Supplementary Information

Synthetic Vaccines Targeting Mincle Through Conjugation of Trehalose Dibehenate

Cameron C. Hanna[a†], Joshua W. C. Maxwell[a,b†], Hendra S. Ismanto[c], Anneliese S. Ashhurst[a,d,e,f], Lukas M. Artner[a], Santosh Rudrawar,[a] Warwick J. Britton[f], Sho Yamasaki[c] and Richard J. Payne[a,b*]

a. School of Chemistry, The University of Sydney, Sydney, NSW 2006, Australia

b. Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science, School of Chemistry, The University of Sydney, NSW 2006, Australia

c. Department of Molecular Immunology, Research Institute for Microbial Diseases, Osaka University, 565-0871 Osaka, Japan

d. School of Medical Sciences, Faculty of Medicine and Health, The University of Sydney, NSW 2006, Australia

e. The University of Sydney Institute for Infectious Diseases, NSW 2006, Australia

f. Tuberculosis Research Program Centenary Institute, The University of Sydney, Camperdown, NSW 2006, Australia

† These authors contributed equally.

*Correspondence and requests for materials should be addressed to Richard J. Payne

Email: richard.payne@sydney.edu.au

General methods and materials

Solution-phase chemistry

All reactions were carried out in dried glassware under an argon atmosphere and at room temperature (22-24 °C) unless otherwise specified. Reactions undertaken at -78 °C utilised a bath of dry ice and acetone. Reactions undertaken at -15 °C utilised a bath of ice, water and sodium chloride. Reactions carried out at 0 °C employed a bath of water and ice. Anhydrous THF, CH_2Cl_2, MeCN, DMF, toluene and MeOH were obtained using a PureSolv® solvent purification system with water detectable only in low ppm levels. Reactions were monitored by thin layer chromatography (TLC) on aluminium backed silica plates (Merck Silica Gel 60 F254). Visualisation of TLC plates was undertaken with an ultraviolet (UV) light at λ = 254 nm and staining with solutions of vanillin, ninhydrin, phosphomolybdic acid (PMA), potassium permanganate or sulfuric acid, followed by exposure of the stained plates to heat. Silica flash column chromatography (Merck Silica Gel 60 40 – 63 μm) was undertaken to purify crude reaction mixtures using solvents as specified. All commercially available reagents were used as obtained from Sigma-Aldrich, Merck or Acros Organics. Amino acids, coupling reagents and resins were obtained from NovaBiochem or GL Biochem and peptide synthesis grade DMF was obtained from Merck or Labscan.

Solid-phase chemistry

Fmoc-strategy solid-phase peptide synthesis (Fmoc-SPPS) procedures were carried out using 2-CTC-functionalised polystyrene resin, Rink amide-functionalised Chemmatrix or polystyrene resin, or Wang-functionalised Chemmatrix resin within fritted syringes (purchased from Torviq). All reagent equivalents are given with respect to the amount of amino acid loaded to resin.

Automated SPPS was performed on either the Biotage Initiator+ Alstra microwave peptide synthesiser or the Syro I Automated Parallel Peptide Synthesiser.

Washing: The resins was filtered and washed with DMF (5 x 5 mL).

Fmoc deprotection: The resin was treated with piperidine/DMF (1:4 v:v, 4 mL, 2 × 4 min).

Coupling of proteinogenic amino acids: Fmoc-AA-OH (0.4 mmol, 4 eq.), *N,N'*-diisopropylcarbodiimide (DIC, 0.4 mmol, 4 eq.), and ethyl (hydroxyimino)cyanoacetate (Oxyma, 0.4 mmol, 4 eq.) were dispensed from concentrated stock solutions in DMF onto the resin, and made up to a final volume of 4 mL with DMF. The reaction was heated to 50 °C and mixed for 12 min.

Capping: The resin was treated with Ac_2O/pyridine (1:9 v/v, 4 mL) for 5 min.

Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H NMR spectra were obtained using a Bruker DRX 400 or DRX 500 at frequencies of 400 MHz or 500 MHz respectively in $CDCl_3$, CD_3OD or DMSO-d_6. Chemical shifts are reported in parts per million (ppm) and coupling constants are provided in Hertz (Hz) where possible. The residual solvent peaks (or tetramethylsilane (TMS)) were used as internal standards. ^{1}H NMR data is reported as follows: chemical shift values (ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br. = broad), coupling constant(s) and relative integral. ^{13}C NMR spectra were obtained using a Bruker DRX 400 or DRX 500 at 100 MHz or 125 MHz in $CDCl_3$, Me_3OD or DMSO-d_6 unless otherwise specified. ^{13}C NMR data is reported as chemical shift values (ppm).

Mass spectrometry (MS)

Low-resolution mass spectra for novel compounds were recorded on a Bruker amaZon SL mass spectrometer (ESI) operating in positive mode or on a Shimadzu 2020 (ESI) mass spectrometer operating in positive mode. High resolution mass spectra were recorded on a Bruker-Daltronics Apex Ultra 7.0T Fourier transform (FTICR) mass spectrometer. Low resolution matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectra were recorded on a Bruker AutoflexTM Speed MALDI-TOF mass spectrometer operating in linear mode using a matrix of 10 mg/mL sinapinic acid in water/MeCN (1:1 v/v).

Liquid Chromatography Mass Spectrometry (LCMS)

LC-MS was performed either on a Shimadzu 2020 LC-MS instrument with an LC-M20A pump, SPD-20A UV/Vis detector and a Shimadzu 2020 (ESI) mass spectrometer operating in positive mode or on a Shimadzu UPLC-MS equipped with the same modules as above but with an SPD-M30A diode array detector. Separations on the LC-MS system were performed on a

Waters Sunfire 5 μm, 2.1 x 150 mm (C18) column. On the UPLC-MS system, separations were performed on a Waters Acquity 1.7 μm, 2.1 x 50 mm (C18) column. These separations were performed using a mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in MeCN (Solvent B) using linear gradients.

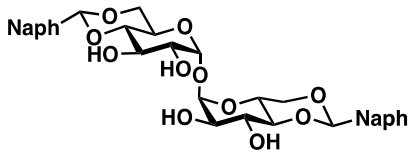
Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

Preparative reverse-phase HPLC was performed using a Waters 500 pump with a 2996 photodiode array detector and a Waters 600 Multisolvent Delivery System. The column used for each purification is described in the relevant experimental section.

Analytical HPLC was performed on a Waters System 2695 separations module with a 2996 photodiode array detector and an Alliance series column heater at 40 °C. Unless otherwise stated, separations were carried out using a Waters Sunfire C18 column (5 μm, 2.1 × 150 mm) at a flow rate of 0.2 mL min^{-1}. Separations were performed using a mobile phase of 0.1% trifluoracetic acid in water (Solvent A) and 0.1% trifluoracetic acid in MeCN (Solvent B) using linear gradients.

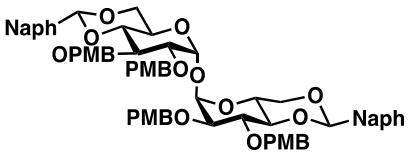
Experimental

Synthesis of 4,6,4′,6′-di-O-(2-naphthyl)methylene-α,α-D-trehalose (S1)



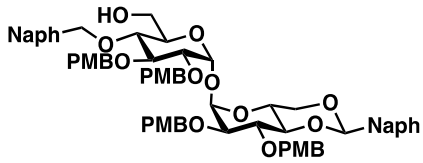
To a solution of commercially available trehalose dihydrate (7.56 g, 20 mmol) in pyridine (50 mL) at 0 ºC was added TMS-Cl (14 mL, 110 mmol) dropwise over 5 min. The mixture was stirred for 18 h at room temperature. On completion of the reaction, the mixture was diluted with water (200 mL) and EtOAc (200 mL), and the organic phase was isolated. The organic phase was washed with water (2 x 100 mL) and dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to afford the crude TMS-protected trehalose as an off-white solid which was used without purification. To a solution of the crude TMS-protected trehalose (8.27 g, 13.38 mmol) in dry CH_2Cl_2 (100 mL) with freshly activated molecular sieves (4A, 2 g, powdered) was added 2-naphthaldehyde (4.59 g, 29.43 mmol). The mixture was stirred under a nitrogen atmosphere for 5 minutes before being cooled to 0 ºC. TMS-OTf (725 μL, 4.0 mmol) was added to the mixture dropwise over 5 minutes. After 3 h, TBAF (53 mL, 53.4 mmol) was added and the reaction mixture stirred for 16 h at rt. The reaction was filtered over celite, extracted with CH_2Cl_2 (2 x 100 mL), concentrated *in vacuo* and purified by column chromatography to afford **S1** as a white solid (7.70 g, 93%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.99 (s, 2H), 7.75–7.50 (m, 6H), 7.68–7.64 (m, 2H), 7.53–7.51 (m, 4H), 5.77 (s, 2H), 5.19 (d, J = 4.0 Hz, 2H), 4.66 (m, 1H), 4.31 (dd, J = 9.5, 5.0 Hz, 2H), 4.20 (dt, J = 9.5, 5.0 Hz, 2H), 4.10 (t, J = 9.5 Hz, 2H), 3.83 (t, J = 10.0 Hz, 2H), 3.70 (dd, J = 9.5, 4.0 Hz, 2H), 3.60 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 138.5, 138.2, 129.9, 129.3, 128.6, 128.5, 126.4, 126.1, 103.8, 100.1, 97.5, 96.0, 80.7, 80.0, 79.8, 76.2, 75.3, 75.2, 74.7, 72.6, 66.4, 66.3; HRMS Calculated for $C_{34}H_{34}O_{11}Na$ $[M+Na]^+$: 641.1999, Found (ESI+): 641.2001 $[M+Na]^+$; IR (ATR) ν_{max} = 3431, 2930, 2860, 1620, 1472, 1176, 1080, 976 cm^{-1}; $[\alpha]_D$: +30 (c 1.0, MeOH); mp 195-198 ºC.

Synthesis of 2,3,2′,3′-Tetra-*O*-(*p*-methoxybenzyl)-4,6,4′,6′-di-*O*-(2-naphthyl)methylene-α,α-D-trehalose (3)



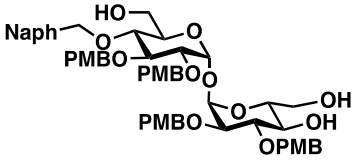
To a solution of compound **S1** (6.00 g, 9.69 mmol) in anhydrous DMF (50 mL) was added NaH (1.16 g, 4.80 mmol) at room temperature. After stirring for 20 minutes, the reaction was cooled to 0 ºC and PMB-Cl (10.48 mL, 77.6 mmol) was added, followed by the addition of TBAI (624 mg, 1.94 mmol). The reaction flask was fitted with a reflux condenser, heated to 100 ºC, and left to stir for 16 h under argon. On completion of the reaction, the mixture was cooled to 0 ºC and quenched with a solution of saturated NH_4Cl (~50 mL). The product was extracted into CH_2Cl_2 (2 x 100 mL), washed with brine (2 x 100 mL) and then water (1 x 100 mL). The organic phase was dried over anhydrous $MgSO_4$, concentrated *in vacuo*, and purified by column chromatography to afford **3** as a white foam (8.31 g, 78%). **^{1}H NMR** ($CDCl_3$, 400 MHz) δ (ppm): δ 7.99 (s, 2H), 7.90–7.80 (m, 7H), 7.65–7.63 (m, 2H), 7.53–7.29 (m, 4H), 7.34–7.27 (m, 8H), 6.83–6.80 (m, 7H), 5.72 (s, 2H), 5.14 (d, *J* = 4.0 Hz, 2H), 4.86 (d, *J* = 10.2 Hz, 2H), 4.80 (d, *J* = 10.7 Hz, 2H), 4.78 (d, *J* = 11.2 Hz, 2H), 4.66 (d, *J* = 11.2 Hz, 2H), 4.30 (dt, *J* = 10.0, 4.7 Hz, 2H), 4.21 (dd, *J* = 10.0, 4.7 Hz, 2H), 4.14 (t, *J* = 9.4 Hz, 2H), 3.80–3.75 (m, 2H), 3.72 (s, 4H), 3.67 (t, *J* = 9.4 Hz, 2H), 3.60 (s, 6H), 3.65–3.60 (m, 2H); **^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 160.0, 159.2, 135.2, 133.7, 133.2, 131.3, 130.4, 130.0, 129.8, 128.4, 128.1, 128.0, 126.5, 126.2, 125.5, 124.2, 115.1, 114.0, 101.0, 95.0, 82.4, 78.9, 78.4, 75.2, 73.5, 69.1, 63.1, 55.2, 55.1; **HRMS** Calculated for $C_{66}H_{67}O_{15}$ $[M+H]^+$: 1098.4402, Found (ESI+): 1098.4409 $[M+H]^+$; **IR** (ATR) ν_{max} = 3010, 2935, 2862, 1614, 1588, 1512, 1250, 1090, 670 cm^{-1}; **$[\alpha]_D$**: +10 (c 1.0, CH_2Cl_2); **mp** 62-64 ºC. These data are in agreement with those reported by Sarpe *et al.*[1]

Synthesis of 2,3,2′,3′-Tetra-*O*-(*p*-methoxybenzyl)-4-*O*-(2-naphthyl)methyl-4′,6′-*O*-(2-naphthyl) methylene-*α*,*α*-D-trehalose (4)



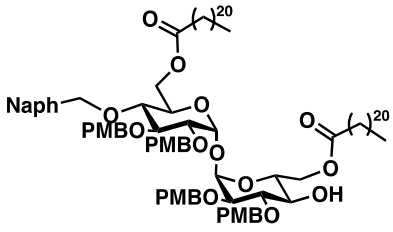
Compound **3** (7.60 g, 6.91 mmol) was dissolved in anhydrous toluene (70 mL) and cooled to -15 °C in a salt/ice/water bath. To the solution was added DIBAL-H (1 M in THF, 34.5 mL, 34.5 mmol) dropwise over 10 min. The reaction was removed from the salt/ice/water bath and allowed to stir for 15 min at rt with close monitoring by TLC. Once finished, the reaction was quickly quenched at -15 °C with MeOH (2 mL), then a mixture of KOH/MeOH (1:10 v/v, 2 mL). To the reaction mixture was added Rochelle's salt (100-200 mg) and was then left to stir at rt for 2 h. The product was extracted into CH_2Cl_2 (100 mL), washed with water (1 x 100 mL), dried over anhydrous $MgSO_4$ and concentrated *in vacuo.* The product was purified by silica gel chromatography to yield compound **4** as a white foam (4.94 g, 65 %). **^{1}H NMR** ($CDCl_3$, 400 MHz) δ (ppm): 8.00-7.77 (m, 7H), 7.72 (s, 1H), 7.62 (dd, *J* = 8.4, 1.5 Hz, 1H), 7.45–7.38 (m, 4H), 7.30–7.19 (m, 9H), 6.82–6.70 (m, 8H), 5.70 (s, 1H), 5.10 (d, *J* = 3.4 Hz, 2H), 5.08 (d, *J* = 3.4 Hz, 1H), 5.02 (d, *J* = 11.0 Hz, 1H), 4.90 (m, 2H), 4.80–4.68 (m, 3H), 4.72–4.59 (m, 4H), 4.25 (td, *J* = 9.9, 5.0 Hz, 1H), 4.20 (dd, *J* = 10.1, 5.0 Hz, 1H), 4.17–4.07 (m, 3H), 3.73–3.70 (m, 1H), 3.70 (s, 6H), 3.65–3.50 (m, 12H); **^{13}C NMR** ($CDCl_3$, 100 MHz) δ (ppm): 160.0, 159.2, 159.1, 159.1, 135.5, 134.9, 133.2, 133.2, 132.8, 132.9, 131.0, 129.8, 130.0, 129.4, 129.1, 129.2, 128.1, 128.3, 127.9, 127.6, 126.6, 126.4, 126.2, 126.2, 126.0, 125.5, 123.9, 113.9, 113.7, 113.7, 113.7, 101.3, 94.5, 93.6, 82.4, 81.2, 79.4, 78.5, 78.3, 77.2, 75.2, 75.1, 73.2, 73.0, 71.2, 69.1, 62.9, 61.6, 55.0, 55.1, 55.1; **HRMS** Calculated for $C_{66}H_{68}O_{15}Na$ $[M+Na]^+$: 1123.4456, Found (ESI+): 1123.4450 $[M+Na]^+$; **IR** (ATR) ν_{max} = 3478, 2929, 1613, 1510, 1465, 1230, 1080, 750, 741 cm^{-1}; **$[\alpha]_D$**: +45 (c 1.0, CH_2Cl_2). These data are in agreement with those reported by Sarpe *et al.*[1]

Synthesis of 2,3,2′,3′-Tetra-*O*-(*p*-methoxybenzyl)-4-*O*-(2-naphthyl)methyl-*α,α*-D-trehalose (5)



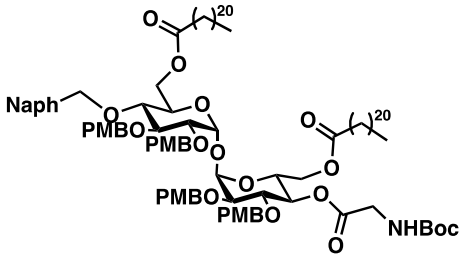
To a solution of compound **4** (3.0 g, 2.72 mmol) in acetic acid (28 mL) was added water dropwise (12 mL) at room temperature. The mixture was sonicated to aid dissolution, after which the solution was stirred at 45 °C for 24 h. On completion of the reaction, the product was extracted into EtOAc (2 x 50 mL), washed with water (1 x 50 mL), dried over anhydrous $MgSO_4$ and concentrated *in vacuo.* The product was purified by silica gel chromatography (eluent 3:7 v/v hexane:EtOAc) to afford compound **5** as a yellow oil (1.83 g, 70%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ (ppm): 7.89 – 7.78 (m, 3H, 3 x ArH), 7.78 – 7.71 (m, 1H, ArH), 7.54 – 7.46 (m, 2H, 2x ArH), 7.44 (dd, J = 8.4, 1.7 Hz, 1H), 7.36 – 7.16 (m, 8H, 8x ArH), 6.96 – 6.72 (m, 8H, 8x ArH), 5.13 (d, J = 3.5 Hz, 1H, H-1'), 5.11 (d, J = 3.6 Hz, 1H, H-1), 5.05 (d, J = 11.2 Hz, 1H), 4.96 – 4.89 (m, 2H), 4.86 – 4.78 (m, 2H), 4.69 (d, J = 11.1 Hz, 1H), 4.67 – 4.59 (m, 4H), 4.13–4.04 (m, 2H, H-3, H-5), 4.01 (dt, J = 9.9, 3.8 Hz, 1H), 3.86 (dd, J = 9.3 Hz, 1H, H-3'), 3.79(s, 3H), 3.78 (s, 3H), 3.74 (s, 3H), 3.68 (d, J = 7.1 Hz, 7H, H-6, H-6'), 3.62 (t, J = 10.1, 8.9 Hz, 1H, H-4), 3.58 – 3.52 (m, 2H, H-2, H-4'), 3.50 (dd, J = 9.6, 3.5 Hz, 1H, H-2').**^{13}C NMR** (100 MHz, $CDCl_3$) δ (ppm): 159.1, 159.0, 159.0, 159.0 (4 x ArO), 135.8, 133.1, 132.8, 130.9, 130.7, 130.0, 129.9 (7 x ArC), 129.5-129.0 (16 x ArC), 128.0, 127.8, 127.5, 126.2, 126.0, 125.8, 125.7 (10x ArC), 93.0 (C-1), 93.0 (C-1'), 81.0 (C-3), 80.4 (C-3'), 79.2 (C-2), 79.1 (C-2'), 77.5 (C-4), 75.1, 74.7, 74.7, 72.5, 72.4 (5x ArC), 72.0 (C-5'), 71.4 (C-5), 70.0 (C-4'), 61.6 (C-6), 61.3 (C-6'), 55.0, 55.0, 54.9, 54.8 (4 x OCH_3). **HRMS** Calculated for $C_{55}H_{62}O_{15}Na$ $[M+Na]^+$: 985.3986. Found (ESI+) 985.3969 $[M+Na]^+$; **IR** (ATR) ν_{max} = 3442, 2921, 2855, 1613, 1513, 1249 cm^{-1}; **$[\alpha]_D$:** +0.67 (c 1.0, CH_2Cl_2).

Synthesis of 6,6'-Di-behenoyl-2,3,2′,3′-Tetra-*O*-(*p*-methoxybenzyl)-4-*O*-(2-naphthyl)methyl-*α*,*α*-D-trehalose (6)



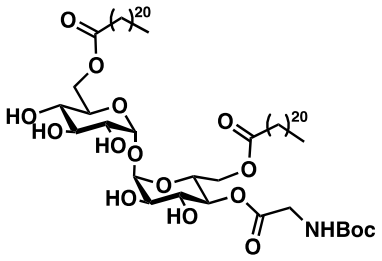
To a solution of compound **5** (1.25 g, 0.77 mmol) in CH_2Cl_2 (75 mL) was added *i*Pr_2NEt (265 µL, 1.54 mmol). The reaction mixture was cooled to 0 °C and behenoyl chloride (552 mg, 1.54 mmol) was added dropwise over 10 min. The reaction was stirred for 4 h at rt with close monitoring by TLC. On completion of the reaction, the solvent was removed *in vacuo*, and the resulting residue was diluted in ethyl acetate (50 mL). The product was washed with water (2 x 50 mL), dried over anhydrous $MgSO_4$, concentrated *in vacuo* and purified by column chromatography (eluent 2:3 v/v hexane:EtOAc) to afford compound **6** as a colourless oil (829 mg, 67%). **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 7.84 – 7.80 (m, 3H, 3 x ArH), 7.71 (s, 1H, ArH), 7.50 – 7.45 (m, 2H, 2 x ArH), 7.41 (m, 1H, ArH), 7.31 – 7.28 (m, 4H, 4 x ArH), 7.25 – 7.23 (m, 4H, 4 x ArH), 6.87 – 6.81 (m, 8H, 8 x ArH), 5.15 – 5.14 (m, 2H, H1, H-1'), 5.03 (d, J = 11.0 Hz, 1H, $ArCH_2$), 4.94 (d, J = 10.5 Hz, 1H, $ArCH_2$), 4.89 (d, J = 10.7 Hz, 1H, $ArCH_2$), 4.82 (d, J = 10.5 Hz, 1H, $ArCH_2$), 4.75 – 4.71 (m, 2H, $ArCH_2$), 4.68 – 4.62 (m, 4H, $ArCH_2$), 4.35 (dd, J = 12.3, 3.8 Hz, 1H, H-6'a), 4.27 – 4.24 (m, 1H, H-5), 4.21 (dd, J = 12.1, 3.7 Hz, 1H, H-6a), 4.14 – 4.11 (m, 2H, H-5', H-6b), 4.06 (m, 1H, H-3), 3.98 (m, 1H, H-6'b), 3.85 (m, 2H, H-3'), 3.81, 3.79, 3.75, 3.70 (4s, 12H, 4 x CH_3O), 3.60 – 3.55 (m, 2H, H-2), 3.50 (m, 1H, H-2'), 3.42 (m, 1H, H-4'), 2.28 (m, 2H, $BehCH_2α$), 2.22 – 2.07 (m, 2H, $BehCH_2α$), 1.61 – 1.48 (m, 4H, 2 x $BehCH_2$), 1.32 – 1.21 (m, 72H, 36 x $BehCH_2$), 0.89 (t, J = 6.8 Hz, 6H, 2 x $BehCH_3$). **^{13}C NMR** (126 MHz, $CDCl_3$) δ (ppm): 174.4, 173.6 (2 x BehCO), 159.5 (3 x ArC), 159.4 (ArC), 135.6, 133.4, 133.2, 131.0, 130.9, 130.2, 130.1, 130.0, 129.9, 129.7, 129.3, 129.3, 128.4, 128.1, 127.8, 127.0, 126.3, 126.2, 126.1, 114.1, 114.1, 114.0, 114.0 (30 x ArC), 94.3 (C-1), 94.1 (C-1'), 81.6 (C-3), 80.3 (C-3'), 79.4 (C-2), 78.8 (C-2'), 77.5 (C-4), 75.5, 75.3, 75.2, 72.8, 72.7 (4 x $PMBCH_2$, $NapCH_2$), 70.2 (C-5'), 70.0 (C-4'), 69.3 (C-5), 63.0 (C-6'), 62.8 (C-6), 55.4, 55.4, 55.4, 55.3 (4 x CH_3O), 34.2, 34.2 (2 x $BehCH_2α$), 32.1 (2 x $BehCH_2$), 29.8 - 29.3 (32 x $BehCH_2$), 25.0 (2 x $BehCH_2$), 25.0, 22.8 (2 x $BehCH_2$), 14.3 (2 x $BehCH_3$). **HRMS** Calculated for $C_{99}H_{146}O_{17}Na$ $[M+Na]^+$: 1630.0452, Found (ESI+) 1630.0452 $[M+Na]^+$; **IR** (ATR) ν_{max} = 2921, 2852, 1737, 1514, 1249 cm^{-1}; **$[\alpha]_D$:** +0.46 (c 1.0, CH_2Cl_2).

Synthesis of 6,6'-dibehenoyl-4-(*tert*-butoxycarbonyl)glycinate-2,3,2′,3′-tetra-*O*-(*p*-methoxybenzyl)-4-*O*-(2-naphthylmethyl)-*α,α*-D-trehalose (7)



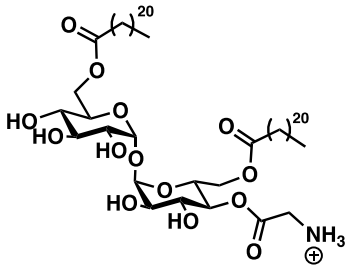
To a stirred solution of compound **6** (1.20 g, 0.74 mmol) in CH_2Cl_2 (20 mL) was added Boc-Gly-OH (1.4 g, 3.80 mmol). Once dissolved, *N,N*'-diisopropylcarbodiimide (600 μL, 3.80 mmol) and 4-dimethylaminopyridine (90 mg, 0.74 mmol) were added to the reaction mixture at 0 °C. The reaction was stirred at 0 °C for 5 min, then at rt for 3 h. On completion, the reaction mixture was diluted in CH_2Cl_2, filtered through celite, and washed with water (1 x 75 mL), sat. aq. $NaHCO_3$ (2 x 75 mL) solution and water again (1 x 75 mL). The organic phase was dried over anhydrous $MgSO_4$ and the solvent was removed *in vacuo*. The crude compound was purified by column chromatography (eluent 1:4 v/v hexane:EtOAc) to afford compound **7** as a colourless oil (926 mg, 70%). **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 7.85 – 7.81 (m, 3H, 3 x ArH), 7.72 (m, 1H, ArH), 7.51 – 7.46 (m, 2H, 2 x ArH), 7.43 (dd, J = 8.4, 1.7 Hz, 1H, ArH), 7.32 – 7.29 (m, 2H, 2 x ArH), 7.25 – 7.20 (m, 6H, 6 x ArH), 6.89 – 6.81 (m, 8H, 8 x ArH), 5.12 (2d, J = 3.5 Hz, 2H, H-1, H-1'), 5.05 – 5.01 (m, 2H, NapCH_2a, H-4'), 4.98 (d, J = 10.5 Hz, 1H, PMBCH_2a), 4.92 (m, 1H, BocNH), 4.86 (d, J = 10.5 Hz, 1H, PMBCH_2b), 4.79 – 4.69 (m, 3H, NapCH_2b, PMBCH_2), 4.66 – 4.59 (m, 4H, 2 x PMBCH_2), 4.27 – 4.22 (m, 2H, H-5, H-6a), 4.20 – 4.14 (m, 2H, H-5', H-6b), 4.10 – 4.04 (m, 2H, H-3, H-6'a), 3.94 (dd, J = 9.4 Hz, 1H, H-3'), 3.82 – 3.78 (m, 9H, GlyCH_2, H-6'b, 2 x CH_3O), 3.73 (s, 3H, CH_3O), 3.71 (s, 3H, CH_3O), 3.62 – 3.57 (m, 3H, H-2, H-2', H-4), 2.35 – 2.09 (m, 4H, 2 x BehCH_2α), 1.59 – 1.50 (m, 4H, 2 x BehCH_2β), 1.46 (s, 9H, $C(CH_3)_3$), 1.32 – 1.22 (m, 72H, 36 x BehCH_2), 0.89 (t, J = 6.9 Hz, 6H, 2 x BehCH_3). **^{13}C NMR** (126 MHz, $CDCl_3$) δ (ppm): 173.6 (BehCO), 173.6 (BehCO), 169.4 (GlyCO), 159.6, 159.5, 159.4, 159.4 (4 x ArC), 155.6 (BocCONH), 135.6, 133.4, 133.2, 130.9, 130.6, 130.0, 129.9, 129.7, 129.4, 129.2, 128.4, 128.1, 127.8, 127.0, 126.3, 126.2, 126.1, 114.1, 114.1, 114.0, 113.9 (30 x ArC), 94.0 (2 x C-1), 81.6 (C-3), 80.0 ($C(CH_3)_3$), 79.4, 78.9 (2 x C-2), 78.2 (C-3'), 77.5 (C-4), 75.5 (PMBCH_2), 75.3 (NapCH_2), 75.0 (PMBCH_2), 73.1 (PMBCH_2), 73.0 (PMBCH_2), 70.6 (C-4'), 69.4 (C-5), 68.1 (C-5'), 62.7 (C-6), 61.7 (C-6'), 55.4, 55.4, 55.3, 55.3 (4 x CH_3O), 42.6 (GlyCH_2), 34.2, 34.1 (2 x BehCα), 32.1 - 29.3 (32 x BehCH_2), 28.4 ($C(CH_3)_3$), 25.0, 24.8 (2 x BehCH_2), 22.8 (2 x BehCH_2), 14.2 (2 x BehCH_3). **HRMS** Calculated for $C_{62}H_{114}O_{18}Na$ $[M+Na]^+$: 1787.1191 Found (ESI+) 1787.1196 $[M+Na]^+$; **IR** (ATR) ν_{max} = 2922, 2852, 1737, 1613, 1514, 1250 cm^{-1}; **[α]$_D$**: +1.37 (c 1.0, CH_2Cl_2).

Synthesis of 6,6'-Di-behenoyl-4-(*tert*-butoxycarbonyl)glycinate-*α,α*-D-trehalose (8)



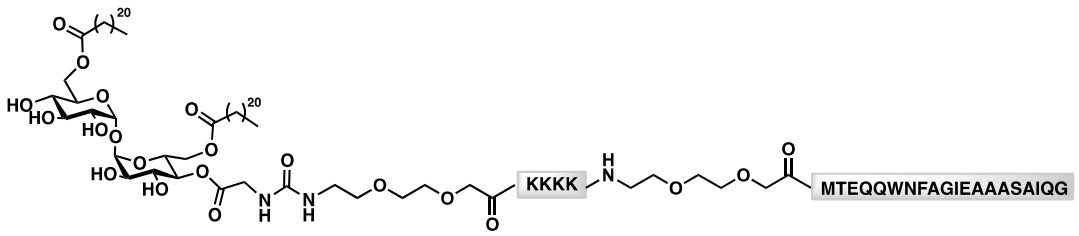
To a solution of compound **7** (400 mg, 0.22 mmol) in a mixture of CH_2Cl_2/H_2O (17/1, v/v, 10 mL), was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 499 mg, 2.2 mmol) at 0 °C. After 30 min, the reaction mixture was warmed to rt and stirred for 24 h. On completion of the reaction, the mixture was diluted with CH_2Cl_2 (50 mL) and quenched with sat. Na_2CO_3 solution (10 mL). The organic phase was separated, washed with sat. Na_2CO_3 solution (1 x 50 mL), dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The crude compound was purified by column chromatography (eluent 1:9 v/v MeOH:CH_2Cl_2) to afford **8** as a colourless oil. (150 mg, 60%). **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 5.47 (m, 1H, BocNH), 5.15 (d, J = 3.4 Hz, 1H, H-1'), 5.12 (d, J = 3.4 Hz, 1H, H-1), 4.89 (t, J = 9.6 Hz, 1H, H-4'), 4.34 – 3.94 (m, 8H, GlyCH_2a, H-3, H-3', H-4, H-5', H-6, H-6'a, H-6'b), 3.81 (dd, J = 17.6, 4.5 Hz, 1H, GlyCH_2b), 3.69 (dd, J = 9.5, 3.6 Hz, 1H, H-2'), 3.61 (dd, J = 9.6, 3.7 Hz, 1H, H-2), 3.40 (dd, J = 8.8 Hz, 1H, H-5), 2.33 – 2.29 (m, 4H, 2 x BehCH_2α), 1.58 (m, 4H, 2 x BehCH_2β), 1.42 (s, 9H, $C(CH_3)_3$), 1.32 – 1.24 (m, 72H, 36 x BehCH_2), 0.87 (t, J = 6.9 Hz, 6H, 2 x BehCH_3). **^{13}C NMR** (126 MHz, $CDCl_3$) δ (ppm): 174.6 (BehCO), 174.0 (BehCO'), 170.2 (GlyCO), 156.8 (BocCONH), 93.3 (C-1'), 93.0 (C-1), 80.8 ($C(CH_3)_3$), 73.2 (C-3), 71.7 (C-2, C-2', C-4'), 71.2 (C-3'), 70.4 (C-5), 70.3 (C-4), 68.2 (C-5'), 63.3 (C-6), 62.0 (C-6'), 42.5 (GlyCH_2), 34.3 (BehCα), 34.1 (BehC'α), 32.1 (2 x BehCH_2), 29.9 - 29.3 (34 x BehCH_2), 28.4 ($C(CH_3)_3$), 25.0 (BehCH_2), 24.9 (BehCH_2), 22.8 (2 x BehCH_2), 14.2 (2 x BehCH_3) **HRMS** Calculated for $C_{63}H_{117}NO_{16}Na$ $[M+Na]^+$: 1166.8265, Found (ESI+) 1166.8271 $[M+Na]^+$; **IR** (ATR) ν_{max} = 3326, 2916, 2898, 1738, 1157 cm^{-1}; **$[\alpha]_D$**: -0.046 (c 1.0, CH_2Cl_2).

Synthesis of 6,6'-Di-behenoyl-4-glycinate-*α,α*-D-trehalose (1)



To a solution of compound **8** (75 mg, 65 μmol) in CH_2Cl_2 (5 mL) at 0 °C was added TFA (5 mL). The reaction was warmed to rt and stirred for 20 min. Following complete conversion of the starting material as judged by TLC, the reaction was diluted with toluene (10 mL) and concentrated *in vacuo* to afford compound **1** as the corresponding trifluoroacetate salt (68 mg, 99%). **^{1}H NMR** (500 MHz, $CDCl_3$) δ (ppm): 5.18 - 5.16 (m, 1H), 5.13 (d, *J* = 3.6 Hz, 1H), 4.92 (t, *J* = 9.8 Hz, 1H), 4.38 – 4.32 (m, 2H), 4.28 – 3.95 (m, 6H), 3.77 – 3.69 (m, 4H), 3.62 (d, *J* = 9.3 Hz, 1H), 3.37 (d, *J* = 8.2 Hz, 1H), 2.31 (dt, *J* = 22.4, 7.6 Hz, 4H), 1.78 (br, 2H), 1.59 – 1.57 (m, 4H), 1.25 (d, *J* = 3.3 Hz, 80H), 0.88 (t, *J* = 6.9 Hz, 6H); **^{13}C NMR** (126 MHz, $CDCl_3$) δ (ppm): 173.9, 173.8, 170.7, 93.4, 93.3, 77.4, 77.2, 76.9, 72.1, 71.9, 71.4, 70.8, 70.5, 68.6, 67.0, 63.3, 34.3, 34.2, 32.1, 30.0 - 29.3 (32 resonances), 25.0, 24.9, 24.9, 22.8, 14.3, 14.3; **HRMS** Calculated for $C_{58}H_{110}NO_{14}$ $[M+H]^+$: 1045.7921, Found (ESI+) 1045.7922 $[M+H]^+$; **IR** (ATR) ν_{max} = 3360, 2931, 2900, 1626, 1471, 1171, 1150, 1080, 979, 772.

Synthesis of TDB-ESAT6$_{(1-20)}$ Conjugate Vaccine (9)



Preloaded Fmoc-Gly-Chemmatrix Wang resin (25 μmol, 0.42 mmol/g) was swollen in dry CH_2Cl_2 for 30 min and then washed with CH_2Cl_2 (5 x 3 mL) and then DMF (5 × 3 mL). The peptide chain was extended to the final amino triethyleneglycolate residue *via* automated solid phase peptide synthesis using a Biotage Initiator+ Alstra microwave peptide synthesiser. The resin (25 μmol scale) was treated with a solution of 4-nitrophenyl chloroformate (25 mg, 0.125 mmol) and *i*Pr$_2$NEt (21 μL, 0.125 mmol) in CH_2Cl_2 (2 mL) for 2 hours, after which it was washed with CH_2Cl_2 (10 x 5 mL). To the resin was then added a solution of compound **1** (28.7 mg, 27.5 μmol) and *i*Pr$_2$NEt (8 μL, 50 μmol) in anhydrous THF (30 μL) and was incubated at 40 ºC for 24 h. Upon completion, the resin was washed with CH_2Cl_2 (5 x 5 mL), DMF (5 x 5 mL) and CH_2Cl_2 (15 x 5 mL). The peptide was cleaved from resin by treatment with an acidic cocktail containing TFA/triisopropylsilane/water (90:5:5 v/v/v) with gentle shaking for 2 h. The cleavage solution was filtered and the resin was washed with TFA (2 x 2 mL). The flow through was concentrated under a stream of nitrogen and then precipitated on addition of cold diethyl ether. The mixture was centrifuged and the supernatant was discarded. The dried pellet was dissolved in water containing 40% MeCN + 0.1% TFA, purified by preparative RP-HPLC (Phenomenex Luna® C18(2) (5 μm, 100Å, 10 x 250 mm), 20-100% B over 40 min, 4 mL min^{-1}, 0.1% TFA + 10% *i*PrOH, 40 °C) and lyophilised to afford the desired TDB-ESAT6(1-20) conjugate **9** as a fluffy off-white solid (1.20 mg, 1.8%). **Analytical HPLC**: Rt 13.2 min (C4 Symmetry Column, 20-100% B over 15 min, 0.2 mL min^{-1}, 0.1% TFA, λ = 214 nm); **ESI-MS** Calculated for $C_{187}H_{316}N_{36}O_{56}S$ $[M+2H]^{2+}$: 1999.4, $[M+3H]^{3+}$: 1333.2, $[M+4H]^{4+}$: 1000.2; Found (ESI+) 1999.4 $[M+2H]^{2+}$, 1333.2 $[M+3H]^{3+}$, 1000.2 $[M+4H]^{4+}$; **MS (MALDI-TOF)** Calculated for $C_{187}H_{316}N_{36}O_{56}SNa$ $[M+Na]^{+}$: 4017.260; Found (MALDI-TOF): 4017.529 $[M+Na]^{+}$, 3176.992 (fragmentation artifact).

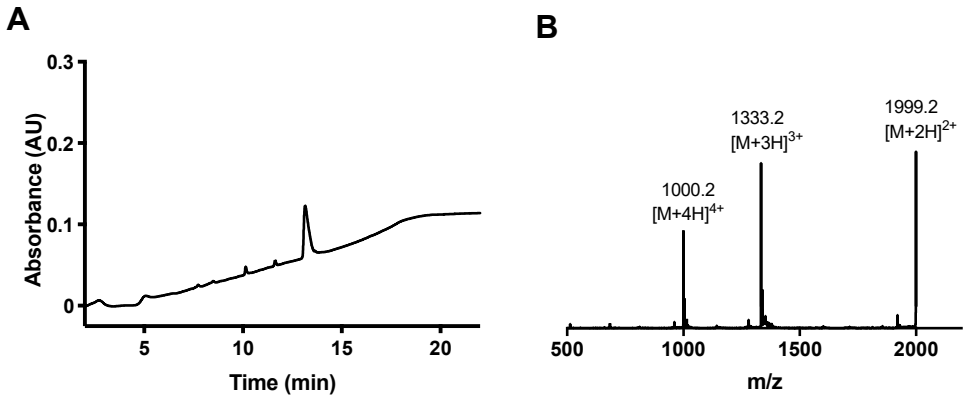


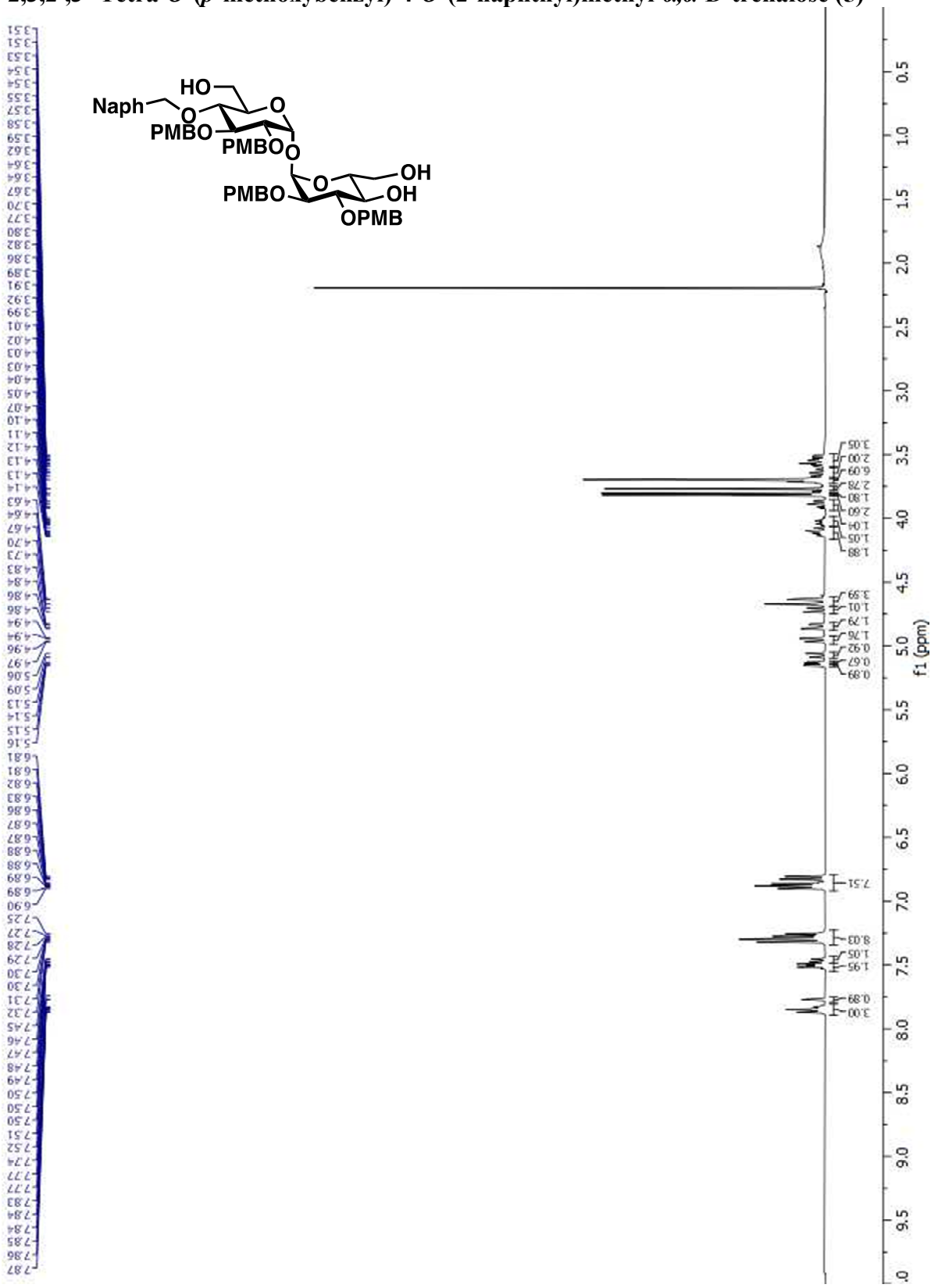
Figure S1. **A**: Analytical HPLC of purified TDB-ESAT6(1-20) conjugate vaccine **10**. **B**: ESI-MS of purified TDB-ESAT6(1-20) conjugate vaccine **10.**

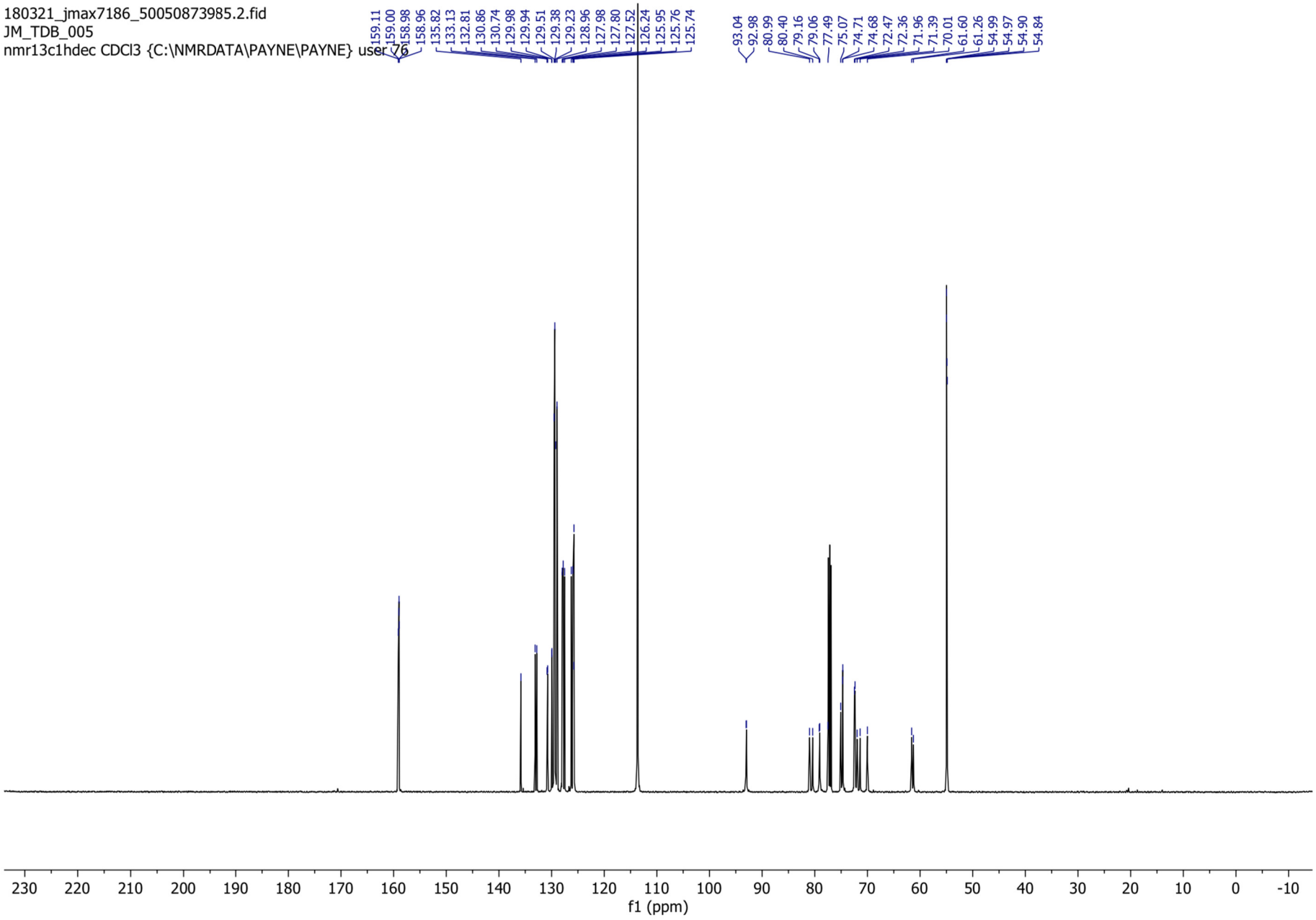
Mincle reporter cell binding assays

Human or mouse Mincle co-expressed with FcRγ in 2B4-NFAT-GFP reporter cells as well as FcRγ only negative control were prepared as described previously.[2] TDB or TDB-ESAT6 $_{(1\text{-}20)}$ (**2**) were dissolved in chloroform:methanol (2:1 v/v) at 0.001, 0.01, 0.1, or 1 nmol and were then added to 96-well plates (20 μL), followed by solvent evaporation. Reporter cells were then added to each well and incubated at 37°C with 5% CO_2 for 20 hours. NFAT-GFP expression was measured by flow cytometry.

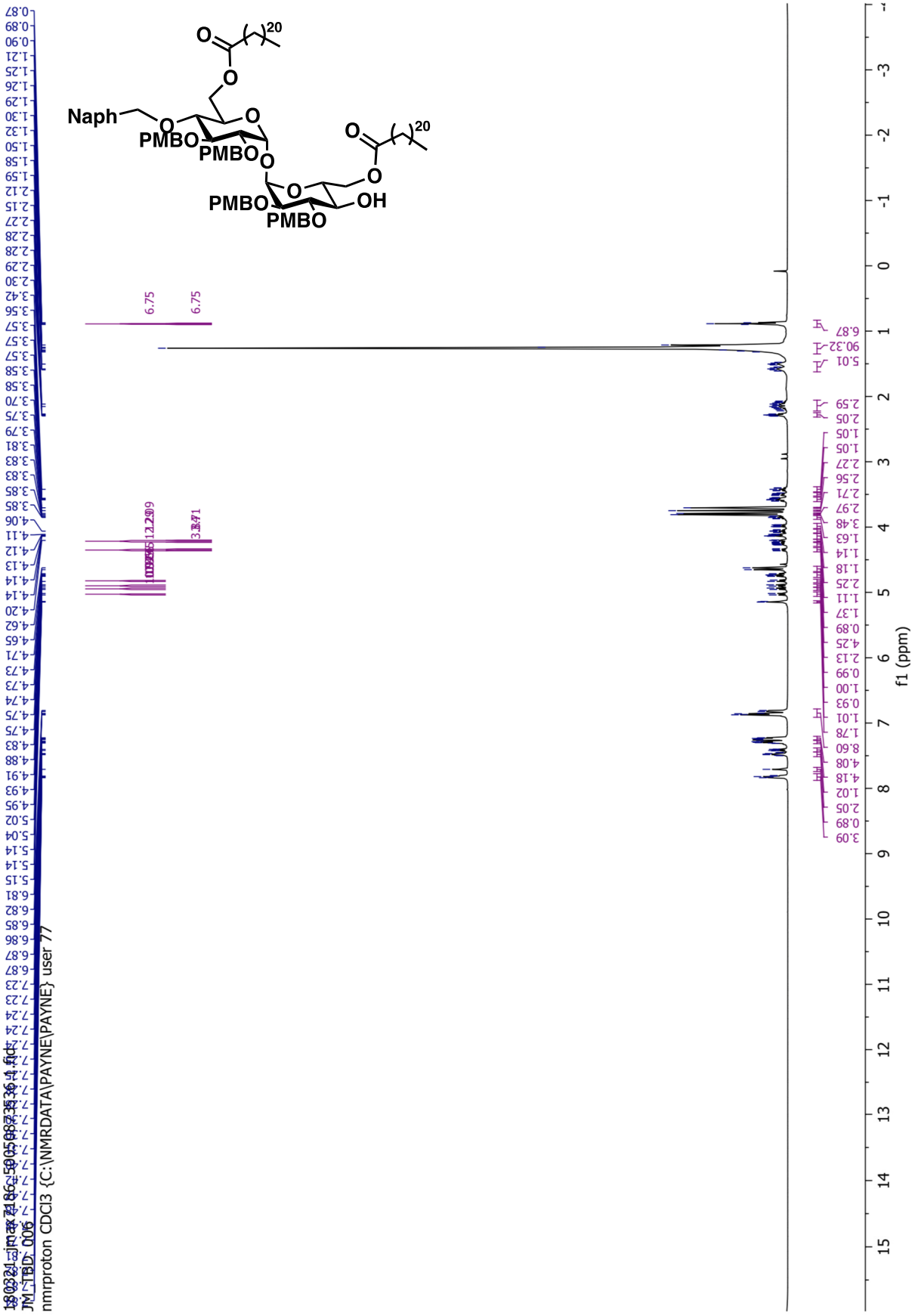
NMR Spectra of novel TDB analogue precursors and C4-functionalised TDB (1)

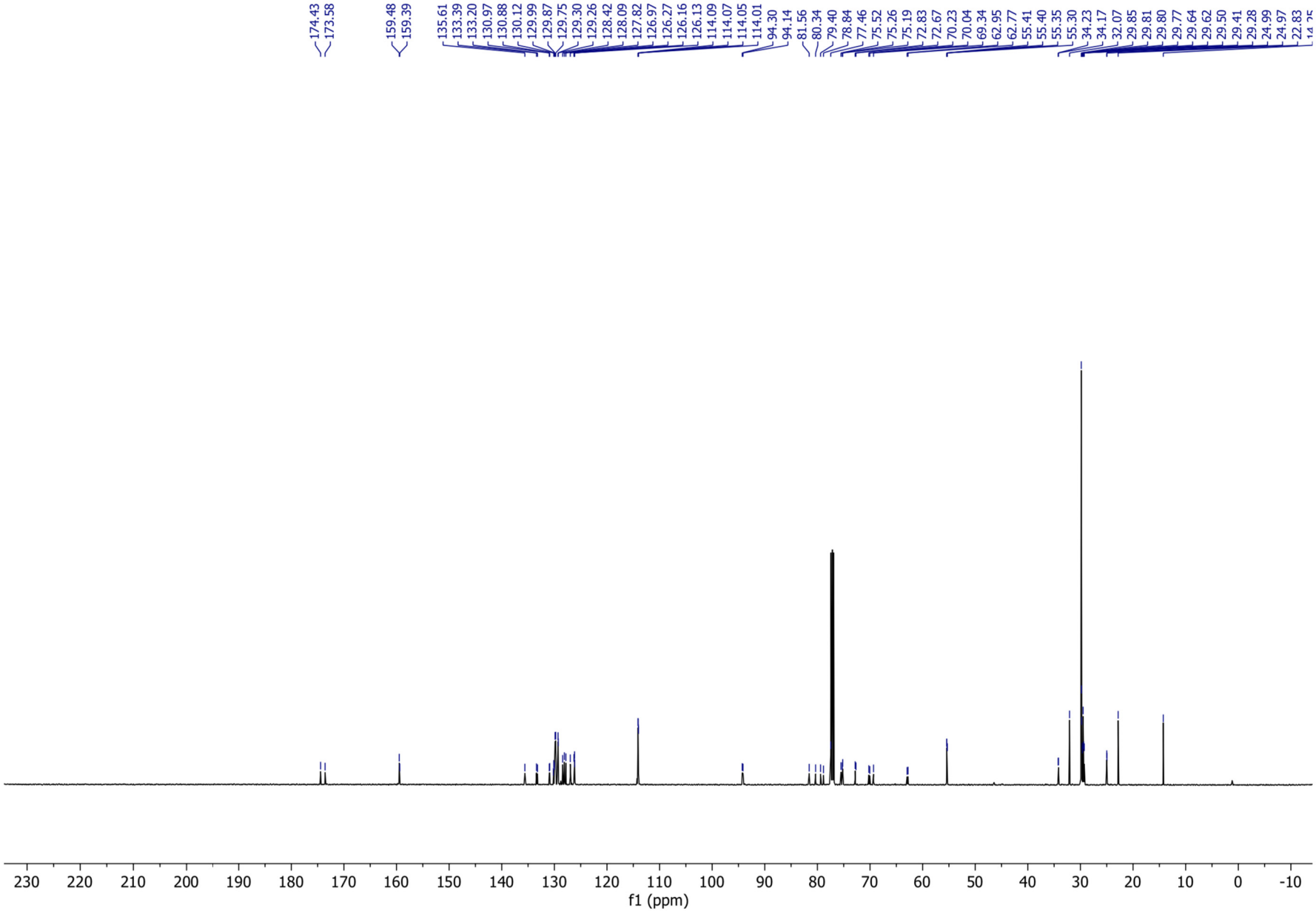
2,3,2′,3′-Tetra-*O*-(*p*-methoxybenzyl)-4-*O*-(2-naphthyl)methyl-*α*,*α*-D-trehalose (5)



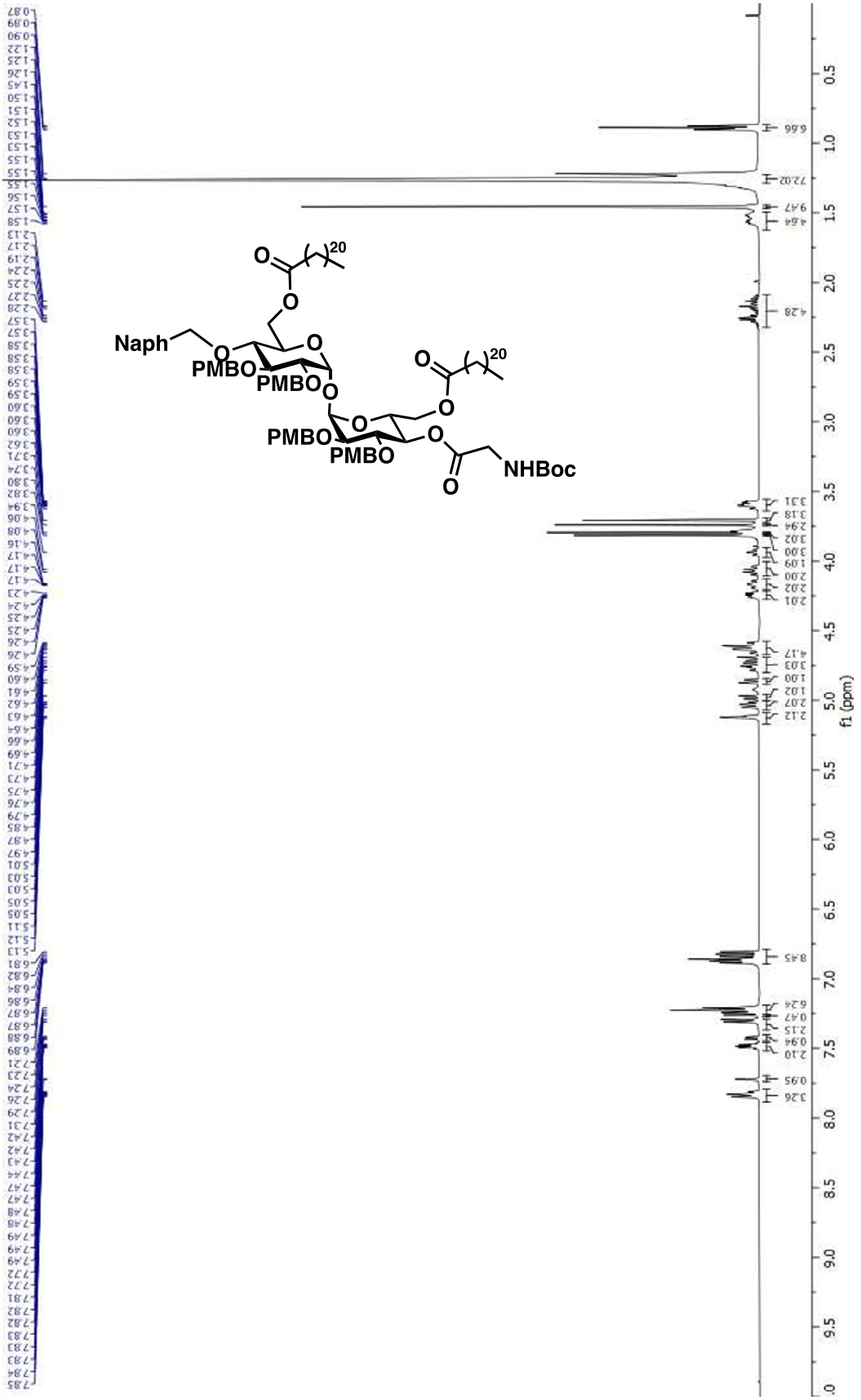

180321_jmax7186_50050873985.2.fid
JM_TDB_005
nmr13c1hdec CDCl3 {C:\NMRDATA\PAYNE\PAYNE} user 76
159.11
159.00
158.98
158.96
135.82
133.13
132.81
130.86
130.74
129.98
129.94
129.51
129.38
129.23
128.96
127.98
127.80
127.52
126.24
125.95
125.76
125.74
93.04
92.98
80.99
80.40
79.16
79.06
77.49
75.07
74.71
74.68
72.47
72.36
71.96
71.39
70.01
61.60
61.26
54.99
54.97
54.90
54.84
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

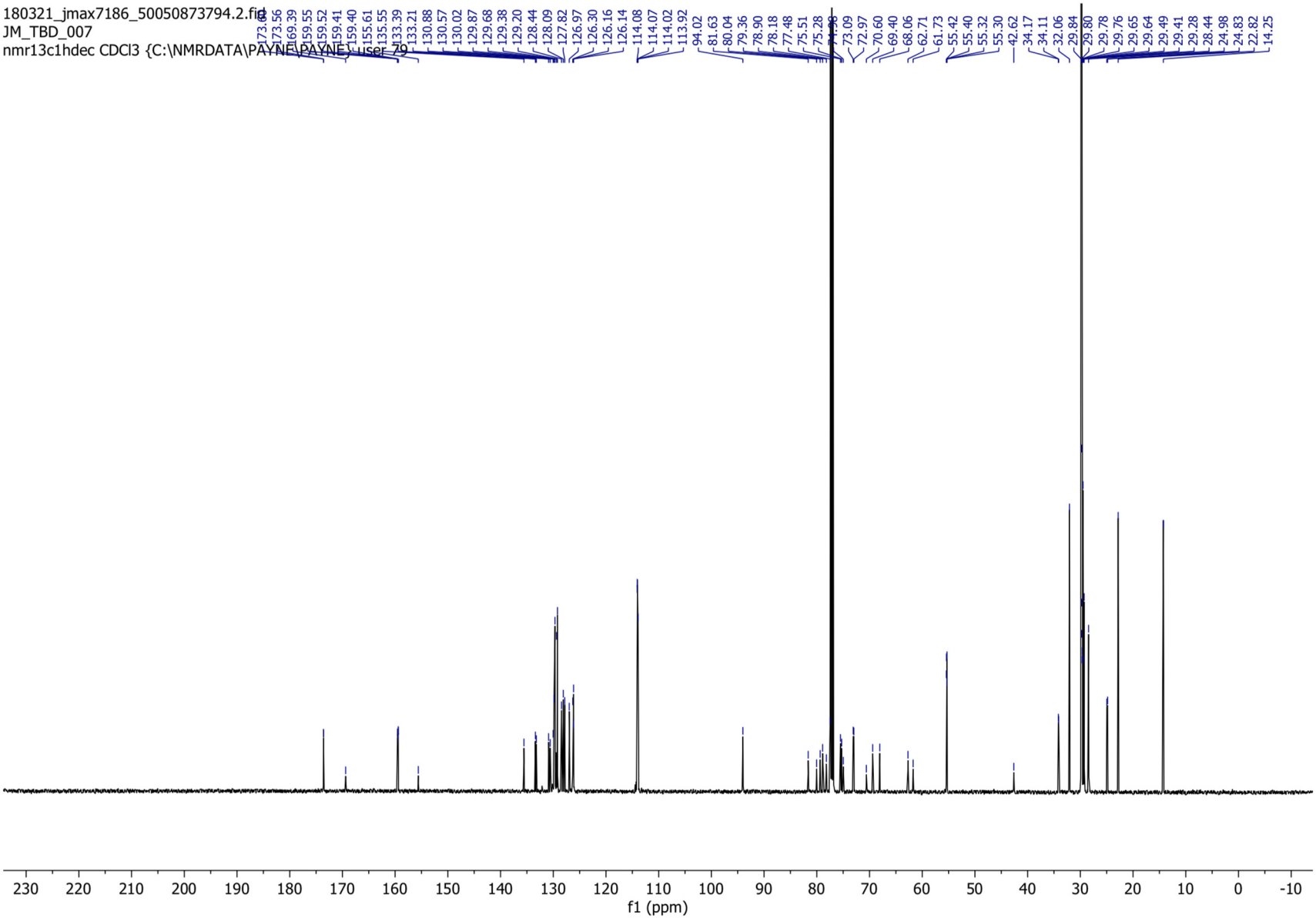
6,6'-Di-behenoyl-2,3,2′,3′-Tetra-*O*-(*p*-methoxybenzyl)-4-*O*-(2-naphthyl)methyl-*α*,*α*-D-trehalose (6)



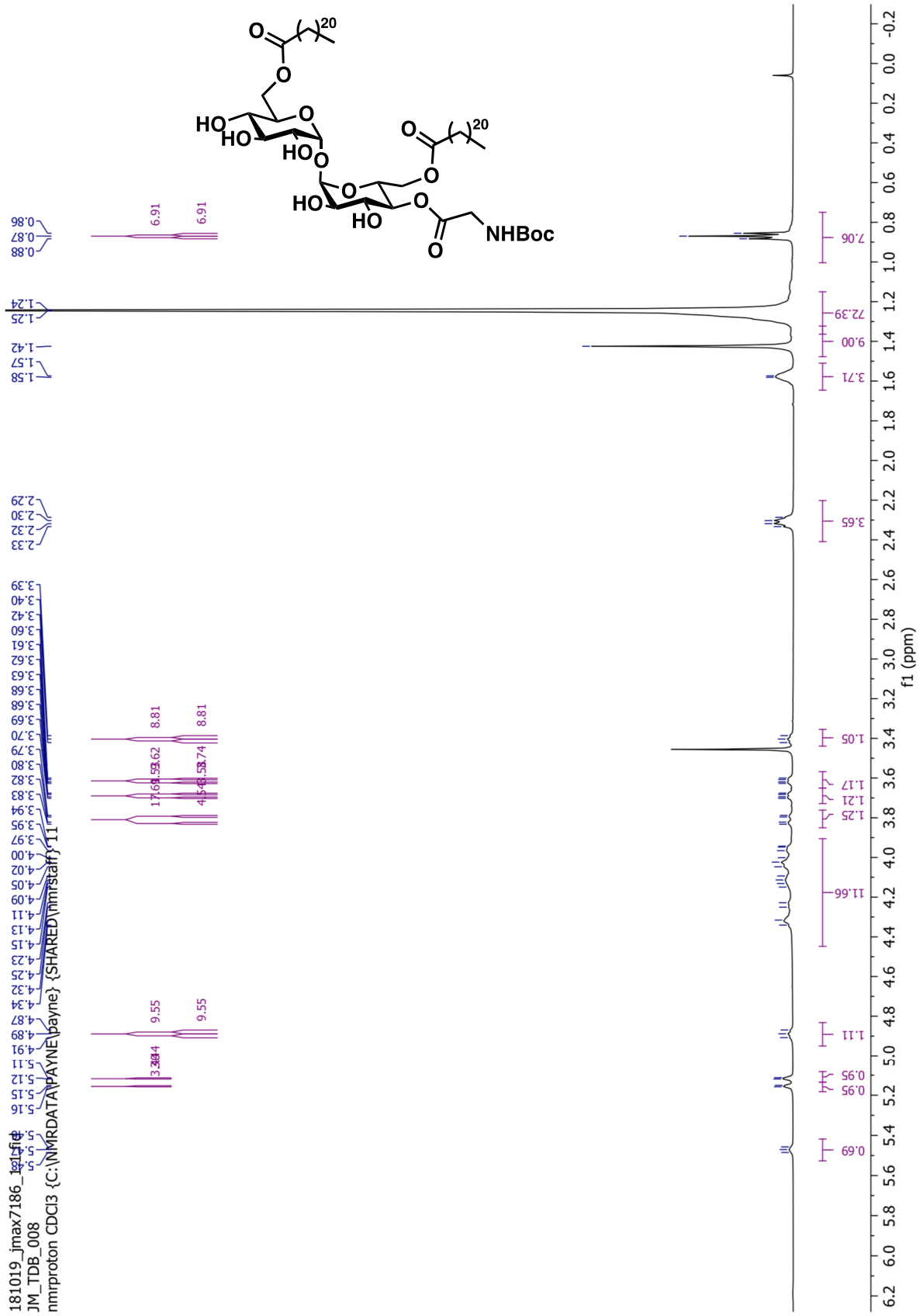

174.43
173.58
159.48
159.39
135.61
133.39
133.20
130.97
130.88
130.12
129.99
129.87
129.75
129.30
129.26
128.42
128.09
127.82
126.97
126.27
126.16
126.13
114.09
114.07
114.05
114.01
94.30
94.14
81.56
80.34
79.40
78.84
77.46
75.52
75.26
75.19
72.83
72.67
70.23
70.04
69.34
62.95
62.77
55.41
55.40
55.35
55.30
34.23
34.17
32.07
29.85
29.81
29.80
29.77
29.64
29.62
29.50
29.41
29.28
24.99
24.97
22.83
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

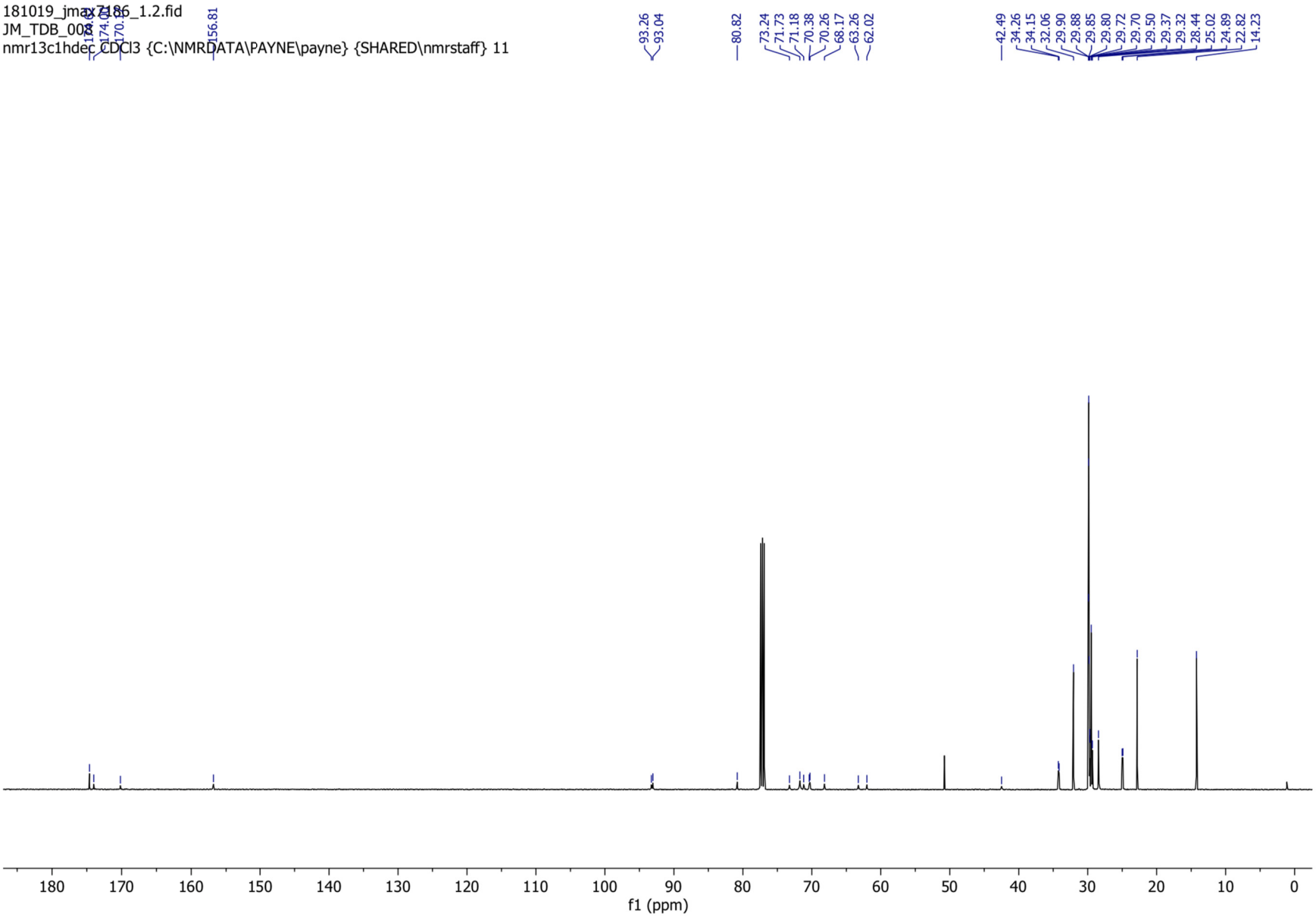
6,6'-dibehenoyl-4-(*tert*-butoxycarbonyl)glycinate-2,3,2′,3′-tetra-*O*-(*p*-methoxybenzyl)-4-*O*-(2-naphthylmethyl)-*α*,*α*-D-trehalose (7)



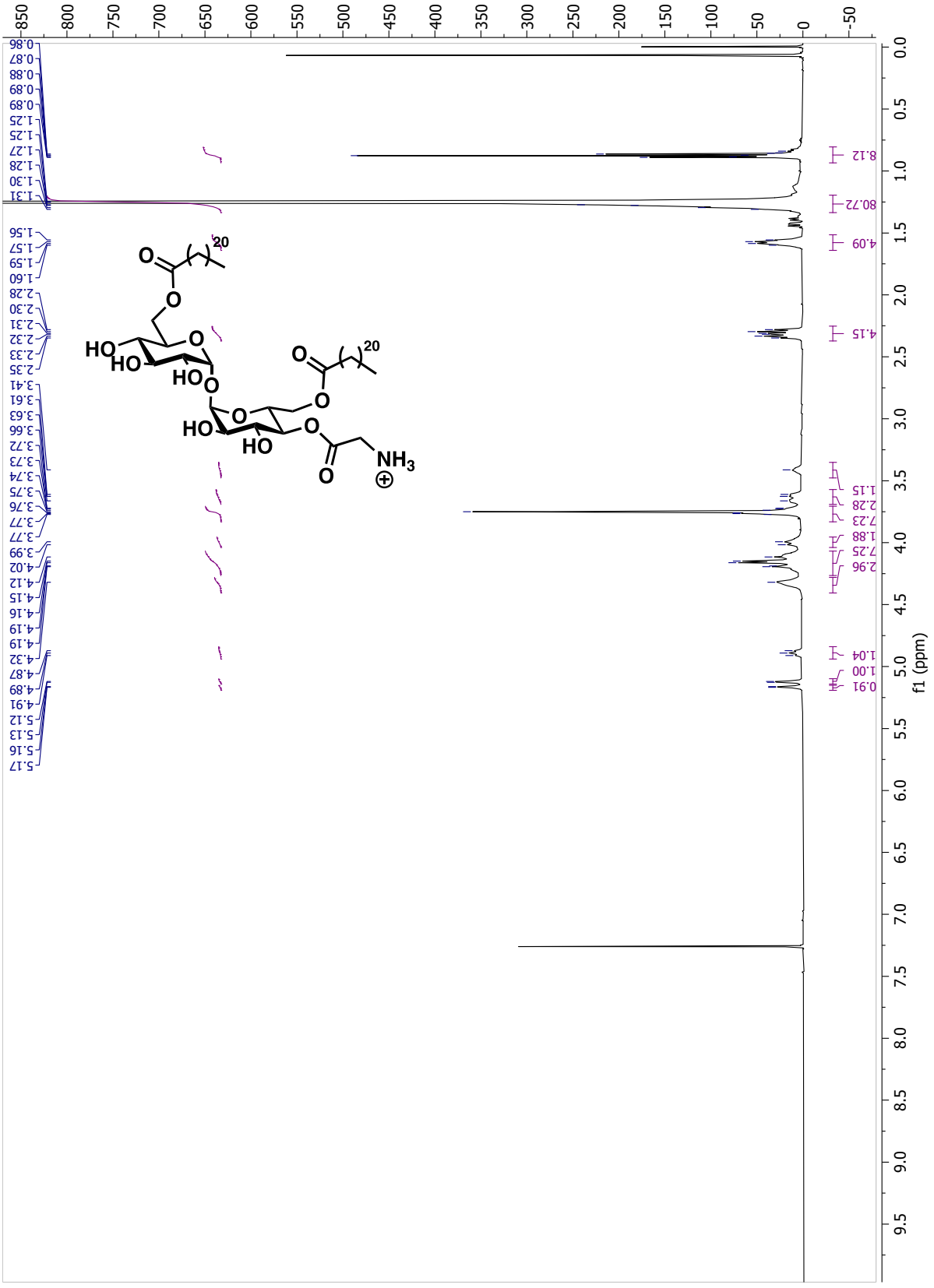

180321_jmax7186_50050873794.2.fid
JM_TBD_007
nmr13c1hdec CDCl3 {C:\NMRDATA\PAYNE\PAYNE\ user 79}
173.68
173.56
169.39
159.55
159.52
159.41
159.40
155.61
135.55
133.39
133.21
130.88
130.57
130.02
129.87
129.68
129.38
129.20
128.44
128.09
127.82
126.97
126.30
126.16
126.14
114.08
114.07
114.02
113.92
94.02
81.63
80.04
79.36
78.90
78.18
77.48
75.51
75.28
73.09
72.97
70.60
69.40
68.06
62.71
61.73
55.42
55.40
55.32
55.30
42.62
34.17
34.11
32.06
29.84
29.80
29.78
29.76
29.65
29.64
29.49
29.41
29.28
28.44
24.98
24.83
22.82
14.25
230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

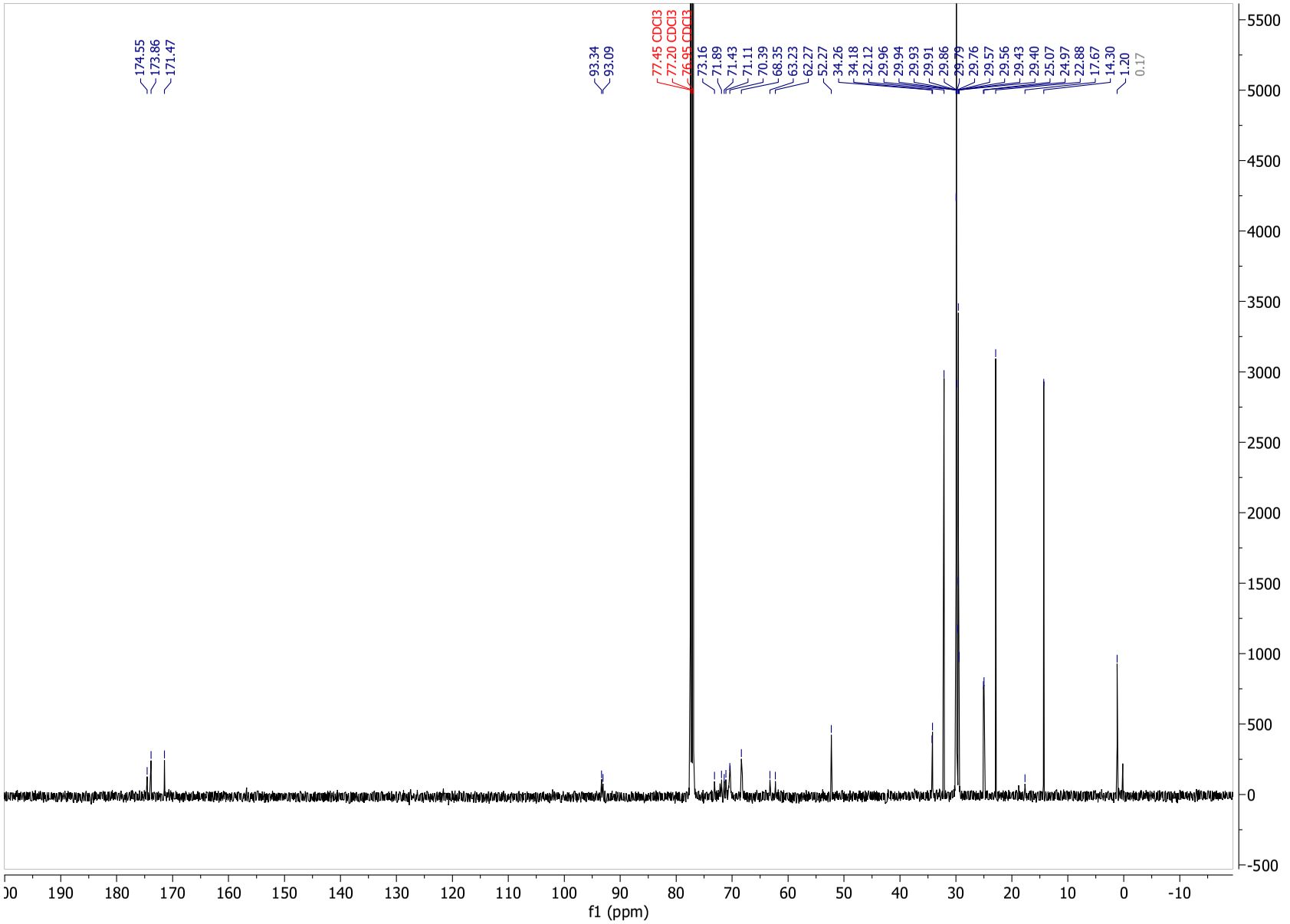
6,6'-Di-behenoyl-4-(*tert*-butoxycarbonyl)glycinate-*α,α*-D-trehalose (8)




181019_jmax7186_1.2.fid
JM_TDB_008
nmr13c1hdec CDCl3 {C:\NMRDATA\PAYNE\payne} {SHARED\nmrstaff} 11
174.04
170.81
156.81
93.26
93.04
80.82
73.24
71.73
71.18
70.38
70.26
68.17
63.26
62.02
42.49
34.26
34.15
32.06
29.90
29.88
29.85
29.80
29.72
29.70
29.50
29.37
29.32
28.44
25.02
24.89
22.82
14.23
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

6,6'-Di-behenoyl-4-glycinate-*α,α*-D-trehalose (1)




174.55
173.86
171.47
93.34
93.09
77.45 CDCl3
77.20 CDCl3
76.95 CDCl3
73.16
71.89
71.43
71.11
70.39
68.35
63.23
62.27
52.27
34.26
34.18
32.12
29.96
29.94
29.93
29.91
29.86
29.79
29.76
29.57
29.56
29.43
29.40
25.07
24.97
22.88
17.67
14.30
1.20
0.17
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

References:

1. Sarpe, V. A., Kulkarni, S. S., *Org. Biomol. Chem.*, 2013, **11**, 6460-6465.

2. E. Ishikawa, T. Ishikawa, Y. S. Morita, K. Toyonaga, H. Yamada, O. Takeuchi, T. Kinoshita, S. Akira, Y. Yoshikai and S. Yamasaki, *J. Exp. Med.*, 2009, **206**, 2879-2888.