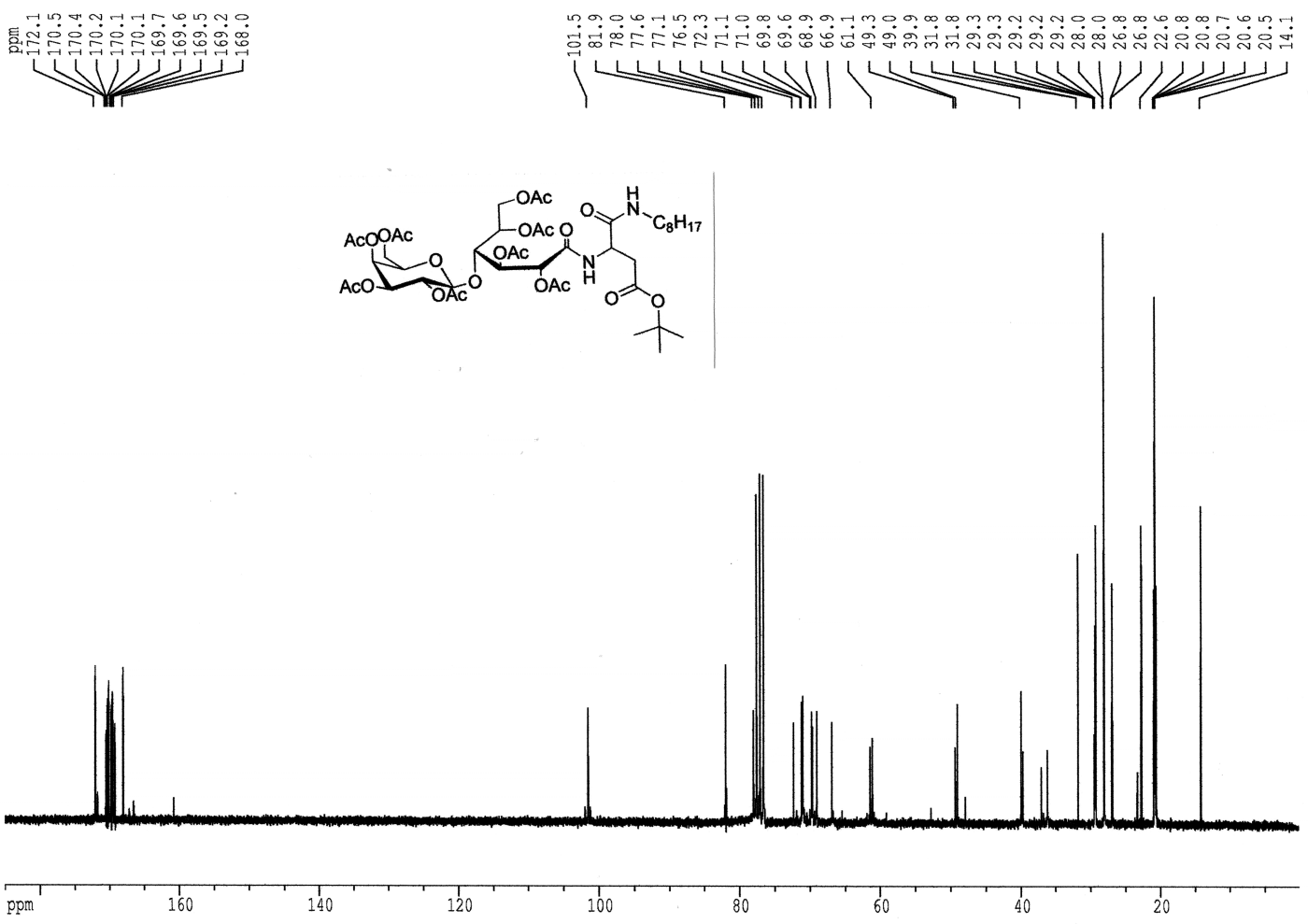

Figure S4. ^{13}C NMR spectrum of LAH (**2**) in $CDCl_3$

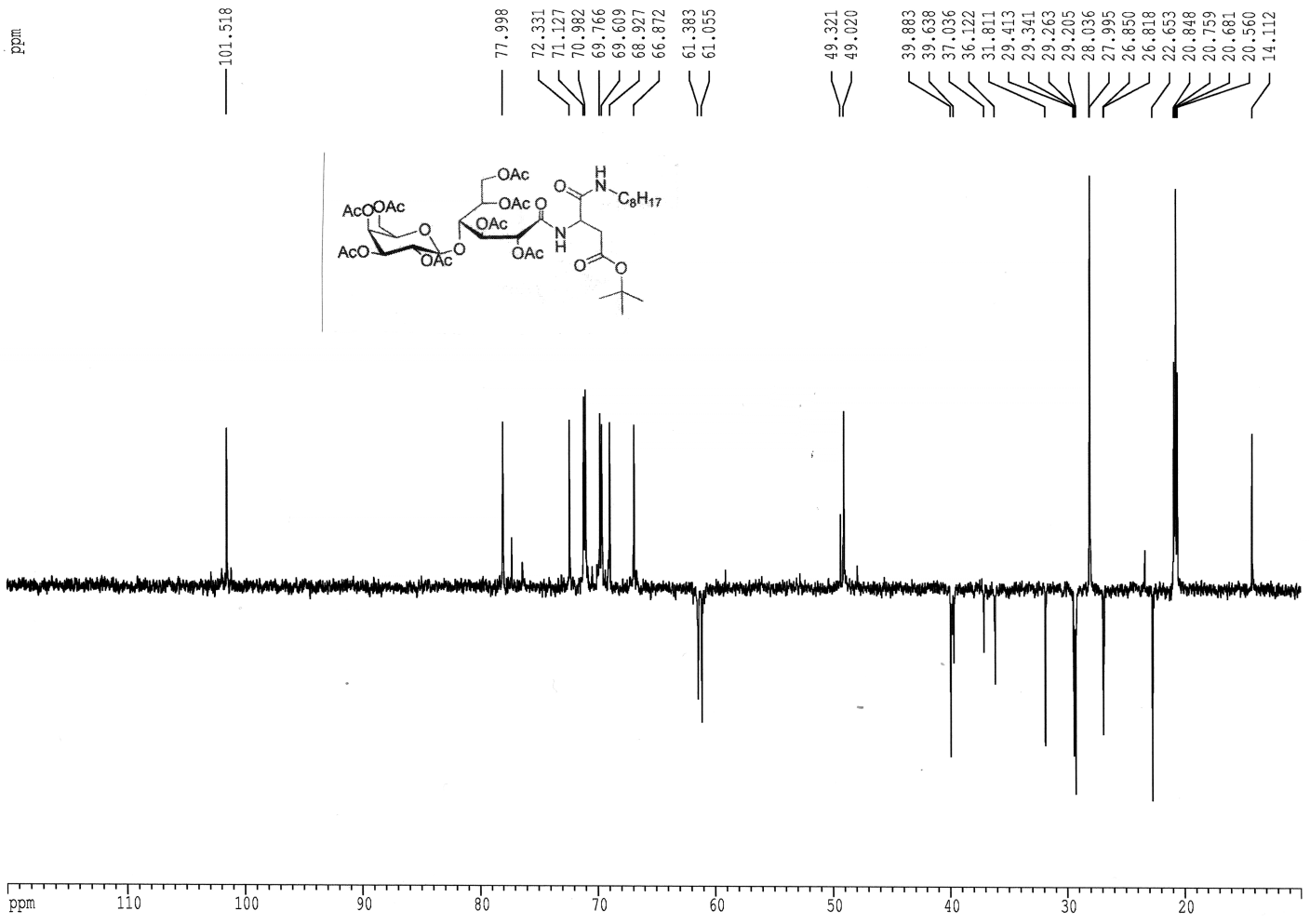

Figure S5. DEPT 135 spectrum of LAH (**2**) in $CDCl_3$

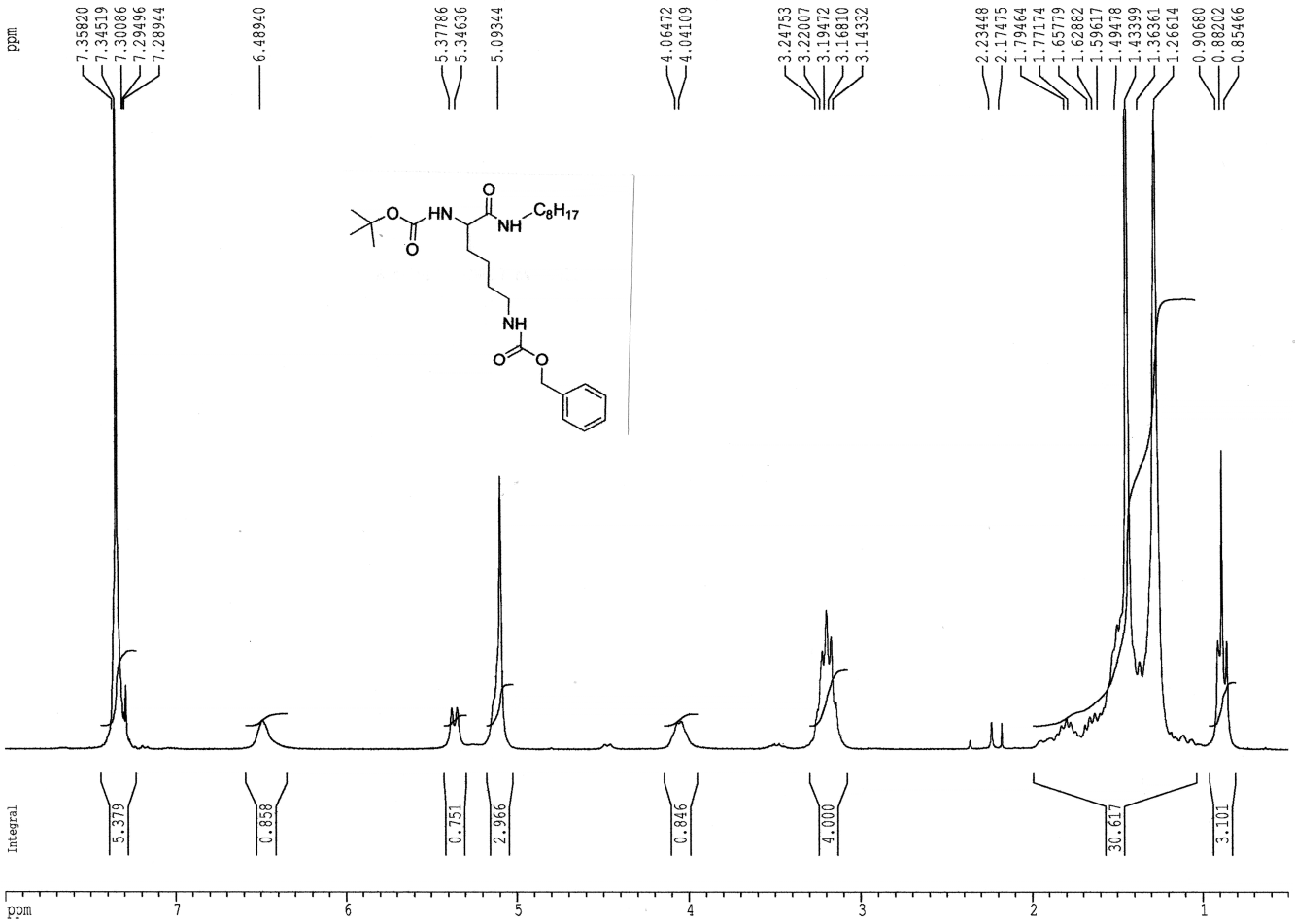

Figure S6. ^{1}H NMR spectrum of **5** in $CDCl_3$

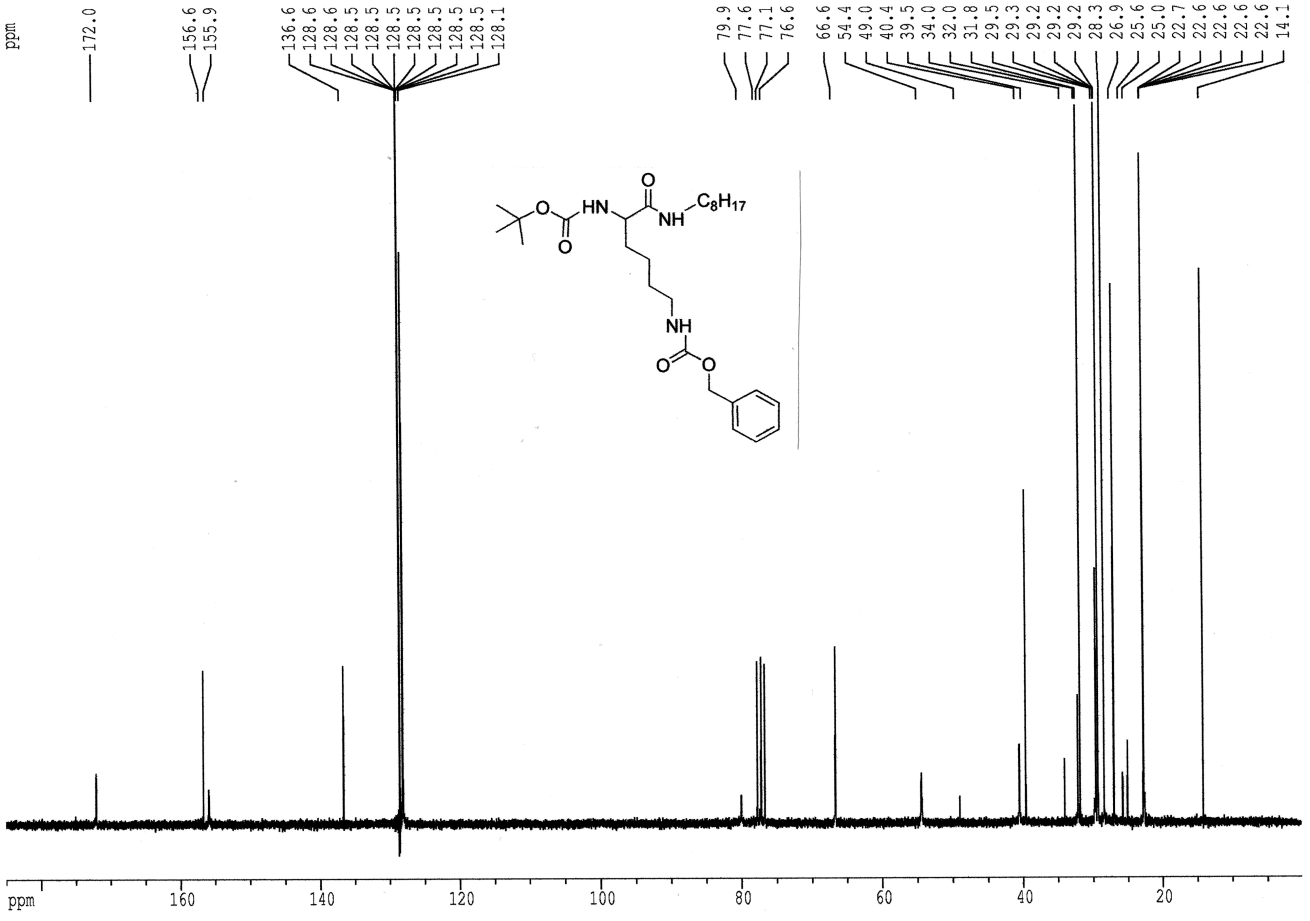

Figure S7. ^{13}C NMR spectrum of **5** in $CDCl_3$

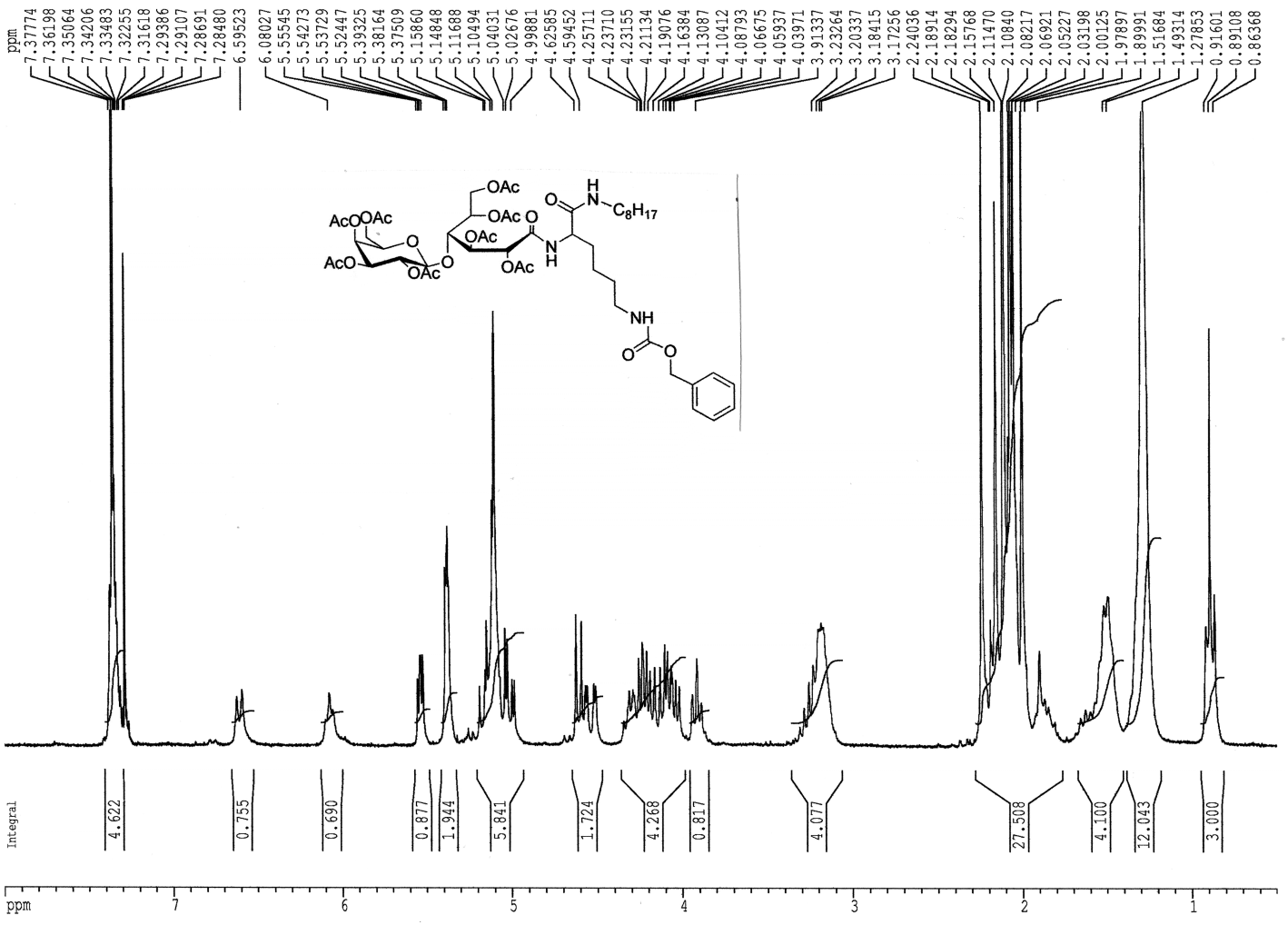

Figure S8. ^{1}H NMR spectrum of LLH (**6**) in $CDCl_3$

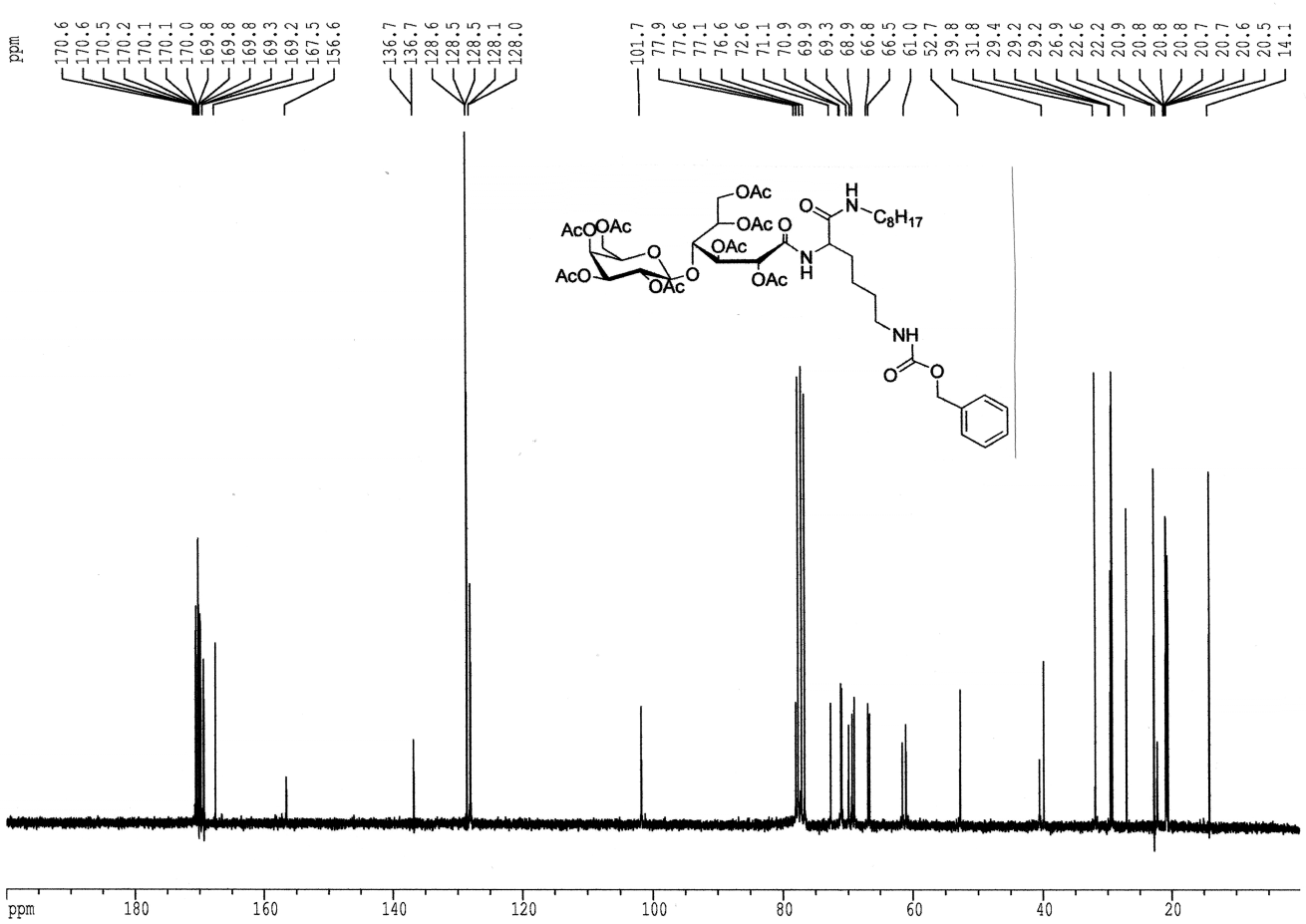

Figure S9. ^{13}C NMR spectrum of LLH (**6**) in $CDCl_3$

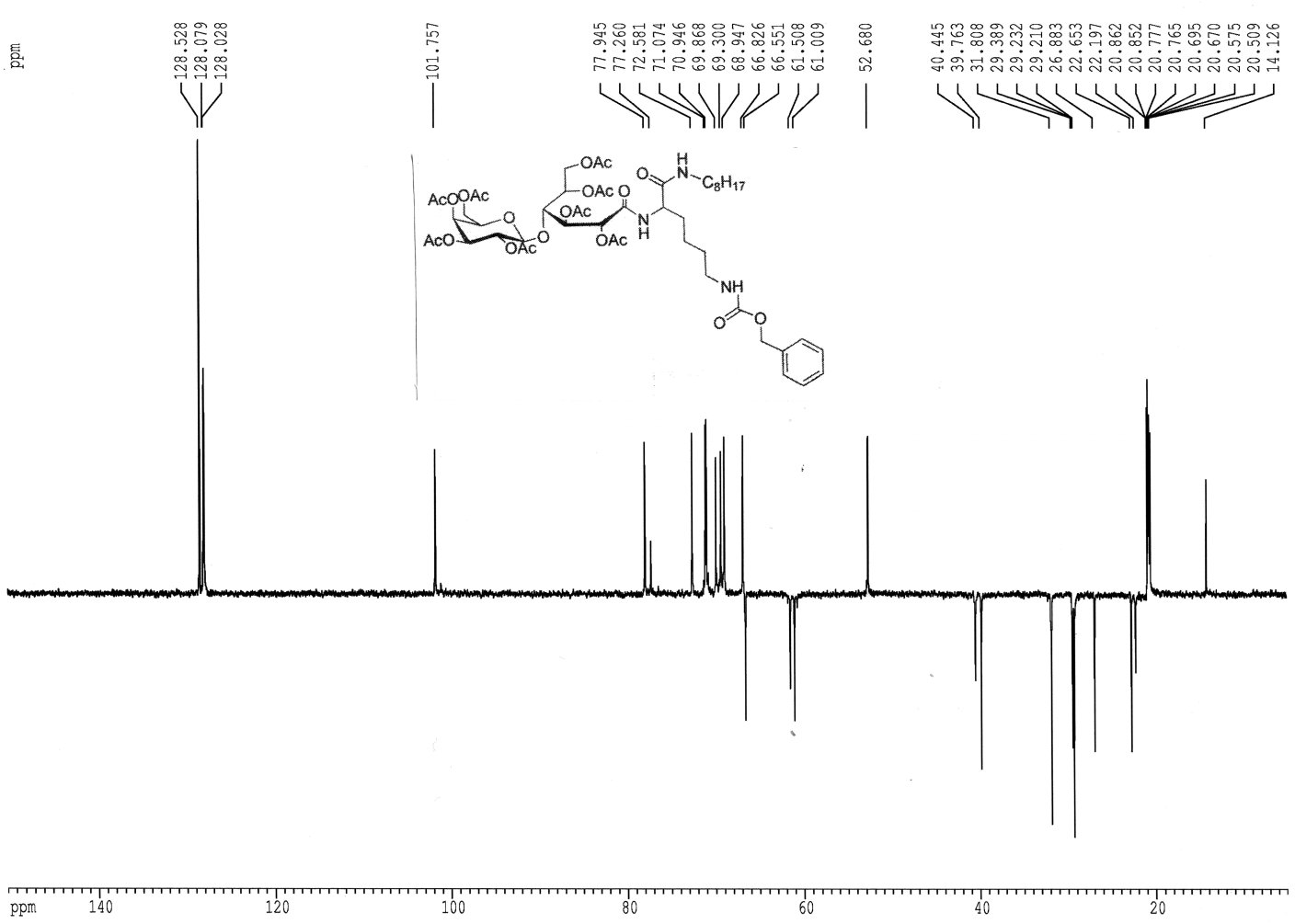

Figure S10. DEPT 135 spectrum of LLH (**6**) in $CDCl_3$

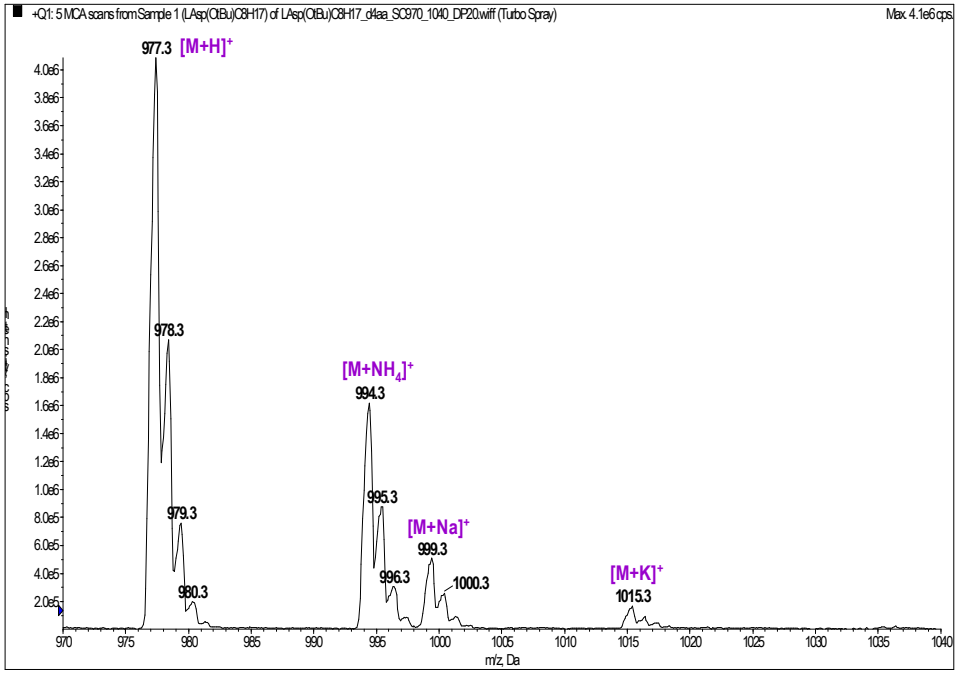

Figure S11. MS spectrum of LAH (**2**)

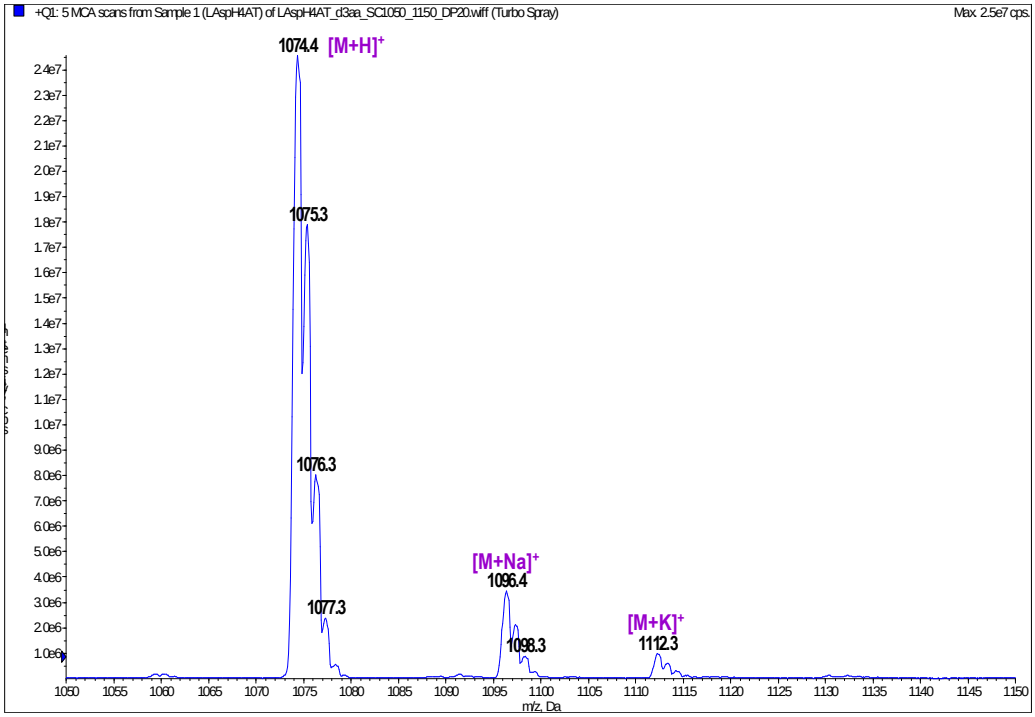

Figure S12. MS spectrum of O-acetylated LAH-4-AT (**3**)

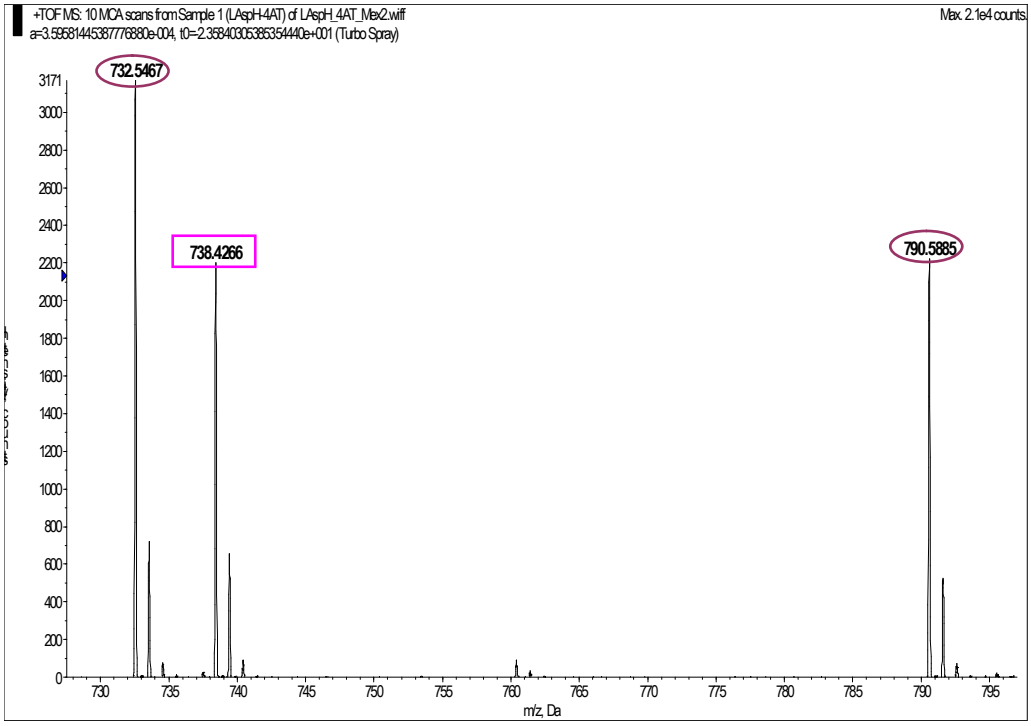

Figure S13. HRMS spectrum of LAH-4-AT (**4**)

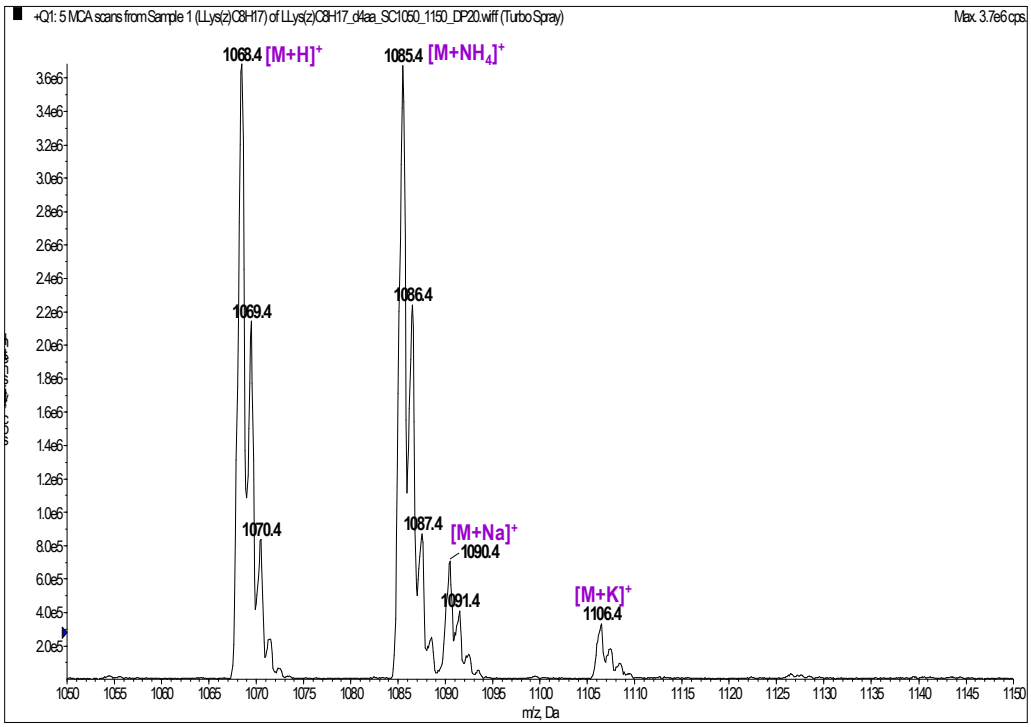

Figure S14. MS spectrum of LLH (**6**)

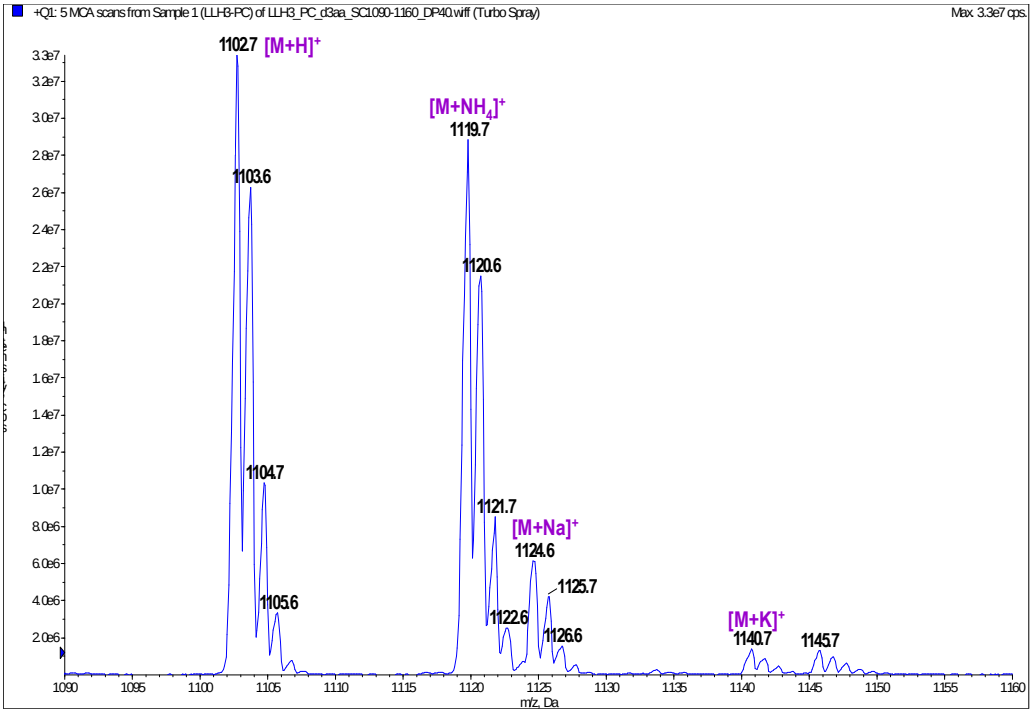

Figure S15. MS spectrum of O-acetylated LLH-3-CP (**7**)

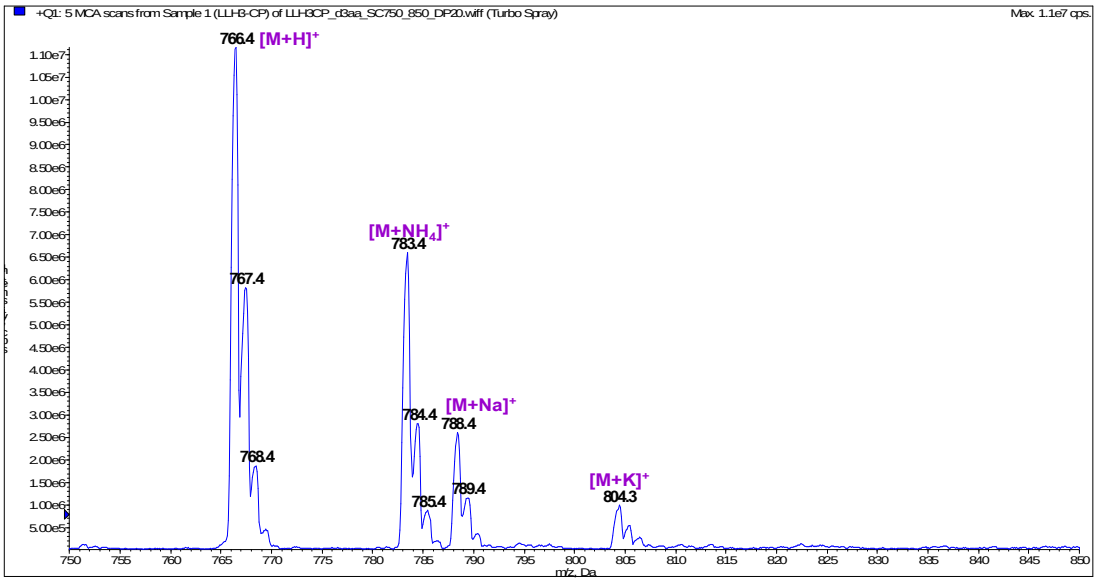

Figure S16. MS spectrum of LLH-3-CP (**8**)

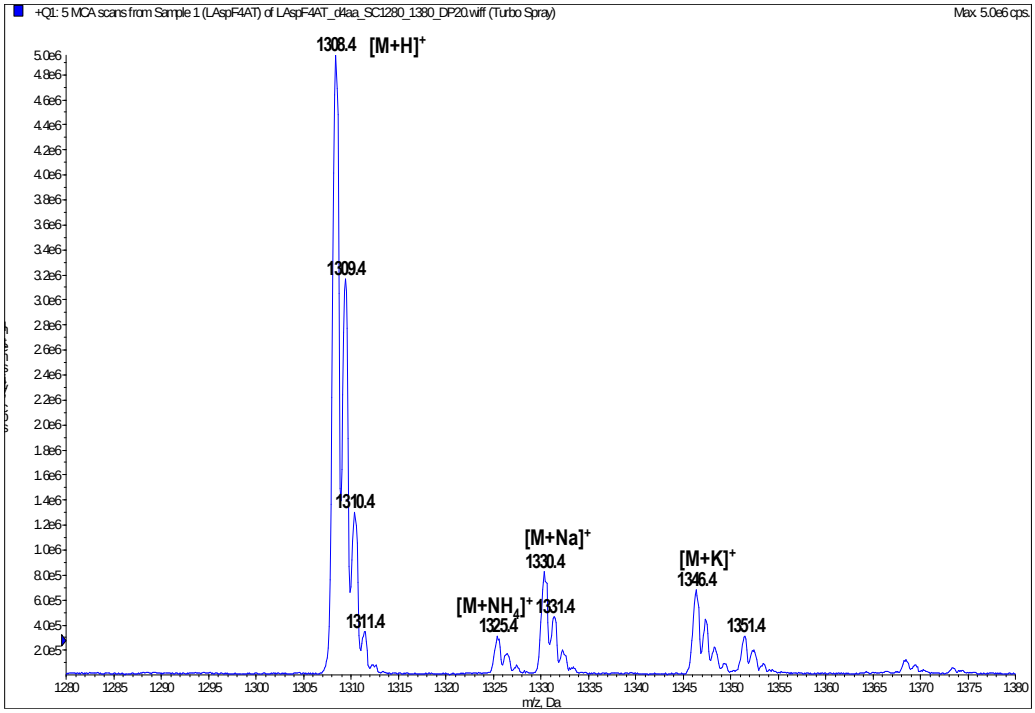

Figure S17. MS spectrum of O-acetylated LAF-4-AT (**10**)

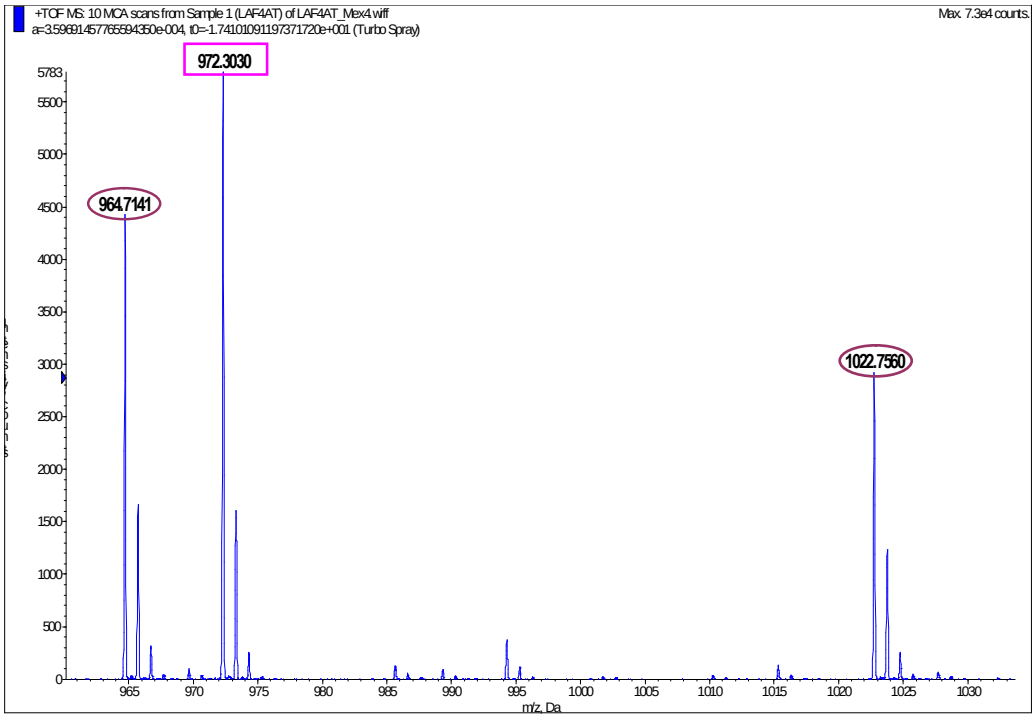

Figure S18. HRMS spectrum of LAF-4-AT (**11**)

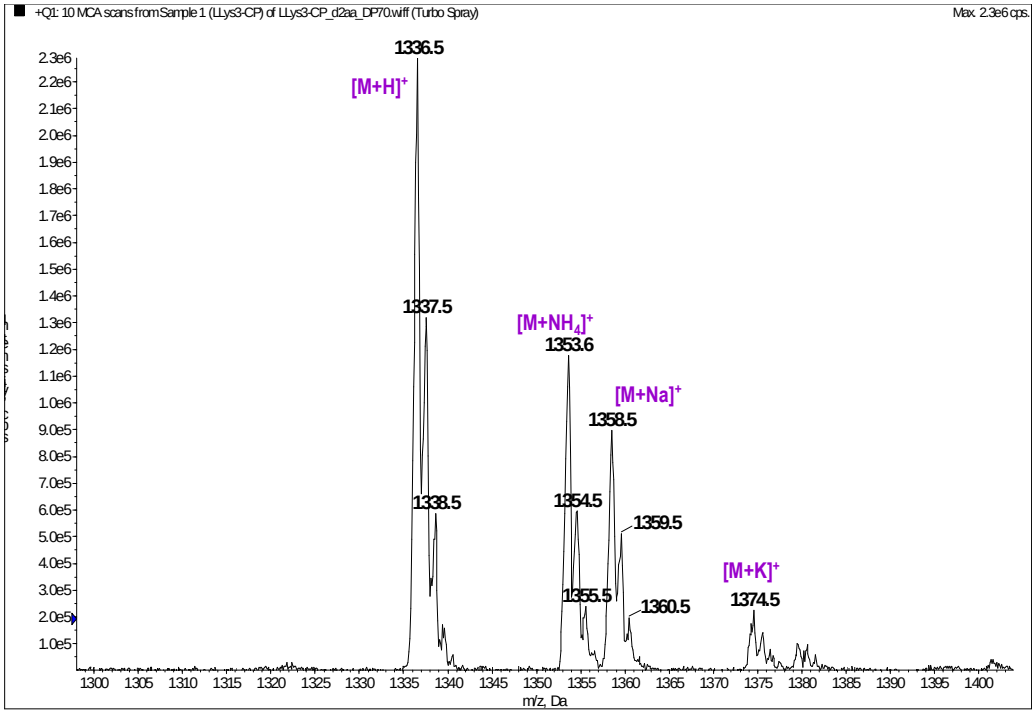

Figure S19. MS spectrum of O-acetylated LLF-3-CP (**13**)

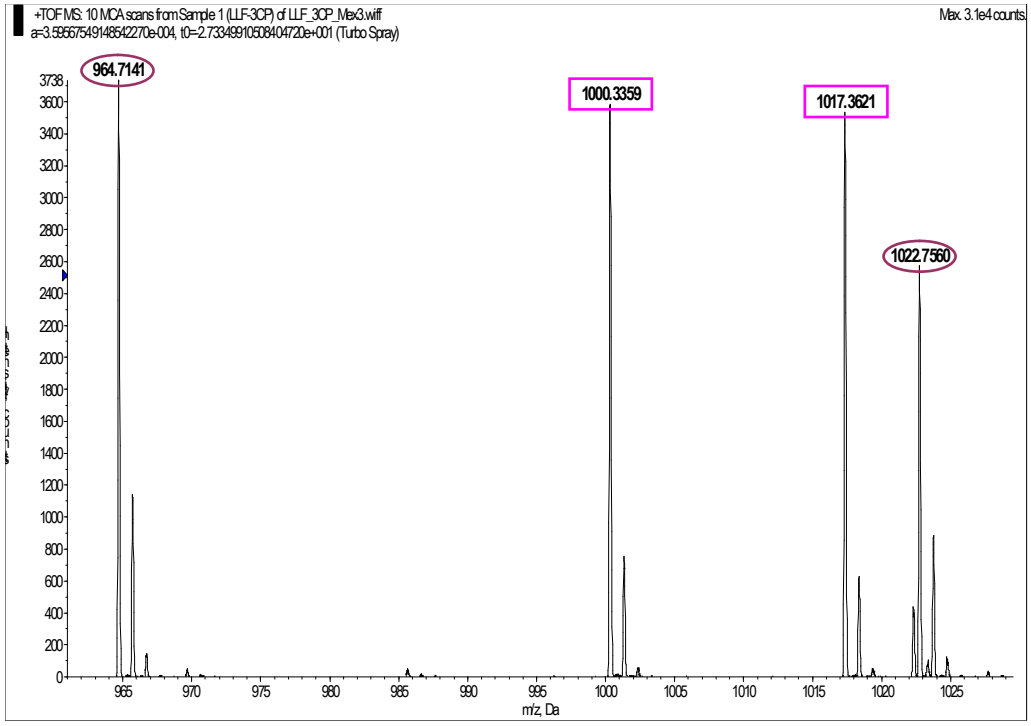

Figure S20. HRMS spectrum of LLF-3-CP (**14**)

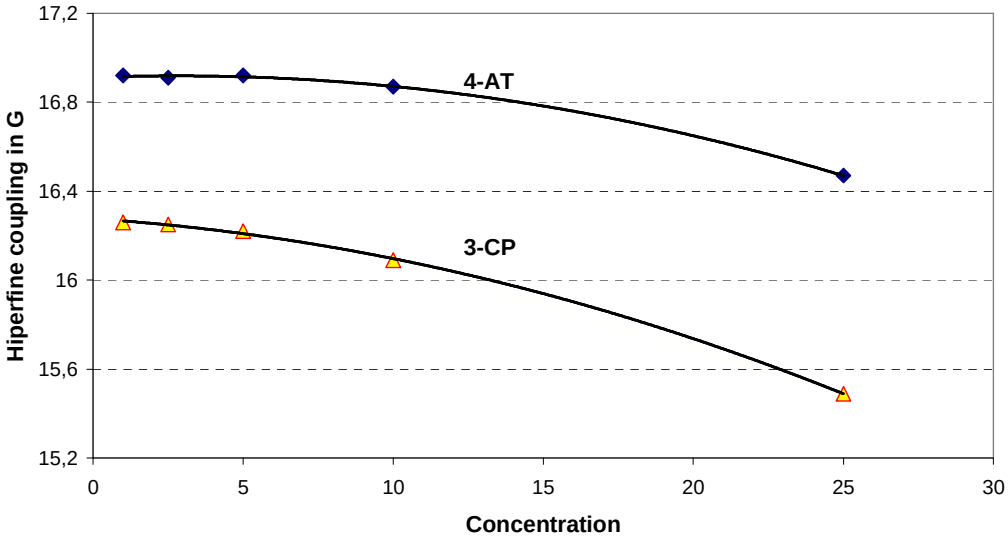

Figure S21. Concentration dependence of the nitrogen coupling for the unconjugated nitroxides.

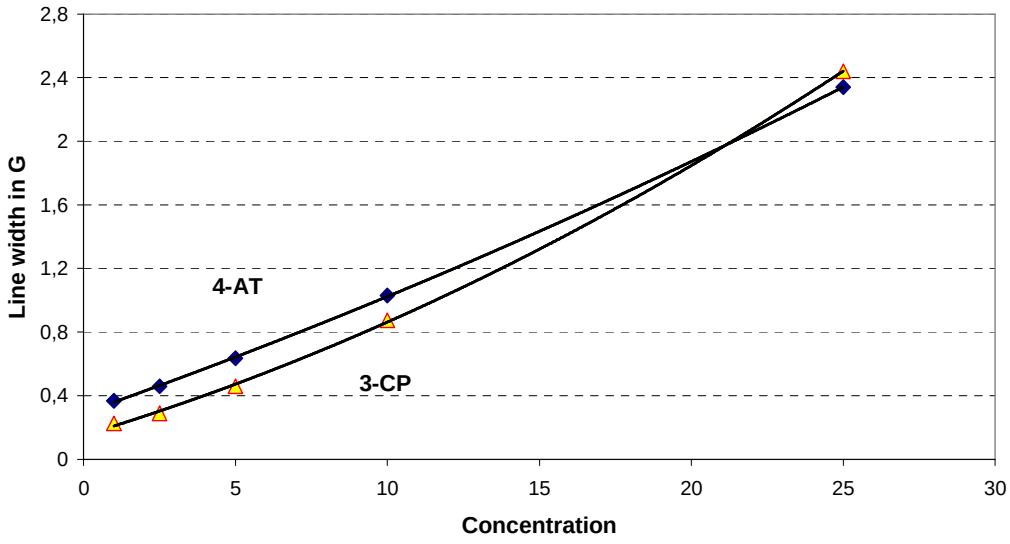

Figure S22. Concentration dependence of the linewidth for the unconjugated nitroxides.

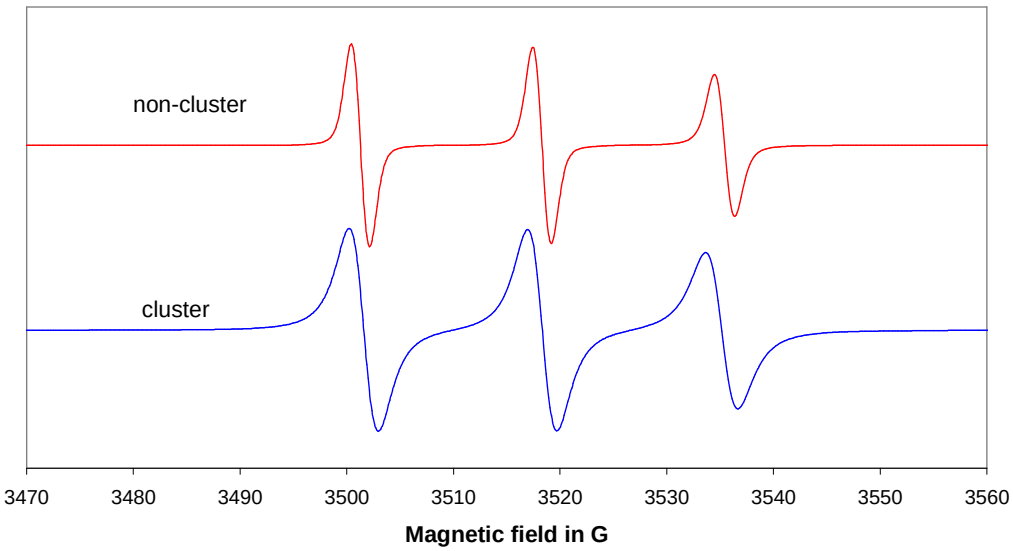

Figure S23. EPR spectra of the non-clustered and clustered LAH-4-AT (**4**)

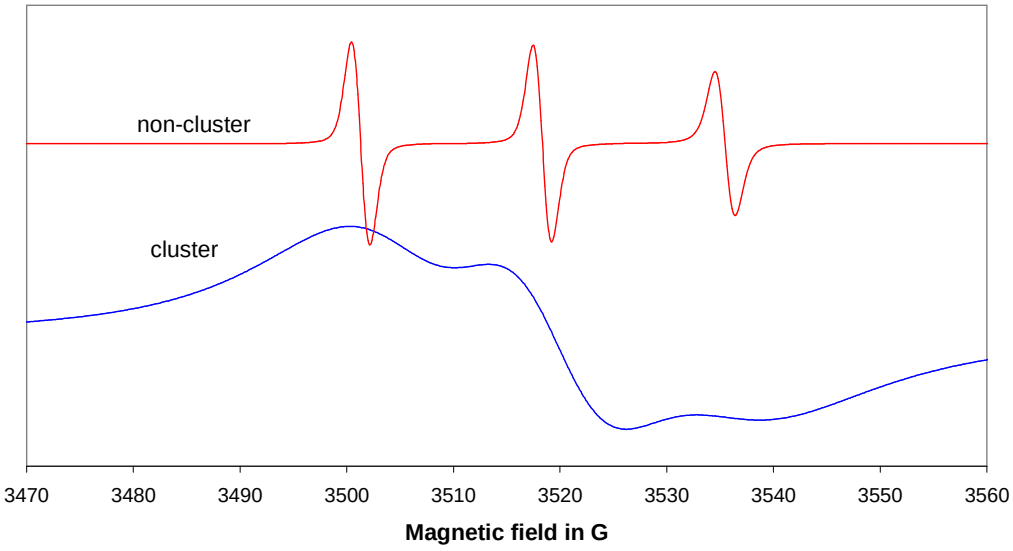

Figure S24. EPR spectra of the non-clustered and clustered LAF-4-AT (**11**)

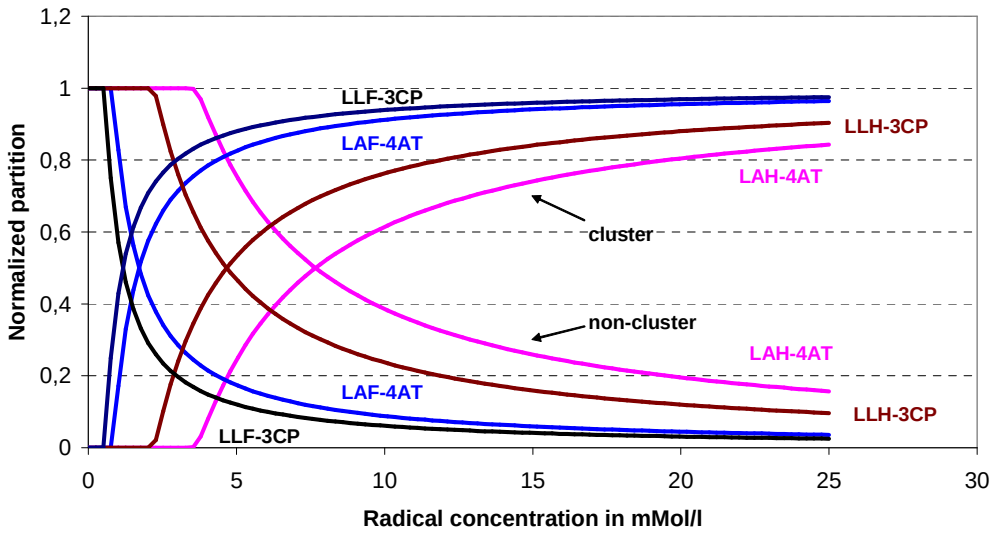

Figure S25. Radical distributions of the non-clustered and clustered radicals as a function of concentrations.

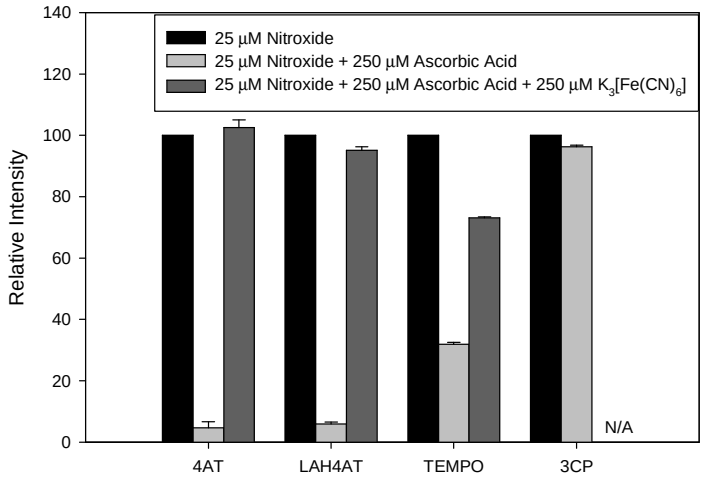

Figure S26. Reduction and restoration of the nitroxide EPR signals by ascorbic acid and potassium ferricyanide. 25 µM of nitroxide was measured by EPR (see experimental for details) over a 30 min period and the signal intensity was found to be stable. 250 µM ascorbic acid was added and the EPR spectra were taken again for 30 min. 250 µM $K_3[Fe(CN)_6]$ was then added to the sample with ascorbic acid and the EPR spectra was taken again for another 30 min. The y-axis corresponds to the relative intensity compared to the 25 µM of nitroxide. n=2.

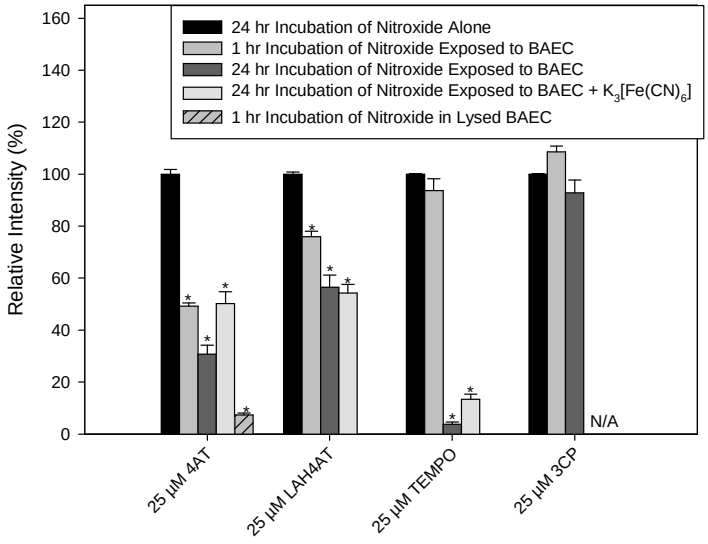

Figure S27. Relative EPR signal intensities of extracellular nitroxides after 1 h and 24 h incubation in the presence and absence of BAEC. EPR spectra were again taken after addition of 250 µM $K_3[Fe(CN)_6]$. Plots are relative to the signal intensity of 25 µM nitroxide in DMEM normalized to 100%. In this figure is also shown the result of lysed cells one hour after being incubated with 4-AT. An EPR signal consistent with the presence of 4-AT compound was observed with a relative intensity of 7.5% while the extracellular concentration is about 50%.