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Design and synthesis of a series of novel, cationic liposaccharide derivatives as potential penetration enhancers for oral drug delivery

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ABSTRACT

Oral delivery remains a challenge for a number of drug candidates with low absorption profile (poor membrane permeability, lack of stability, solubility issues or susceptibility to enzymatic degradation) and various methodologies have been investigated to overcome it. The approach presented here consists of associating, by ion-pairing, a hydrophilic, ionizable model drug to a series of synthetic counter-ionic entities with a controlled degree of lipophilicity in order to enhance its penetration of biomembranes and offer some protection against in situ degradation, while preserving its biologically active chemical structure. We report herein the synthesis of a series of positively charged potential penetration enhancers designed from D-glucose, 2-aminododecanoic acid, and additional lipophilic amino acids, and converted afterward into quaternary ammoniums in an optimized, innovative one-step reaction. Each liposaccharide derivative synthesized was fully characterized and their ability to generate micelles in solution was assessed by isothermal titration calorimetry.

1. Introduction

Oral delivery is the method of choice for drug administration as it generally results in lower production costs, ease of administration, and better patient compliance. Unfortunately, a large number of molecules with promising therapeutic potential fail to reach the oral drug market by lack of absorption and poor oral bioavailability, leading to their abandonment in early clinical or research phase, or to their development for parenteral administration only. Often, this poor bioavailability is linked to high hydrophilicity and/or low enzymatic resistance and common approaches to overcome these issues aim at increasing the lipophilicity of these molecules (log *P*), thereby facilitating their penetration across the intestinal epithelium by passive diffusion.[1–3]

Improvements to the lipophilicity can be achieved by chemically modifying the structure of the active compound (derivation by esterification or lipidation, for example), which can also lead to improved resistance to enzymatic degradation, however additionally carries the risk of altering the overall biological properties (reduction or loss of activity).[4]

An alternative consists in formulating the active compound with penetration enhancing excipients, such as surfactants or ion-pairing agents.[5,6] Ross et al.[7] and Mrestani et al.[2] have previously reported improvement in permeability and absorption of β-lactam antibiotics when associated with cationic lipophilic entities such as quaternary ammonium bromides or chlorides. Recently, we investigated the effect of a series of positively charged glucolipids on the membrane permeability of piperacillin in Caco-2 monolayers, a common in vitro model of intestinal epithelium.[8] Significant increases in apparent permeability coefficients were observed, notably with a glucose-based derivative containing a 12-carbon long lipid chain, however the permeability values were still low ($<10^{-6}$ cm s^{-1}), indicating that if administered orally, these formulations would unlikely be fully absorbed.[9] Furthermore, the pH gradient residing in the gastro-intestinal tract might cause the drug–glucolipid entities to dissociate as one of the residue could be partially neutralized, negatively affecting the permeability enhancing effect of the designed glucolipids.

In an attempt to design more suitable and efficient liposaccharide-based penetration enhancers, a novel series of compounds were synthesized. Based on our in vitro results, the D-glucose unit was preserved as well as the dodecanoic (C_{12}) lipoamino acid residue. An additional lipophilic moiety, namely leucine, phenylalanine (two of the most lipophilic natural amino acids)[†] or a second C_{12}, was introduced. The N-terminal amino group was then converted into a quaternary ammonium, hence

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[†] http://www.sigmaaldrich.com/life-science/metabolomics/learning-center/amino-acid-reference-chart.html.

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ensuring that the resulting cationic liposaccharide derivative remains positively charged in basic conditions (intestine). The amphoteric character of the designed charged liposaccharides is responsible for their surfactant properties—hence their alternative descriptive of 'sugar surfactants'—but also makes them prone to aggregate or micellize in solution.[10,11] Measurements by isothermal titration calorimetry (ITC) were therefore performed to determine the critical aggregate concentration (CAC) of each entity. Energy parameters were also calculated—enthalpy of micellization (ΔH), entropy (ΔS), and Gibbs free energy (ΔG).

2. Results and discussion

2.1. Chemistry

The series of liposaccharide derivatives were prepared in six steps from peracetylated β-D-glucosyl azide **1**[12] as presented in Scheme 1. The tributylphosphine used as a reducing agent by Falconer et al.[12] to yield peracetylated glucose amine **2**, and later its Boc-protected conjugate **3**, proved to yield several by-products, relatively difficult to isolate, hence a new coupling pathway was investigated. The azido residue was reduced by catalytic hydrogenation to afford β-D-glucosyl amine **2**, which was next reacted with (*R*,*S*)-N^{α}-Boc-dodecanoic acid (Boc-C_{12}) in the presence of diisopropylethylamine (DIPEA) and *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU).[12,13] The *tert*-butyloxycarbonyl protecting group was then removed with trifluoroacetic acid to yield amine **4** in nearly quantitative yield. The coupling of the second amino acid residue was performed similarly to give the peracetylated diastereomeric liposaccharide derivatives **6a**–**c**, with yields ranging from 59 to 65%. Due to the reactivity of the free amino residues with the acetyl group present on the glucose scaffold, compounds **6a**–**c** were subsequently used without further purification.

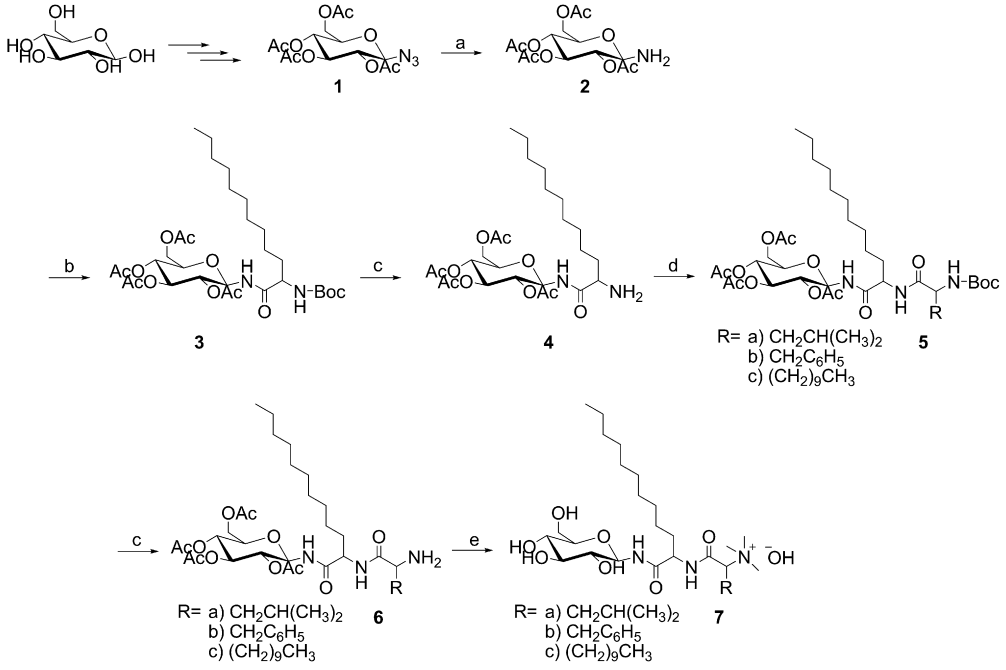
General O-deacetylation methods report the use of bases such as NH_3[14] or triethylamine[15] in methanol, NaOH/pyridine/ethanol,[16] K_2CO_3 in methanol,[17] and various alkali,[18] e.g., sodium methoxide (Zemplén conditions). However, these conventional bases tend to accelerate the *O*–*N*-acyl migration when utilized in polar solvents such as methanol.[19] Anion exchange Amberlite resin IRA-400[HO^-] was considered as an alternative to carry out the deacetylation of amines **6a**–**c**,[20] but did not prevent the formation of the acetamide by-product.

We then looked at ways to perform both the deacetylation and the alkylation into ammoniums in one step. The use of an excess of alkyl halide in the presence of inorganic bases such as K_2CO_3 or NaOH was attempted;[21,22] however, the organic and inorganic salts generated by this method proved very difficult to separate, due to comparable solubility in most common solvents. Another method published by Cowling et al.[23] reported using the sterically hindered organic base 2,6-lutidine to generate methylated quaternary ammonium derivatives from amines, but this proved unsuccessful when attempted with compounds **6a**–**c**.

We therefore developed an easy, practical methodology to prepare our target methylated quaternary ammoniums by reacting excess methyl iodide in the presence of Amberlite resin IRA-400[HO^-] in methanol. This novel approach allows both reactions (deacetylation and alkylation) to be carried out simultaneously and requires only a simple filtration to remove the resin. The excess methyl iodide was evaporated along with the solvent to afford the titled products in nearly quantitative yields. Additionally, the Amberlite resin can easily be reused after regeneration with 1 M NaOH and distilled water. Ammoniums **7a**–**c** were prepared following this new method and later lyophilized from acetonitrile/water.

2.2. Critical aggregation concentration (CAC)

Since the designed liposaccharide derivatives possess both hydrophilic and lipophilic moieties, they are susceptible to either aggregation or micellization under aqueous conditions, which could potentially influence their interactions with the drugs they would be associated to, both ex vivo and in situ. Determining their critical aggregation concentration (CAC) in aqueous solution was therefore an important step in predicting the type of interactions which would occur when associating the liposaccharide derivative to a model drug. In the sub-aggregate range of concentrations, ion-



Scheme 1. Synthesis of a series of positively charged liposaccharide derivatives and subsequent quaternisation. Reagents and conditions: (a) H_2, 10% Pd/C, DCM, 12 h; (b) N^{α}-Boc-aminododecanoic acid (1.75 equiv), HBTU 2 equiv, DIPEA (3.5 equiv), DCM, 24 h; (c) TFA/DCM (1:1), 1 h; (d) N^{α}-Boc-NH–CH(R)–COOH (2 equiv), HBTU (2.2 equiv), DIPEA (4 equiv), DCM, 24 h; (e) CH_3I, CH_3OH, Amberlite IRA-400[HO^-], 48 h.

pairing is usually a favored process, despite possibilities of premicellar aggregation.

When reaching the CAC and above, the hydrophobic intramolecular interactions between the lipid chains of the liposaccharide derivatives become predominant and can lead to phenomena of aggregation and/or micellization, both strongly impacting the permeation into and through biological membranes.[24–26]

Measurements of CAC also provide some insights into the potential toxicity of the designed penetration enhancers, notably likelihood to cause disruptions to biological membranes due to their surfactant properties.[27,28] A number of quantification methods of the critical micellar concentration (CMC) of ionic or non-ionic surfactants in aqueous systems have been reported.[29] In a previous study on sugar/lipoamino acid conjugates, Ross et al. determined the CMC of synthetic ionic surfactants using isothermal titration calorimetry (ITC), a sensitive, routine method which additionally provides access to thermodynamic parameters such as enthalpy and entropy of micellization.[7] Using a similar approach, we conducted ITC measurements for compounds **7a**–**c** in deionized water; CAC values were then determined according to the method of Van Os, where the CAC is represented by a change of slope in the cumulative enthalpy plot.[30]

The calorimetric titration of **7c**, comprising two C_{12} moieties, is presented in Figure 1. The CAC value of **7c** was found to be 0.275±0.010 mM, which is in agreement with the 0.01–10 mM concentration range commonly observed for glycolipids.[7,31–33]

When examining conjugates **7a** and **7b**, variations in heat exchanged were detected but the enthalpy of aggregate formation was so low (<0.5 kcal mol^{-1}) that the CAC could not be accurately estimated. Modifications to the experimental conditions (injection volumes (5–15 μL) and duration (10–30 s), starting concentrations (4–8 mM)) did not yield better results. It was hypothesized that either these two liposaccharide derivatives did not aggregate or that the aggregates formed were not stable enough in water to be detected by ITC.

Characterization of the aggregates was also attempted separately using Zetasizer measurements in water (results not shown). The high polydispersity indexes observed in the broad range of concentrations used for all liposaccharides (from 0.1 mM to 8 mM) did not enable to conclude on the CAC of the aggregates formed.

The ITC findings can be related to the lipophilicity of the designed molecules: using the log *P* predictor of ACD/Labs (v12.01) estimates of partition coefficients values were obtained for **7a**, **7b** and **7c**, and found to be, respectively, 0.64 (±0.68), 1.69 (±0.71), and 3.44 (±0.68); similar values were obtained with the ALogPS 2.1 program (Virtual Computational Chemistry Laboratory 2007).[34] Based on these theoretical values, **7c** would be more likely to undergo aggregation in water-based media than **7a** or **7b**, owing to its significantly higher lipophilicity.

As mentioned above, ITC additionally allows direct observation of enthalpy from the heat/titration plots. The measured enthalpy reflects the contribution of individual interactions occurring between the liposaccharide molecules and is a function of compound concentration (mM) in the calorimeter cell.

For **7c** ΔH_{mic} was found to be −2.60±0.10 kcal mol^{-1} (−10.88±0.41 kJ mol^{-1}). The negative sign indicates an exothermic process in which intermolecular interactions between the aggregating molecules overpower the solvation process. Negative enthalpy can generally be attributed to increased van der Waals and hydrophobic interactions, which actively contribute to micelle formation.[35]

To determine if the binding process observed is thermodynamically favored, the Gibbs free energy of aggregation (ΔG_{agg}) needs to be considered. It can be calculated by Eq. 1:

$$\Delta G_{agg} = RT \ln x_{CAC} \quad (1)$$

where R is the gas constant (8.314 J K^{-1} mol^{-1}), T the absolute temperature (K), and x_{CAC} the CAC value in mole fraction units.

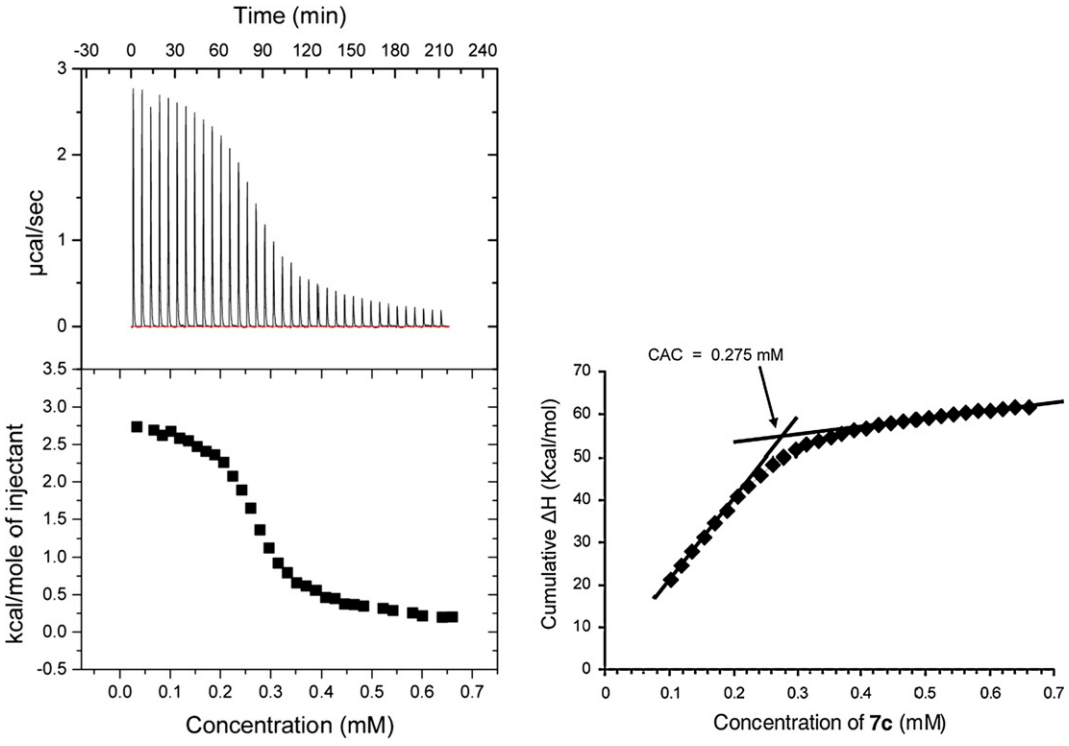


Figure 1. Calorimetric titration of compound **7c**, picturing the sequence of injections (left top panel) and enthalpy curve (left lower panel). Cumulative enthalpy values were then plotted versus concentration (right) to determine the CAC (defined as the concentration at which the two tangents at the extremities of the curve intersect).[7]

The negative value found for **7c**, specifically ΔG_{agg}=−7.52 ±0.03 kcal mol^{-1} (−31.46±0.12 kJ mol^{-1}), shows that favorable changes are occurring during the aggregation process, leading to the formation of stabilized entities in the aqueous environment.

The determination of ΔG_{agg} and ΔH_{agg} leads to the calculation of the entropy of micellization, ΔS_{agg}, using Eq. 2:

$$\Delta S_{agg} = \frac{\Delta H_{agg} - \Delta G_{agg}}{T} \quad (2)$$

Like enthalpy, entropy is strongly dependent on temperature. In the case of **7c**, ΔS_{mic} was found to be 15.9±0.4 cal K^{-1} mol^{-1} (66.5±1.6 J K^{-1} mol^{-1}). The positive entropy change implies a decrease in the general degree of order in the system, which can be partially explained by the desolvation process associated with the pairing of molecules.[36] The release of solvent molecules (water) from the forming aggregates along with a reduction of the degree of freedom of the aggregated amphiphiles usually leads to an increase in entropy.[37,38] The size and shape of the aggregates as well as their distribution in the solvent could also influence the entropy.

When looking at $-T\Delta S_{mic}$ though, the negative value returned contributes to lowering the Gibbs free energy, thereby favoring aggregation process.

3. Conclusion

Three novel liposaccharide derivatives containing a quaternary ammonium residue at the N-terminus were successfully prepared using a novel, efficient strategy to carry out the glucose deacetylation and the quaternisation of the free amino group in one step utilizing Amberlite IRA-400[HO$^-$] combined with excess alkyl halide. This methodology avoided the *O*–*N*-acyl migration side reaction commonly observed when undertaking the deacetylation of amino sugars and greatly simplified the isolation of the expected products with no further purification required.

The ability of the synthesized amphiphilic molecules to aggregate in aqueous solution was assessed by isothermal titration calorimetry. The critical aggregation concentration and thermodynamics parameters associated with the aggregation process (ΔG_{mic}, ΔH_{mic} and ΔS_{mic}) were determined for one of the liposaccharide derivatives, **7c**, comprising of two C_{12} moieties (aminododecanoic acid). The relatively low CAC value found (0.275 mM) suggested a good aptitude of that amphiphile to aggregate; this was confirmed by determination of the thermodynamical parameters, which indicated a dual contribution of both enthalpy and entropy to the aggregation process. However, such results could not be experimentally obtained for the other two liposaccharide derivatives, **7a** and **7b**, suggesting that either aggregation was not occurring or that the aggregates formed were not stable enough to be assessed.

Further investigations, including in vitro and in vivo evaluations, of the synthesized liposaccharide derivatives will be carried out to examine their biological effect when admixed to ionizable model drugs and their capacity to act as potential oral penetration enhancers.

4. Experimental section

4.1. Chemicals and equipment

Peptide synthesis solvent, *N*,*N*-dimethylformamide (DMF), and reagents, trifluoroacetic acid (TFA) and diisopropylethylamine (DIPEA), were purchased from Auspep (Melbourne, VIC, Australia). *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and di-*tert*-butyl dicarbonate (Boc_2O) were obtained from GL Biochem Ltd (Shanghai, China). N^{α}-Boc-protected amino acids were supplied by Novabiochem (Läufelfingen, Switzerland).

Palladium (10% wt on carbon) was purchased from Lancaster Synthesis (Lancashire, England) and Amberlite IR 400 [$^-$OH] was provided by the British Drug Houses (BDH) Ltd (England). Ultra pure gases (N_2, H_2, Ar) were supplied by BOC Gases (Brisbane, QLD, Australia). Silica for flash chromatography (Silica gel 60, 230–400 mesh) was obtained from Lomb Scientific (Taren Point, NSW, Australia). Deuterated solvents (d_1-$CDCl_3$ and DMSO-d_6) were manufactured by Cambridge Isotope Laboratories Inc. (Andover, MA, USA). All other reagents were purchased in analytical grade or higher purity from Sigma–Aldrich (Castle Hill, NSW, Australia) or Merck Pty (Kilsyth, VIC, Australia). Solvents were freshly distilled and dried prior to use and all moisture-sensitive reactions were carried out under inert atmosphere (N_2/Ar) using oven-dried glassware.

Infrared measurements were performed on an IR spectrometer Spectrum 2000 (Perkin Elmer Pty Ltd, Glen Waverley, VIC, Australia), at a resolution of 4 cm^{-1} ATR. ^{1}H and ^{13}C NMR were recorded on a Bruker Avance 500 spectrometer (Bruker Biospin, Rheinstetten, Germany) at 500 MHz and 298 K. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane with chemical shifts referenced to the residual $CHCl_3$ peak (δ 7.24 ppm) or DMSO peak (δ 2.50 ppm). Mass chromatograms were acquired in acetonitrile/water+0.1% acetic acid on a PE Sciex API3000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) operating in positive-ion electrospray mode (ESI-MS). Results were viewed using the Analyst 1.4 software provided. High-resolution mass spectrometry (HRMS) data were obtained on a Qstar Pulsar instrument (Applied Biosystems) operating in positive-ion electrospray mode. Isothermal titration calorimetry (ITC) experiments were conducted on a MicroCal VP-ITC calorimeter (MicroCal, Northampton, MA, USA) at 37 °C in deionized water (pH 6.0) and data computed using Origin software 5.0 and VP viewer 2000 software.

4.2. Synthesis

*4.2.1. N-[2-(N′-tert-Butyloxycarbonyl)amino-D,L-dodecanoyl]-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl amine (**3**).* Peracetylated β-D-glucosyl azide (**1**) (1.00 g, 2.68 mmol) was dissolved in dry dichloromethane (20 mL). Palladium catalyst (0.1 g, 10% on carbon) was added and the mixture was stirred under hydrogen for 18 h. Upon completion, the solution was filtered through Celite and concentrated under reduced pressure to 20 mL. Separately, N^{α}-Boc-aminododecanoic acid[34] (1.47 g, 4.67 mmol), HBTU (0.97 g, 5.13 mmol), and DIPEA (1.20 g, 1.62 mL, and 9.30 mmol) were dissolved in dry DCM (20 mL) and allowed to react for 15 min before being added to the amine solution and stirred for 12 h at room temperature. The solution was then evaporated and the residue was washed successively with 5% HCl (2×50 mL), saturated solution of sodium bicarbonate (2×50 mL), brine (2×25 mL), dried over $MgSO_4$, and filtered off. The residual solvent was evaporated under vacuum and the crude product was purified by column chromatography to give **3** as a colorless oil (1.30 g, 76%); R_f=0.29 ethyl acetate/hexane 1:2 (v/v); ESI-MS, *m*/*z*: 645 [M+H]$^+$, 667 [M+Na]$^+$. ^{1}H NMR (500 MHz, $CDCl_3$) (δ): 7.02–6.84 (1H, m, amide NH), 5.31–5.18 (2H, m, H-1 and H-3 (sugar)), 5.01 (1H, t, *J*=9.8 Hz, H-2 (sugar)), 4.91–4.86 (1H, m, H-5 (sugar)), 4.30–4.24 (1H, m, H-6$_a$ (sugar)), 4.02–4.01 (2H, m, H-6$_b$ (sugar) and α-CH (LAA)), 3.82–3.77 (1H, m, H-4 (sugar)), 2.01, 1.97, 1.96, 1.95 (12H, 4s, 4CH_3CO (sugar)), 1.92–1.68 (2H, m, β-CH_2 (lipid)), 1.44–1.41 (9H, m, Boc), 1.22–1.19 (16H, m, 8CH_2 (lipid)), 0.81 (3H, t, *J*=6.7 Hz, CH_3 (lipid)).

*4.2.2. N-(2-Amino-D,L-dodecanoyl)-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl amine (**4**).* Compound **3** (1.93 g, 3.00 mmol) was dissolved in DCM/TFA (50 mL, 1:1) and stirred for 1 h. The solvent was removed under vacuum and the residue was left overnight under vacuum to remove the excess TFA, then neutralized with aqueous

sodium bicarbonate (25 mL), and extracted with DCM (100 mL) to yield **4** as a colorless oil (1.50 g, 92%); ESI-MS, MS, *m*/*z*: 545 $[M+H]^+$, 567 $[M+Na]^+$. ^1H NMR (500 MHz, $CDCl_3$) (δ): 7.97–7.89 (1H, m, amide NH), 5.31–5.18 (2H, m, H-1 and H-3 (sugar)), 5.09–4.93 (2H, m, H-2 and H-5 (sugar)), 4.30–4.16 (1H, m, H-6_a (sugar)), 4.14–4.08 (1H, m, H-6_b (sugar)), 4.06–4.02 (1H, m, H-4 (sugar)), 3.82–3.77 (1H, m, α-CH (lipid)), 2.11, 2.08, 2.06, 2.03 (12H, 4s, $4CH_3CO$ (sugar)), 1.82–1.79 (2H, m, β-CH_2 (lipid)), 1.25–1.22 (16H, m, $8CH_2$ (lipid)), 0.72 (3H, t, *J*=6.7 Hz, CH_3 (lipid)).

*4.2.3. N-[2-(N′-(N″-(tert-Butyloxycarbonyl)-L-leucyl) amino-D,L-dodecanoyl)]-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl amine (**5a**).* N^{α}-Boc-leucine (0.62 g, 2.49 mmol), HBTU (0.51 g, 2.69 mmol) and DIPEA (0.86 mL, 4.96 mmol) were dissolved in dry DCM (50 mL) and compound **4** (0.67 g, 1.23 mmol) was added. The reaction mixture was stirred at room temperature for 12 h. After evaporation, the residue was washed with 5% HCl solution (2×25 mL), aqueous sodium bicarbonate (2×25 mL) and brine (2×25 mL), dried over $MgSO_4$, and concentrated under vacuum. Purification by flash chromatography (ethyl acetate/hexane 1:2+1% triethylamine), R_f=0.3; (1:1 mixture of diastereomers) yielded **5a** as a colorless oil (0.72 g, 65%). ESI-MS, *m*/*z*: 780 $[M+Na]^+$. IR (film): γ=3291, 2930, 2859, 1751, 1703, 1649, 1506, 1366, 1218, 1165, 1035, 907, 567, 598 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) (δ): 7.07–7.00 (1H, m, amide NH), 6.73–8.69 (1H, m, amide NH), 5.30–5.21 (2H, m, H-1 and H-3 (sugar)), 5.04–5.00 (1H, m, H-5 (sugar)), 4.92–4.88 (1H, m, H-2 (sugar)), 4.28–4.24 (2H, m, H-6_a (sugar) and α-CH (lipid)), 4.04–4.08 (2H, m, H-6_b (sugar), and α-CH (Leu)), 3.81–3.76 (1H, m, H-4 (sugar)), 2.01, 1.97, 1.96, 1.98 (12H, 4s, $4CH_3CO$ (sugar)), 1.82–1.79 (1H, m, $CH(CH_3)_2$ (Leu)), 1.75–1.74 (2H, m, β-CH_2 (lipid)), 1.64–1.59 (2H, m, β-CH_2 (lipid)), 1.41–1.39 (9H, m, $C(CH_3)_3$ (Boc)), 1.20–1.18 (16H, m, $8CH_2$ (lipid)), 0.92–0.88 (6H, m, $2CH_3$ (Leu)), 0.83 (3H, t, *J*=6.7 Hz, CH_3 (lipid)). ^{13}C NMR (500 MHz, $CDCl_3$) (δ): 172.7, 172.5, 172.1, 172.0, 170.6, 170.4, 170.4, 169.7, 169.7, 169.3, 155.9, 155.8, 80.3, 80.1, 78.0, 77.9, 73.8, 73.5, 73.4, 72.7, 72.6, 70.4, 70.2, 68.1, 68.0, 67.6, 61.6, 61.5, 53.1, 52.7, 31.8, 31.7, 31.7, 31.6, 31.4, 29.4, 29.2, 29.1, 29.1, 28.2, 28.1, 25.1, 25.1, 24.5, 24.5, 22.9, 22.8, 22.5, 21.7, 21.6, 20.5, 20.4, 20.4, 13.9. HRMS calcd for $C_{37}H_{63}N_3Na$: 780.4259, found: 780.4253.

*4.2.4. N-[2-(N′-(N″-(tert-Butyloxycarbonyl)-L-phenylalanyl)amino-D,L-dodecanoyl)]-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl amine (**5b**).* Compound **5b** was prepared following the procedure described for compound **5a**, using N^{α}-Boc-phenylalanine (0.66 g, 2.49 mmol) as starting material instead. R_f=0.3 ethyl acetate/hexane, 1:2 (v/v). The title product (1:1 mixture of diastereomers) was isolated as a colorless oil by flash chromatography (0.60 g, 62%); ESI-MS, *m*/*z*: 792 $[M+H]^+$, 814 $[M+Na]^+$. IR (film): γ=3316, 2927, 2856, 1748, 1687, 1651, 1529, 1453, 1367, 1219, 1167, 1035, 908, 743, 699, 599, 567 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) (δ): 7.33–7.22 (5H, m, Ph), 7.07–6.98 (1H, m, amide NH), 6.40–7.38 (1H, m, amide NH), 5.32–5.26 (2H, m, H-1 and H-3 (sugar)), 5.18–5.11 (1H, m, H-5 (sugar)), 5.07 (1H, t, *J*=11.6 Hz, H-2 (sugar)), 4.32–4.29 (3H, m, H-6_a (sugar), α-CH (lipid)), α-CH (Phe), 4.09 (1H, dd, *J*=11.6, 9.8 Hz, H-6_b (sugar)), 3.80–3.75 (1H, m, H-4 (sugar)), 3.13–3.09 (2H, m, CH_2Ph), 2.01, 1.97, 1.96, 1.98 (12H, 4s, $4CH_3CO$ (sugar)), 1.71–1.69 (2H, m, β-CH_2 (lipid)), 1.44–1.41 (9H, m, $C(CH_3)_3$ (Boc)), 1.25–1.22 (16H, m, $8CH_2$ (lipid)), 0.81 (3H, t, *J*=6.7 Hz, CH_3 (lipid)). ^{13}C NMR (500 MHz, $CDCl_3$) (δ): 171.8, 171.6, 170.5, 170.4, 169.8, 169.4, 136.7, 136.4, 129.2, 129.1, 129.0, 128.8, 128.7, 128.6, 127.2, 126.8, 126.5, 126.3, 126.0, 125.9, 125.1, 124.3, 80.4, 78.0, 75.5, 73.5, 73.4, 72.7, 72.6, 70.5, 70.2, 68.0, 61.5, 53.4, 31.7, 29.4, 29.2, 29.2, 29.1, 28.2, 28.1, 25.1, 22.5, 20.6, 20.5, 20.4. HRMS calcd for $C_{39}H_{61}N_5Na$: 814.4204, found: 814.4209.

*4.2.5. N-[2-(N′-(2-(N″-(tert-Butyloxycarbonyl)amino-D,L-dodecanoyl)amino-D,L-dodecanoyl))]-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl amine (**5c**).* Compound **5c** was prepared following the procedure described for compound **5a**, using N^{α}-Boc-amino-dodecanoic acid (0.78 g, 2.49 mmol) as starting material instead; R_f=0.3 ethyl acetate/hexane, 1:2 (v/v). The title product (1:1 mixture of diastereomers) was isolated as a colorless oil by flash chromatography (0.62 g, 68%); ESI-MS, *m*/*z*: 842 $[M+H]^+$, 864 $[M+Na]^+$. IR (film): γ=3288, 3075, 2925, 2855, 1752, 1703, 1647, 1546, 1502, 1456, 1366, 1219, 1167, 1037, 982, 907, 865, 778, 698, 643, 598 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) (δ): 6.86–6.59 (1H, m, amide NH), 6.53–6.51 (1H, m, amide NH), 5.29–5.20 (2H, m, H-1 and H-3 (sugar)), 5.05–4.98 (2H, m, H-2 and H-5 (sugar)), 4.86–4.84 (1H, dd, *J*=2.1, 1.8 Hz, H-6_a (sugar)), 4.05–3.98 (3H, m, H-6_b (sugar), 2α-CH (lipid)), 3.97–3.94 (1H, m, H-4 (sugar)), 2.01, 1.97, 1.96, 1.98 (12H, 4s, $4CH_3CO$ (sugar)), 1.86–1.82 (2H, m, β-CH_2 (lipid)), 1.56–1.54 (2H, m, β-CH_2 (lipid)), 1.46–1.42 (9H, m, $C(CH_3)_3$ (Boc)), 1.24–1.21 (32H, m, $16CH_2$ (lipid)), 0.86–0.83 (6H, m, $2CH_3$ (lipid)). ^{13}C NMR (500 MHz, $CDCl_3$) (δ): 172.4, 172.1, 171.9, 170.9, 170.5, 170.4, 169.8, 169.7, 169.4, 78.2, 78.1, 78.0, 73.5, 72.8, 72.7, 72.5, 70.4, 70.1, 68.1, 68.0, 61.5, 61.5, 60.2, 53.2, 31.7, 31.3, 31.2, 29.4, 29.3, 29.3, 29.2, 29.1, 28.2, 28.1, 25.6, 25.5, 25.3, 25.2, 22.5, 20.9, 20.6, 20.6, 20.5, 20.4, 14.0, 13.9. HRMS calcd for $C_{43}H_{75}N_3Na$: 864.5198, found: 864.5192.

*4.2.6. N-[2-(N′-L-Leucyl)amino-D,L-dodecanoyl]-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylamine (**6a**).* Compound **5a** (0.60 g, 0.79 mmol) was dissolved in TFA/DCM (1:1) and stirred for 1 h. The mixture was diluted with DCM (50 mL), evaporated, and washed with sodium bicarbonate solution (2×25 mL), the organic layer was separated, dried over $MgSO_4$, filtered, and evaporated under vacuum to yield compound **6a** as a colorless oil (0.48 g, 93%), 1:1 mixture of diastereomers; R_f=0.12 (ethyl acetate/hexane 4:1, ninhydrin dip); ESI-MS, *m*/*z*: 658 $[M+H]^+$. IR (film): γ=3292, 2955, 2923, 2854, 1659, 1543, 1465, 1367, 1259, 1201, 1080, 1036, 889, 719, 651, 568 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) (δ): 7.64–7.63 (1H, m, amide NH), 7.17–7.14 (1H, m, amide NH), 5.29 (2H, s, NH_2), 5.27–5.24 (2H, m, H-1 and H-3 (sugar)), 5.22–5.18 (1H, t, *J*=9.4 Hz, H-2 (sugar)), 5.07–5.01 (1H, m, H-5 (sugar)), 4.95–4.88 (1H, m, H-4 (sugar)), 4.33–4.24 (2H, m, H-6_a and H-6_b (sugar)), 4.07–4.03 (1H, m, α-CH (lipid)), 3.80–3.76 (1H, m, α-CH (Leu)), 2.01, 1.97, 1.96, 1.98 (12H, 4s, $4CH_3CO$ (sugar)), 1.88–1.78 (2H, m, β-CH_2 (Leu)), 1.71–1.69 (2H, m, β-CH_2 (lipid)), 1.60–1.58 (1H, m, $CH(CH_3)_2$ (Leu)), 1.32–1.23 (16H, m, $8CH_2$ (lipid)), 0.96–0.91 (6H, m, $2CH_3$ (Leu)), 0.86 (3H, t, *J*=6.7 Hz, CH_3 (lipid)). ^{13}C NMR (500 MHz, $CDCl_3$) (δ): 176.2, 172.4, 172.2, 170.8, 170.6, 170.5, 170.5, 169.8, 169.7, 169.4, 169.3, 78.1, 78.0, 73.5, 73.52, 72.6, 72.6, 70.7, 70.1, 68.0, 61.5, 61.5, 53.3, 53.2, 52.5, 43.6, 43.2, 31.7, 30.5, 29.4, 29.40, 29.3, 29.2, 29.1, 29.0, 25.4, 25.3, 24.7, 23.2, 23.2, 22.5, 21.2, 20.6, 20.4, 13.9. HRMS calcd for $C_{32}H_{56}N_3O_{11}$: 658.3915, found: 658.3909.

*4.2.7. N-[2-(N′-L-Phenylalanyl)amino-D,L-dodecanoyl]-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl amine (**6b**).* Compound **6b** was prepared following the procedure described for compound **6a**, using compound **5b** (2.00 g, 2.52 mmol) as a starting material instead; R_f=0.12 (ethyl acetate/hexane 4:1, ninhydrin dip); compound **6b** was obtained as a colorless oil in a 1:1 mixture of diastereomers (1.65 g, 95%); ESI-MS, *m*/*z*: 692$[M+H]^+$, 714 $[M+Na]^+$. IR (film): γ=3305, 3062, 2925, 2855, 1747, 1652, 1530, 1438, 1368, 1220, 1034, 908, 844, 745, 699, 648, 599 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) (δ): 7.33–7.32 (1H, m, amide NH), 7.7.31–7.29 (1H, m, amide NH), 7.28–7.22 (5H, m, Ph), 5.58–522 (2H, m, H-1 and H-3 (sugar)), 4.99–4.90 (1H, t, *J*=9.5 Hz, H-5 (sugar)), 4.56–4.49 (1H, t, *J*=9.5 Hz, H-2 (sugar)), 4.35–4.24 (2H, m, H-6_a and H-4 (sugar)), 4.05–3.84 (1H, dd, *J*=2.3, 1.9 Hz, H-6_b (sugar)), 3.78–3.64 (2H, m, α-CH (lipid) and α-CH (Phe)), 3.13–3.05 (2H, m, CH_2Ph), 2.03, 2.00, 1.99, 1.97 (12H, 4s, $4CH_3CO$ (sugar)), 1.71–1.65 (2H, m, β-CH_2 (lipid)), 1.26–1.23 (16H, m, $8CH_2$ (lipid)), 0.84 (3H, t, *J*=5.8 Hz, CH_3 (lipid)). ^{13}C NMR (500 MHz, $CDCl_3$) (δ): 174.8, 172.3, 172.1, 170.8, 170.6, 170.5, 170.1,

169.8, 169.3, 169.3, 137.6, 137.2, 129.1, 129.0, 128.76, 128.70, 129.86, 126.82, 78.0, 74.0, 73.5, 73.5, 72.7, 72.6, 70.7, 70.2, 70.1, 68.7, 68.4, 68.1, 68.05, 68.02, 61.7, 61.5, 61.5, 31.7, 30.6, 29.4, 29.4, 29.3, 29.3, 29.2, 29.2, 29.18, 29.5, 25.3, 22.5, 20.6, 20.5, 20.5, 13.9. HRMS calcd for $C_{35}H_{53}N_3NaO_{11}$: 714.3578, found: 714.3572.

*4.2.8. N-[2-(N′-(2-Amino-D,L-dodecanoyl)amino-D,L-dodecanoyl)]-2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl amine (**6c**).* Compound **6c** was prepared following the procedure described for compound **6a** using compound **5c** (0.50 g, 0.59 mmol) as a starting material instead; R_f=0.12 (ethyl acetate/hexane, 4:1, ninhydrin dip); compound **6c** was obtained as a colorless oil in a 1:1 mixture of diastereomers (0.41 g, 95%) of **6c**; ESI-MS, MS, *m*/*z*: 742 $[M+H]^+$. IR (film): γ=3294, 3072, 2923, 2854, 1748, 1663, 1534, 1466, 1438, 1368, 1222, 1138, 1036, 983, 908, 835, 800, 720, 600, 559 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) (δ): 7.63–7.62 (1H, m, amide NH), 7.13–7.03 (1H, m, amide NH), 5.29–5.17 (2H, m, H-1 and H-3 (sugar)), 5.13 (1H, t, *J*=9.2 Hz, H-2 (sugar)), 5.10–5.00 (1H, m, H-5 (sugar)), 4.94–4.87 (1H, m, H-4 (sugar)), 4.34–4.23 (2H, m, H-6_a and H-6_b (sugar)), 4.06–4.03 (1H, dd, *J*=11.8, 12.0 Hz, α-CH (lipid)), 3.79–3.76 (1H, m, α-CH (lipid)), 2.01, 1.97, 1.96, 1.98 (12H, 4s, $4CH_3CO$ (sugar)), 1.86–1.77 (4H, m, 2β-CH_2 (lipid)), 1.25–1.21 (32H, m, 2×8(CH_2) (lipid)), 0.86–0.81 (6H, m, $2CH_3$ (lipid)). ^{13}C NMR (500 MHz, $CDCl_3$) (δ): 172.6, 172.2, 170.8, 170.5, 169.8, 169.7, 169.4, 78.4, 78.1, 78.0, 73.5, 73.4, 72.6, 72.3, 70.7, 70.0, 68.0, 61.5, 53.5, 53.1, 52.6, 31.7, 31.6, 30.5, 29.4, 29.4, 29.4, 29.3, 29.2, 29.1, 29.0, 25.6, 25.5, 25.4, 22.5, 22.2, 20.6, 20.5, 20.4, 19.9, 13.9. HRMS calcd for $C_{38}H_{68}N_3O_{11}$: 742.4854, found: 742.4850.

*4.2.9. N-[2-(N′-(N″,N″,N″-Trimethyl-L-leucyl)amino-D,L-dodecanoyl)]-2,3,4,6-tetrahydroxy-β-D-glucopyranosyl amine hydroxide (**7a**).* Compound **6a** (0.51 g, 0.77 mmol) was dissolved in methanol (30 mL). Ion exchange resin [Amberlite IRA-400 ($^-$OH)] (8 g) and methyl iodide (77 mmol, 2.22 mL) were added and the mixture was stirred for 48 h. The resin was removed by filtration and solvent was evaporated under vacuum to give an oil, which was dissolved in (50 mL) acetonitrile/water (1:1) and then lyophilized to give compound **7a** (1:1 mixture of diastereomers) as a white powder (0.38 g, 90%); mp 168–168.5 °C; ESI-MS, *m*/*z*: 532 $[M-OH]^+$. IR (film): γ=3263, 2924, 1682, 1665, 1554, 1079, 1041, 1019, 672 cm^{-1}. ^1H NMR (500 MHz, DMSO) (δ): 7.45–7.43 (1H, m, amide NH), 7.29–7.28 (1H, m, amide NH), 5.12–4.98 (2H, m, H-1 and H-3 (sugar)), 4.63 (1H, d, *J*=8.7 Hz, H-2 (sugar)), 4.33–4.24 (1H, m, H-5 (sugar)), 4.21–4.18 (1H, dd, *J*=11.8, 10.2 Hz, H-6_a (sugar)), 3.73–3.60 (1H, m, H-6_b (sugar)), 3.51–3.46 (2H, m, α-CH (lipid) and α-CH (Leu)), 3.10 (9H, d, *J*=5.1 Hz, $3CH_3$ (quatern)), 1.89–1.84 (1H, m, $CH(CH_3)_2$ (Leu)), 1.69–1.62 (2H, m, β-CH_2 (lipid)), 1.62–1.34 (2H, m, β-CH_2 (Leu)), 1.32–1.21 (16H, m, $8CH_2$ (lipid)), 0.95–0.87 (6H, m, $2CH_3$ (Leu)), 0.83 (3H, t, *J*=1.1 Hz, CH_3 (lipid)). ^{13}C NMR (500 MHz, DMSO-d_6) (δ): 171.28, 165.7, 165.5, 80.1, 79.98, 79.0, 78.8, 77.7, 72.6, 72.5, 72.4, 70.3, 70.3, 54.7, 54.7, 52.1, 51.8, 31.6, 31.6, 29.3, 29.3, 29.2, 29.23, 29.06, 29.05, 29.03, 28.9, 28.7, 25.4, 25.2, 24.6, 24.0, 22.4, 21.1, 21.0, 14.3. HRMS calcd for $C_{27}H_{54}N_3O_7^+$: 532.3956, found: 532.3956.

*4.2.10. N-[2-(N′-(N″,N″,N″-Trimethyl-L-phenylalanyl) amino-D,L-dodecanoyl)]-2,3,4,6-tetrahydroxy-β-D-glucopyranosyl amine hydroxide (**7b**).* Compound **7b** was prepared following the procedure described for compound **7a** except using compound **6b** (0.55 g, 0.79 mmol) as starting material instead, yielding **7b** (1:1 mixture of diastereomers) as a white powder (0.41 g, 90%); mp 137–137.5 °C; ESI-MS, *m*/*z*: 566 $[M-OH]^+$. IR (film): γ=3277, 3062, 2923, 1667, 1544, 1077, 1037, 698 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6) (δ): 7.66–7.65 (1H, m, amide NH), 7.53–7.47 (1H, m, amide NH), 7.42–7.30 (5H, m, Ph), 4.60–4.55 (1H, t, *J*=12.9 Hz, H-2 (sugar)), 4.42–4.28 (2H, m, H-6_a and H-6_b (sugar)), 3.96–3.91 (1H, m, α-CH (lipid)), 3.63–3.59 (1H, m, α-CH (Phe)), 3.36–3.34 (2H, m, CH_2Ph), 3.22–3.20 (9H, s, $3CH_3$ (quatern)), 1.52–1.50 (2H, m, β-CH_2 (lipid)), 1.24–1.21 (16H, m, $8CH_2$ (lipid)), 0.91 (3H, t, *J*=10.8 Hz, CH_3 (lipid)). ^{13}C NMR (500 MHz, DMSO-d_6) (δ): 171.6, 164.9, 164.7, 164.72, 134.6, 129.7, 129.3, 129.2, 129.1, 128.7, 127.8, 127.6, 127.4, 80.1, 79.9, 79.0, 78.8, 77.7, 77.7, 74.7, 74.3, 72.8, 72.5, 70.2, 61.3, 61.2, 54.7, 54.7, 54.7, 53.5, 53.0, 52.4, 52.3, 52.1, 47.7, 32.4, 32.1, 31.9, 31.8, 31.6, 31.6, 29.4, 29.3, 29.32, 29.3, 29.2, 29.1, 29.09, 29.06, 29.00, 28.9, 25.2, 25.1, 24.9, 22.4, 14.3, 14.2. HRMS calcd for $C_{30}H_{52}N_3O_7^+$: 566.3805, found: 566.3800.

*4.2.11. N-[2-(N′-(2-(N″,N″,N″-Trimethylamino))-D,L-dodecanoyl)amino-D,L-dodecanoyl]-2,3,4,6-tetrahydroxy-β-D-glucopyranosyl amine hydroxide (**7c**).* Compound **7c** was prepared by following the procedure described for compound **7a** using compound **6c** (0.30 g, 0.40 mmol) as starting material instead, yielding **7c** (1:1 mixture of diastereomers) as a white powder (0.24 g, 97%); mp 130–130.5 °C. ESI-MS, MS, *m*/*z*: 616 $[M-OH]^+$. IR (film): γ=3263, 2956, 2921, 2852, 1673, 1554, 1376, 1081, 1040, 719 cm^{-1}. ^1H NMR (500 MHz, DMSO-d_6) (δ): 7.28–7.21 (1H, m, amide NH), 7.20–7.18 (1H, m, amide NH), 4.60–4.53 (1H, m, H-5 (sugar)), 4.19–4.07 (3H, m, H-4, H-6_a, and H-6_b (sugar)), 3.63–3.58 (2H, m, 2α-CH (lipid)), 3.16–3.09 (9H, m, $3CH_3$ (quatern)), 1.83–1.82 (2H, m, β-CH_2 (lipid)), 1.66–1.65 (2H, m, β-CH_2 (lipid)), 1.37–1.21 (32H, m, 2×8CH_2 (lipid)), 0.85–0.82 (6H, m, $2CH_3$ (lipid)). ^{13}C NMR (500 MHz, DMSO-d_6) (δ): 175.5, 166.3, 166.1, 166.1, 80.5, 80.4, 79.0, 78.8, 77.8, 77.58, 77.44, 77.2, 72.4, 72.2, 70.8, 70.4, 52.1, 51.7, 31.7, 31.6, 29.6, 29.5, 29.4, 29.4, 29.3, 29.2, 29.1, 29.0, 28.9, 26.1, 26.0, 25.8, 25.6, 22.4, 14.3, 14. HRMS calcd $C_{33}H_{66}N_3O_7^+$: 616.4895, found: 616.4895.

4.3. Isothermal titration calorimetry (ITC)

ITC measurements were performed at 37 °C. Aliquots of a solution containing the compound in degassed, deionized water (pH 6.0) were injected into the microcalorimeter sample cell (1.4395 mL) loaded with water using a stirrer/syringe rotating at 300 rpm. Sequences were built as follows: title compound concentration, 4 mM; number of injections, 30; injection duration, 20 s; injection volume, 10 μL. For compounds **7a** and **7b**, additional conditions were tested, varying concentrations (4–8 mM), injection durations (10, 20, 30 s), and volumes (5, 10, 15 μL). All measurements were performed in triplicate.

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