Approach Toward a Generic Treatment of Gram-Negative Infections: Synthesis of Haptens for Catalytic Antibody Mediated Cleavage of the Interglycosidic Bond in Lipid A

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In order to develop a generic treatment for infections with Gram-negative bacteria, we developed a synthesis of 2-acylamino-deoxynojirimycin derivatives (**17**, **18**, **19** and **20**), which will be used as haptens for raising catalytic antibodies capable of hydrolyzing the interglycosidic bond in the lipid A moiety of endotoxins. A key intermediate in the preparation of compounds **17**, **18**, **19** and **20** is 3,4,6-tri-*O*-benzyl-2-[(benzyloxycarbonyl)amino]-2-deoxy-D-glucono-δ-lactam (**6**), which was prepared from known 3,4,6-tri-*O*-benzyl-2-[(benzyloxycarbonyl)amino]-2-deoxy-D-glucosamine (**1**) in four steps in 47% overall yield. Antibodies were generated against 2-[(6-aminohexanoyl)amino]-2-deoxy-D-glucono-δ-lactam (**17**) coup-led to the carrier protein bovine serum albumin.

Introduction

Endotoxins are complex lipopolysaccharides (LPS) situated in the outer membrane of the cell wall of Gram-negative bacteria.[1] High concentrations of LPS due to an invasive infection with Gram-negative bacteria cause a strong immune response in higher animals, leading to pathological effects such as fever, shock, multiple organ failure and, in the worst case, death. Most of the biological activities of LPS are sited in the small terminal disaccharide phospholipid moiety known as lipid A (Figure 1).[2] Lipid A has a basic structure composed of a polyacylated β(1−6) disaccharide of glucosamine 1,4′-bisphosphate.[3] The number and structure of the fatty acid chains influence its endotoxic activity.[4]

Although sepsis by Gram-negative bacteria is commonly accepted as a serious problem, no adequate drug has yet been developed.[5] Despite treatment with antibiotics, bacterial sepsis has a high mortality rate (up to 80%)[6][7] because bacteria are capable of developing resistance to antibiotics. In addition, certain antibiotics induce an enhanced release of endotoxin from bacteria.[8] Antibodies to LPS (either monoclonal or polyclonal) may be used as immunotherapeutic agents for the treatment of sepsis.[9] A major drawback of these antibodies is their specificity and lack of cross-reactivity and cross-protectivity.[10] Our approach towards a therapy for endotoxemia is based on catalytic antibodies capable of degrading lipid A. In this context Balreddy et al.[11] reported the synthesis of phosphonate ana-

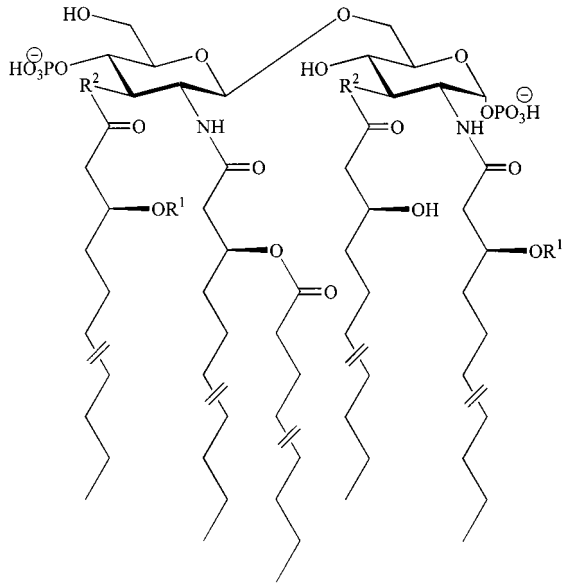

Figure 1. Generalized structure of lipid A, R^1 = acyl group or hydrogen; R^2 = O or NH

logues of lipid X, which were used as haptens for raising antibodies for enzymatic hydrolysis of the fatty acid chains in lipid A. Catalytic antibodies capable of hydrolyzing the glycosidic bond in lipid A should produce the corresponding nontoxic monosaccharides. Advantageously, a catalytic antibody acts as an enzyme (in this case a glycosidase), which displays rate enhancement, specificity and turnover, i.e., the ability to catalyze the reaction of many substrate molecules. In contrast, antibodies that merely bind LPS should be administered stoichiometrically.

The active site of our catalytic antibodies should stabilize the delocalized positive charge and the half-chair conformation of the high-energy oxocarbenium ion formed during cleavage of the glycosidic bond.[12] The first antibody glycosidases were generated against haptens based on transition

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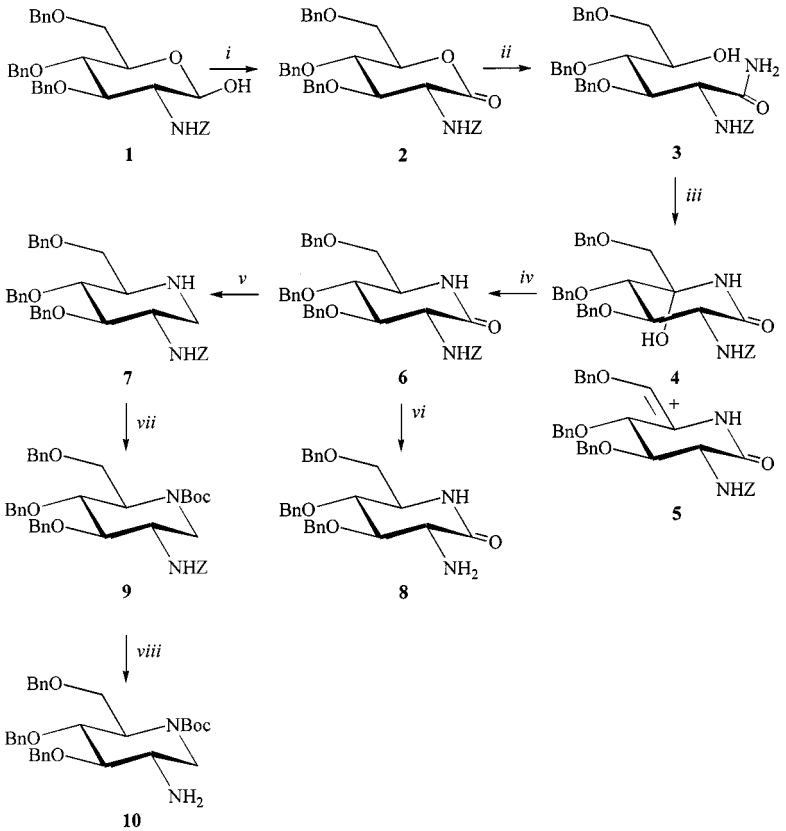
state glycosidase inhibitors such as nojirimycin, isofagomine and castanospermine.[13] In these aminocyclitols the (protonated) nitrogen atom is at or near the anomeric center and is anticipated to mimic the electronic charge developing in the transition state. Here we report the synthesis of 2-acylamido-deoxynojirimycin derivatives, containing an acyl chain function at the 2-amino function, which mimics the native lipid A and which is used for linking to a carrier protein. Currently we use these derivatives as haptens for the generation of antibodies with glycosidase activity towards lipid A.

Results and Discussion

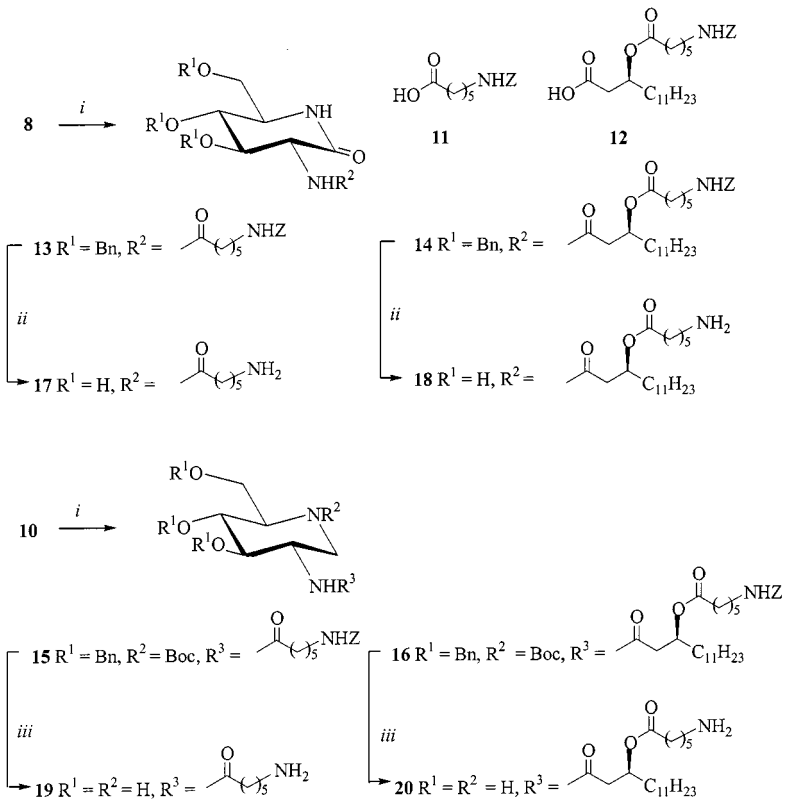
Synthesis of Haptens

Several groups have reported the synthesis of haptens for the generation of antibodies capable of catalyzing the hydrolysis or formation of glycosidic bonds.[13] The design of these haptens is based on cyclic amine glycosidase inhibitors such as nojirimycin, isofagomine and castanospermine. Analogously, our haptens are based on the known synthetic hexosaminidase inhibitor 2-acetamido-1,2,5-trideoxy-1,5-imino-D-glucitol. Various syntheses of this compound have been reported. Fleet et al. described a 17-step synthesis starting from D-glucose.[14] Kappes and Legler obtained this molecule in an 11-step synthesis starting from *N*-acetyl-D-glucosamine.[15] Wong and co-workers described a semi-enzymatic synthesis using fructose 1,6-diphosphate aldolase in combination with catalytic reductive amination.[16] Furneaux et al. described its preparation from *N*-acetyl-D-glucosamine in a six step synthesis in 10% overall yield.[17] Furthermore, it has also been synthesized starting from 1-deoxynojirimycin.[18][19] Recently, Vasella and co-workers reported a synthesis which is based on the facile transformation of sugar lactones to azasugars,[20] as originally described by Pandit and co-workers.[21] Our approach towards the haptens is based on this transformation.

Oxidation of the readily available 3,4,6-tri-*O*-benzyl-*N*-benzyloxycarbonyl-glucosamine[22] (**1**) (Scheme 1) with DMSO/Ac_2O afforded lactone **2** in almost quantitative yield (99%). Subsequent reaction with methanolic ammonia resulted in the hydroxy-amide **3** in high yield (97%). Oxidation of **3** with DMSO/Ac_2O resulted in a mixture of compounds, from which the desired hydroxylactam **4** could be isolated in 58% yield. The corresponding ido-isomer was formed in 3% yield. The other main reaction product (31%) turned out to be the alkene **5**. It is interesting to note that ring closure of the keto-amide occurred without treatment with methanolic ammonia, which is in contrast to the results obtained by Pandit and co-workers[21] for the corresponding glucono derivative. Reduction of **4** with sodium cyanoborohydride afforded the lactam **6** in 85% yield, in which the gluco-configuration at C-5 was firmly established by ^1H NMR spectroscopy. The interesting intermediate lactam **6**, having a highly polarized bond,[23] can be regarded



Scheme 1. Conditions: (i) DMSO, Ac_2O, 99%; (ii) 4 N NH_3/MeOH, 97%; (iii) DMSO, Ac_2O, 58%; (iv) $NaCNBH_3$, HCO_2H, CH_3CN, 85%; (v) $BH_3 \cdot THF$, 78%; (vi) isopropanol, EtOAc, H_2O, formic acid, 5% Pd/C, H_2, 74%; (vii) DCM, TEA, Boc_2O, 99%; (viii) as (vi) 89%



Scheme 2. Conditions: (i) **11**/**12**, PyBOP, DiPEA, DCM; (**13**, 97%), (**14**, 76%), (**15**, 97%), (**16**, 93%); (ii) isopropanol, EtOAc, formic acid, 10% Pd/C, H_2; (**17**, 88%), (**18**, quantitative); (iii) a) DCM/TFA, 1:1 (v/v); b) as (ii); (**19**, quantitative), (**20**, 73%)

as a transition state analogue for the hydrolysis of glycosidic linkages. D-Glucono-δ-lactam has been proposed by others for use as a hapten in order to raise catalytic antibodies.[21b,c] This makes **6**, after further modification, an attractive hapten for the generation of monoclonal antibodies with glycosidase activity (vide infra). Reduction of **6** with $LiAlH_4$, as described by Pandit and co-workers,[21] resulted in a complex mixture of compounds. Fortunately, reduction with BH_3·THF[24] afforded the desired 1,2-dideoxynojirimycin derivative **7** in good yield (78%).

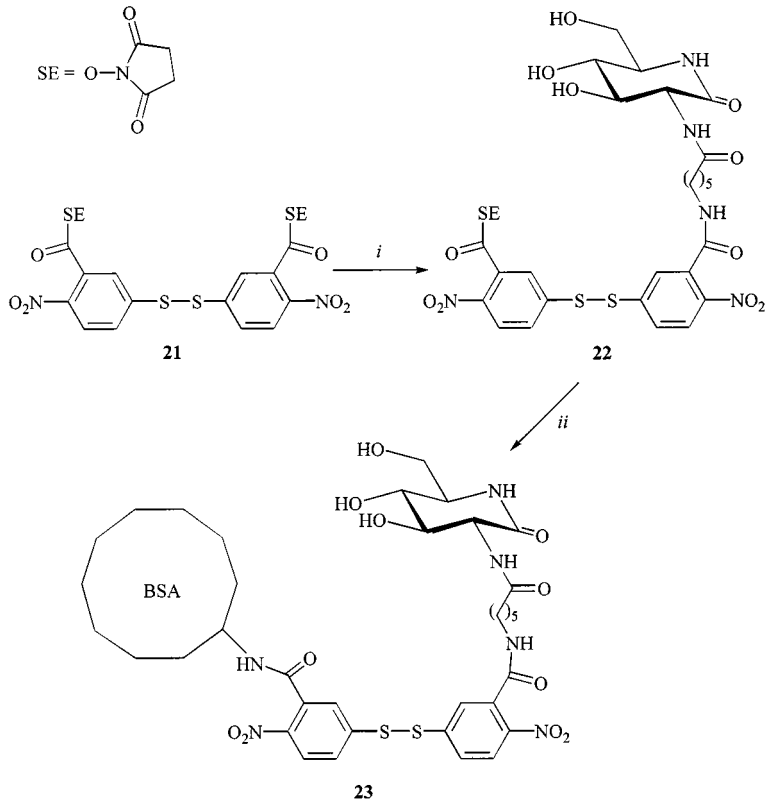
Both the lactam **6** and the 1,2-dideoxynojirimycin derivative **7** were used for the preparation of haptens. The *Z*-group in **6** was selectively removed by hydrogenation over 5% Pd/C (Degussa type) in a mixture of isopropyl alcohol, ethyl acetate, water and formic acid, giving the 2-amino-lactam derivative **8** in 74% yield, which can be coupled with fatty acids. Prior to removal of the *Z*-group, the endocyclic nitrogen in **7** was protected with a Boc-group,[25] giving **9** in excellent yield. After selective removal of the *Z*-group the 2-amino-1,2-dideoxynojirimycin derivative **10** was obtained in 89% yield.

As a first approach to introduce an acyl chain into lactam **8**, a relatively short acyl chain was introduced, derived from the carboxylic acid **11** (Scheme 2), carrying a protected amino group for coupling to a carrier protein. Various coupling conditions were evaluated. Coupling of **8** with the *N*-hydroxysuccinimidyl ester of **11** did not result in the desired acyl derivative. Activation of **11** with dicyclohexylcarbodiimide was not successful either, but PyBOP mediated coupling[26] of **11** with **8** afforded the protected hapten **13** in excellent yield. Analogously, the protected hapten **14** was obtained from **8**, by using the fatty acid **12**, which resembles the acyl chain at C-2 in the nonreducing end of lipid A. Haptens **15** and **16** were obtained by coupling of **10** with **11** and **12**, respectively. Haptens **13** and **14** were deprotected in one step by catalytic hydrogenation on Pd/C, affording the desired haptens **17** and **18**, respectively, which gave satisfactory analytical data. Compounds **15** and **16** were deprotected in two steps: by removal of the Boc group with trifluoroacetic acid, followed by catalytic hydrogenation, giving haptens **19** and **20**, respectively.

Immunochemistry

Preliminary immunochemical experiments were carried out with hapten **17**. To this end, the compound was conjugated to BSA (Scheme 3), through 5,5′-dithiobis(2-nitrobenzoic acid, succinimidyl diester) (**21**) as linker. Reaction of **17** with 2 equivalents of **21** afforded the monosubstituted derivative **22** in 41% yield. Subsequent coupling of **22** with BSA gave the desired immunoconjugate **23**. The hapten density of the conjugate was determined to be 6 haptens per BSA (overall coupling efficiency: 10%, determined after reduction of the BSA conjugate with TCEP and spectrophotometric quantification of the thionitrophenolate ion).

Balb/c mice were immunized with **23**, and hybridomas were prepared from the spleen cells by using standard hy-



Scheme 3. Conditions: (i) **17**, DMF, 16 h, 41%; (ii) BSA, PBS/DMF, 30 h, 25%, (hapten/BSA 6/1)

bridoma protocols.[27] We obtained a total of 24 stable hybrids, producing polyclonal antibodies that exhibited binding specificity for **23** over background binding for BSA in ELISA assays. Preliminary screening experiments showed several of the polyclonal antibodies to be active in the assay of 4-nitrophenyl-*N*-acetyl-β-D-glucosamine hydrolysis. Currently, derived monoclonal antibodies are being investigated for their catalytic properties.

Conclusion

In conclusion, we have synthesized four pseudo-sugar-aza-glycosides as potential antigens for glycoside-hydrolyzing antibodies. Future studies will aim at further development of such antibodies.

Experimental Section

General Remarks: Toluene (Merck) was distilled from P_2O_5. Dichloromethane (Biosolve Ltd.) and acetonitrile (Biosolve Ltd.) were freshly distilled from CaH_2. Methyl sulfoxide (DMSO, Fluka) was stirred overnight with CaH_2 and distilled under reduced pressure. Methanol (Merck) was dried by refluxing with magnesium methoxide and then distilled. *N*,*N*-Diisopropylethylamine (DiPEA, Acros Chimica NV) was distilled from *p*-toluenesulfonyl chloride and redistilled from potassium hydroxide pellets. ^1H and ^{13}C NMR spectra were recorded on a Varian VXR-400S (399.9/100.6 MHz). ^1H and ^{13}C NMR chemical shifts are given in ppm (δ) relative to tetramethylsilane (δ = 0.00), CD_2HOD (δ = 3.307), [D_6]DMSO (δ = 2.525), [D_4]MeOH (δ = 49.0) or [D_6]DMSO (δ = 39.6) as internal standard. The purity of the compounds was established by ^1H NMR spectroscopy: > 95% in all cases. Mass spectra were recorded with a VG Quattro II triple quadropole mass spectrometer (Fisons Instruments, Altrincham, UK). Column chromatography was performed on Silica gel 60 (220−440 mesh ASTM, Fluka). TLC-analysis was performed with silica gel TLC-cards (Fluka) with detection by UV-absorption (254 nm) where applicable and charring with 20% H_2SO_4 in MeOH or ammonium molybdate (25 g/L) and ceric ammonium sulfate (10 g/L) in 20% H_2SO_4. Prior to reactions that require anhydrous conditions, traces of water were removed by co-evaporation with toluene. Polytetrafluoroethylene (PFTE) filters were purchased from Alltech (Breda, The Netherlands). 4-Nitrophenyl-*N*-acetyl-β-D-glucosamine, and 5,5′-dithiobis(2-nitrobenzoic acid, succinimidyl diester) were purchased from Molecular Probes, Inc. (Leiden, The Netherlands).

3,4,6-*Tri*-*O*-benzyl-2-[(benzyloxycarbonyl)amino]-2-deoxy-D-glucono-δ-lactone (2): Acetic anhydride (15 mL) was added under nitrogen atmosphere to a solution of 3,4,6-tri-*O*-benzyl-2-[(benzyloxycarbonyl)amino]-2-deoxy-D-glucosamine **(1)** (4.00 g, 6.86 mmol) in anhydrous DMSO (25 mL). The mixture was stirred for 16 h at ambient temperature. TLC analysis showed complete conversion of starting material into a compound with R_f = 0.57 (ethyl acetate/hexane, 1:2, v/v). The reaction mixture was diluted with diethyl ether (150 mL), washed thoroughly with aqueous 10% $NaHCO_3$ (3 × 100 mL) and twice with saturated NaCl (2 × 200 mL) solution. After drying on Na_2SO_4, the organic layer was concentrated in vacuo. The crude product was purified by silica gel column chromatography. Elution was performed with hexane/ethyl acetate (4:1 → 2:1, v/v). Yield 3.95 g (99%). − ^1H NMR ($CDCl_3$): δ = 3.69 (2 H, br. s, H_2-6), 4.01 (2 H, br. s, H-3, H-4), 4.09 (1 H, t, $J_{2,NH}$ = 7.4 Hz, H-2), 4.37 (1 H, br. s, H-5), 4.50 (2 H, dd, *J* = 12.0 Hz, CH_2 Bn), 4.58−4.81 (4 H, m, *J* = 11.2 Hz,

J = 11.8 Hz, CH_2 Bn), 5.07 (2 H, dd, J = 12.1 Hz, CH_2Z), 5.46 (1 H, d, $J_{2,NH}$ = 7.4 Hz, NH), 7.15−7.40 (20 H, m, CH-arom Bn/*Z*). − $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 56.42 (C-2), 67.33 (CH_2Z), 68.12 (C-6), 73.63, 74.51, 74.74 (3 × CH_2 Bn), 76.22 (C-3), 79.01 (C-5), 79.54 (C-4), 127.73−128.59 (CH-arom Bn/*Z*), 136.11, 137.53, 137.57, 137.68 (4 × Cq Bn/*Z*), 156.21 (C=O *Z*), 168.91 (C=O lactone).

3,4,6-*Tri*-*O*-benzyl-[(2-benzyloxycarbonyl)amino]-2-deoxy-D-glucono-amide (3): A solution of **2** (5.32 g, 9.16 mmol) in anhydrous methanolic ammonia (4 N, 75 mL) was stirred for 1 h after which it was concentrated in vacuo. TLC analysis showed complete conversion of starting material into a compound with R_f = 0.58 (ethyl acetate/hexane, 2:1, v/v). Traces of ammonia in the residue were removed by co-evaporation with toluene (1 × 10 mL). Purification of the residue was accomplished by silica gel column chromatography (hexane/ethyl acetate 2:1 → 1:1, v/v). Yield 5.30 g (97%). Melting point 193.1−195.8°C (dec.). − ^1H NMR ($CDCl_3$): δ = 3.07 (1 H, br. s, OH-5), 3.56 (1 H, dd, $J_{5,6}$ = 5.0 Hz, $J_{6,6'}$ = 10 Hz, H-6), 3.63 (1 H, dd, $J_{5,6'}$ = 5.3 Hz, $J_{6,6'}$ = 10 Hz, H-6′), 3.66 (1 H, dd, $J_{3,4}$ = $J_{4,5}$ = 7.1 Hz, H-4), 3.95 (1 H, br. s, H-5), 4.39 (1 H, bd, $J_{2,3}$ = $J_{3,4}$ = 7.0 Hz, H-3), 4.41−4.69 (6 H, m, CH_2 Bn), 4.52 (1 H, bd, $J_{2,NH}$ = 5.9 Hz, H-2), 5.03 (2 H, dd, CH_2Z), 5.95 (1 H, br. s, NH), 6.09 (1 H, d, $J_{2,NH}$ = 7.0 Hz, NH), 6.38 (1 H, br. s, NH), 7.18−7.28 (20 H, m, CH-arom Bn/*Z*). − $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 54.95 (C-2), 67.08 (CH_2Z), 70.60 (C-5), 70.90 (C-6), 70.34, 74.26, 74.67 (3 × CH_2 Bn), 79.15 (C-3), 79.20 (C-4), 127.53−128.40 (CH-arom Bn/*Z*), 136.03, 137.50, 137.66, 137.94 (4 × Cq Bn/*Z*), 156.43 (C=O *Z*), 173.03 (C=O lactam). − ES-MS; *m/z*: 599.4, $[M + H]^+$; monoisotopic MW calculated for $C_{35}H_{38}N_2O_7$ = 598.3.

3,4,6-*Tri*-*O*-benzyl-2-[(benzyloxycarbonyl)amino]-5-dehydro-2-deoxy-5-hydroxy-D-glucono-δ-lactam (4): Acetic anhydride (12 mL) was added under nitrogen atmosphere to a solution of **3** (3.22 g, 5.39 mmol) in anhydrous DMSO (20 mL). The mixture was stirred for 16 h at ambient temperature. TLC analysis showed complete conversion of starting material into a mixture of products. The reaction mixture was diluted with diethyl ether (150 mL), washed thoroughly with aqueous 10% $NaHCO_3$ (3 × 50 mL) solution and with saturated NaCl (1 × 50 mL) solution. After drying on Na_2SO_4, the organic layer was concentrated in vacuo. The crude product was purified by silica gel column chromatography. Elution was performed with hexane/ethyl acetate (2:1 → 1:2, v/v). Yield 3.14 g (58%). R_f = 0.44 (ethyl acetate/hexane, 2:1, v/v). Melting point 147.5°C (dec.). − ^1H NMR ($CDCl_3$): δ = 3.34 (2 H, dd, $J_{5,6}$ = 9.7 Hz, H_2–6), 3.79 (1 H, d, $J_{3,4}$ = 9.5 Hz, H-4), 3.95 (1 H, br. s, OH-5), 4.04 (1 H, dd, $J_{2,NH}$ = 8.5 Hz, $J_{2,3}$ = 8.7 Hz, H-2), 4.21 (1 H, dd, $J_{2,3}$ = 9.0 Hz, $J_{3,4}$ = 9.1 Hz, H-3), 4.40−4.90 (6 H, m, 3 × CH_2 Bn), 5.07 (2 H, dd, CH_2Z), 5.50 (1 H, bd, $J_{2,NH}$ = 7.5 Hz, NH), 6.56 (1 H, s, NH cyclic), 7.20−7.35 (20 H, m, CH-arom Bn/*Z*). − $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 57.36 (C-2), 67.38 (CH_2Z), 72.52 (C-6), 73.75, 75.18, 75.40 (3 × CH_2 Bn), 78.27 (C-3), 78.39 (C-4), 82.17 (C-5), 128.07−128.63 (CH-arom Bn/*Z*), 156.84 (C=O *Z*), 169.49 (C=O lactam). − ES-MS; *m/z*: 597.3, $[M + H]^+$; monoisotopic MW calculated for $C_{35}H_{36}N_2O_7$ = 596.3.

3,4,6-*Tri*-*O*-benzyl-2-[(benzyloxycarbonyl)amino]-2-deoxy-D-glucono-δ-lactam (6): $NaCNBH_3$ (0.218 g, 3.44 mmol) was added to a solution of **4** (2.11 g, 3.54 mmol) in anhydrous acetonitrile (36 mL) and formic acid (4 mL). The reaction mixture was refluxed for 2 h. TLC analysis indicated the complete disappearance of the starting material. After cooling to 0°C, the mixture was quenched by the addition of aqueous HCl (1.0 N, 3.4 mL). After stirring for 15 min, the mixture was poured into a stirred mixture of EtOAc/10% aqueous $NaHCO_3$ (200 mL, 1:1, v/v). The aqueous layer was separated and extracted with EtOAc (3 × 50 mL). The organic fractions were combined and washed with saturated aqueous NaCl (50 mL) solution. After drying on Na_2SO_4, the organic layer was concentrated in vacuo, and the crude product was purified by silica gel column chromatography. Elution was performed with hexane/ethyl acetate (2:1 → 1:4, v/v). Yield 1.75 g (85%). R_f = 0.39 (ethyl acetate/hexane, 2:1, v/v). − ^1H NMR ([D_6]DMSO): δ = 3.37 (1 H, m, $J_{5,6}$ = 2.8 Hz, $J_{5,6'}$ = 3.9 Hz, $J_{4,5}$ = 9.1 Hz, H-5), 3.56 (2 H, ddd, $J_{5,6}$ = 2.8 Hz, $J_{5,6'}$ = 3.5 Hz, $J_{6,6'}$ = 9.8 Hz, H_2-6), 3.83 (1 H, dd, $J_{4,5}$ = 9.1 Hz, $J_{3,4}$ = 9.8 Hz, H-4), 3.89 (1 H, dd, $J_{3,4}$ = 9.8 Hz, $J_{2,3}$ = 9.1 Hz, H-3), 3.99 (1 H, t, $J_{2,3}$ = 9.1 Hz, H-2), 4.44−4.78 (6 H, m, CH_2 Bn), 5.08 (2 H, dd, CH_2Z), 7.22−7.37 (20 H, m, CH-arom Bn/*Z*), 7.64 (1 H, d, $J_{2,NH}$ = 8.7 Hz, NH), 7.74 (1 H, s, NH lactam). − $^{13}C\{^1H\}$ NMR ([D_6]DMSO): δ = 54.24 (C-5), 55.61 (C-2), 65.34 (CH_2Z), 68.88 (C-6), 72.37, 73.49, 73.70 (3 × CH_2 Bn), 77.07 (C-4), 80.43 (C-3), 127.41−128.66 (CH-arom Bn/*Z*), 147.21, 138.11, 138.14, 138.41 (4 × C_q Bn/*Z*), 156.43 (C=O *Z*), 168.59 (C=O lactam). − ES-MS; *m/z*: 581.2, $[M + H]^+$; monoisotopic MW calculated for $C_{35}H_{36}N_2O_6$ = 580.3.

3,4,6-*Tri*-*O*-benzyl-2-[(benzyloxycarbonyl)amino]-1,2,5-trideoxy-1,5-imino-D-glucitol (7): To neat **6** (108 mg, 0.186 mmol) was added BH_3·THF (1 M in THF, 1.4 mL). The resulting solution was stirred for 16 h at ambient temperature under a stream of nitrogen. The solution was cooled in ice and the reaction was quenched by the addition of aqueous HCl (6 N, 0.5 mL). After stirring for 30 min at room temperature, the pH of the mixture was brought to 10. The organic layer was separated and the water layer was extracted six times with ethyl acetate. After drying on Na_2SO_4, the organic layer was concentrated. The crude product was purified by silica gel column chromatography. Elution was performed with DCM/ethyl acetate (100:0 → 50:50, v/v). Yield 82 mg (78%). R_f = 0.28 (DCM/ethyl acetate, 1:1, v/v). − ^1H NMR ($CDCl_3$): δ = 1.78 (1 H, br. s, NH cyclic), 2.38 (1 H, dd, $J_{1ax,2}$ = 10 Hz, $J_{1ax,1eq}$ = 12 Hz, H-1ax), 2.71 (1 H, m, $J_{5,6}$ = 5 Hz, $J_{5,6'}$ = 8 Hz, $J_{4,5}$ = 9 Hz, H-5), 3.26 (1 H, dd, $J_{1eq,2}$ = 4 Hz, $J_{1ax,eq}$ = 12 Hz, H-1eq), 3.32 (1 H, t, $J_{2,3}$ = $J_{3,4}$ = 9 Hz, H-3), 3.47 (1 H, t, $J_{3,4}$ = $J_{4,5}$ = 9 Hz, H-4), 3.62 (3 H, m, H-2, H_2−6), 4.42−4.83 (7 H, m, 3 × CH_2 Bn, NH), 5.05 (2 H, dd, CH_2Z), 7.20−7.37 (20 H, m, CH-arom Bn/*Z*). − $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 48.77 (C-1), 54.11 (C-2), 59.80 (C-5), 66.78 (CH_2Z), 69.81 (C-6) 73.49, 74.63, 74.93 (3 × CH_2 Bn), 80.60 (C-3), 83.61 (C-4), 127.85−128.62 (CH-arom Bn/*Z*), 136.61, 138.08, 138.29 (Cq Bn/*Z*), 156.08 (C=O *Z*). − ES-MS; *m/z*: 567.3, $[M + H]^+$; monoisotopic MW calculated for $C_{35}H_{38}N_2O_5$ = 566.3.

2-Amino-3,4,6-*tri*-*O*-benzyl-2-deoxy-D-glucono-δ-lactam (8): Pd/C (5%, Degussa type E101 NO/W, 66 mg) was added to a solution of **6** (51 mg, 88 μmol) in a mixture of formic acid/water/ethyl acetate/isopropyl alcohol (3 mL, 3/3/33/60, v/v/v/v). Hydrogen was passed through the stirred mixture for 2 h. TLC analysis indicated the complete conversion of the starting material into a compound with R_f = 0.25 (ethyl acetate/hexane, 4:1, v/v). The mixture was passed over a short column containing a layer of glass wool and a layer of hyflo. Concentration of the filtrate in vacuo yielded 29 mg of a yellow syrup (74%). − ^1H NMR ($CDCl_3$): δ = 3.30 (1 H, dd, $J_{6,6'}$ = 9.7 Hz, $J_{5,6}$ = 7.8 Hz, H-6), 3.53 (3 H, m, H-2, H-5, H-6′), 3.63 (1 H, t, $J_{3,4}$ = 9.2 Hz, $J_{4,5}$ = 8.6 Hz, H-4), 4.28 (3 H, br. s, NH_3^+), 4.35−4.90 (6 H, m, CH_2 Bn), 6.62 (1 H, s, NH lactam), 7.17−7.34 (15 H, m, CH-arom Bn). − $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 54.79 (C-2), 55.80 (C-5), 70.67 (C-6), 73.45, 74.87, 75.25 (3 × CH_2 Bn), 77.34 (C-4), 83.67 (C-3), 127.66−128.60 (CH-arom Bn), 137.39, 137.51, 137.57 (3 × Cq Bn), 171.24 (C=O lactam). − ES-MS; *m/z*: 447.2, $[M + H]^+$; monoisotopic MW calculated for $C_{27}H_{30}N_2O_4$ = 446.2.

3,4,6-*Tri*-*O*-benzyl-2-[(benzyloxycarbonyl)amino]-1,5-[(*tert*-butyloxycarbonyl)imino]-1,2,5-trideoxy-D-glucitol (9): Triethylamine (36 μL, 258 μmol) and BOC anhydride (45 mg, 206 μmol) were added to a solution of **7** (98 mg, 173 μmol) in anhydrous DCM (1.0 mL). The solution was stirred for 46 h. TLC analysis showed complete conversion of starting material into a compound with R_f = 0.47 (ethyl acetate/hexane, 1:4, v/v). The reaction mixture was concentrated and the crude product was purified by silica gel column chromatography. Elution was performed with DCM/MeOH (100:0 → 96.5:3.5, v/v). Yield 115 mg (99%). − ^{1}H NMR ([D$_5$]pyridine): δ = 1.48 (9 H, s, *t*Bu), 3.43 (1 H, d, H-1ax), 3.88 (3 H, m, H$_2$-6, H-3), 4.05 (1 H, br. s, H-4), 4.21 (1 H, br. s, H-2), 4.45 (1 H, d, H-1eq), 4.52−4.80 (6 H, m, 3 × CH$_2$ Bn), 5.25 (3 H, m, H-5, CH$_2$Z), 6.85 (1 H, d, NH), 7.24−7.39 (20 H, CH-arom Bn/*Z*). − ^{13}C{^{1}H} NMR ([D$_5$]pyridine): δ = 27.67 (CH$_3$*t*Bu), 39.8 (C-1), 48.16 (C-2), 51.5 (C-5), 65.91 (*C*H$_2$Z), 67.22 (C-6), 70.94, 71.53, 72.34 (3 × CH$_2$ Bn), 73.29 (C-4), 74.98 (C-3), 79.16 (Cq *t*Bu), 127.28−128.24 (CH-arom Bn/*Z*), 135.38, 137.68, 137.88, 138.49 (Cq Bn/*Z*), 155.45, 155.55 (C=O Boc/*Z*). − ES-MS; *m/z*: 667.3, [M + H]$^+$; monoisotopic MW calculated for $C_{40}H_{46}N_2O_7$ = 666.3.

2-Amino-3,4,6-*tri*-*O*-benzyl-1,5-[(*tert*-butyloxycarbonyl)imino]-1,2,5-trideoxy-D-glucitol (10): Compound **9** (210 mg, 315 μmol) was deprotected as described for compound **8**. Yield 135 mg (89%). R_f = 0.40 (MeOH/DCM, 5:95, v/v). − ^{1}H NMR (CDCl$_3$): δ = 1.45 (9 H, s, *t*Bu), 1.79 (2 H, br. s, NH$_2$), 3.02 (1 H, br. s, H-2), 3.27 (1 H, d, H-1ax), 3.60 (1 H, br. s, H-3), 3.69 (2 H, dd, H$_2$-6), 3.77 (1 H, s, H-4), 3.82 (1 H, bd, H-1eq), 4.42−4.62 (6 H, m, 3 × CH$_2$ Bn), 4.65 (1 H, br. s, H-5), 7.19−7.33 (15 H, m, CH-arom Bn). − ^{13}C{^{1}H} NMR (CDCl$_3$): δ = 28.47 (CH$_3$ *t*Bu), 43.09 (C-1), 49.36 (C-2), 52.91 (C-5), 67.73 (C-6), 71.43, 72.03, 72.81 (3 × CH$_2$ Bn), 73.56 (C-4), 77.66 (C-3), 79.83 (Cq *t*Bu), 127.49−128.44 (CH-arom Bn), 137.96, 138.14, 138.47 (3 × Cq Bn), 156.20 (C=O Boc). − ES-MS; *m/z*: 533.4, [M + H]$^+$; monoisotopic MW calculated for $C_{32}H_{40}N_2O_5$ = 532.3.

3,4,6-*Tri*-*O*-benzyl-2-[(6-benzyloxycarbonyl)aminohexanoylamino]-deoxy-D-glucono-δ-lactam (13): To a stirred mixture of 6-(benzyloxycarbonylamino)hexanoic acid (**11**) (7 mg, 28 μmol), PyBOP (14.3 mg, 27 μmol) and DiPEA (5 μL, 29 μmol) in DCM (260 μL) was added a solution of **8** (12 mg, 27 μmol) in DCM (260 μL). After 30 min, TLC analysis indicated the complete conversion of starting material into a compound with R_f = 0.36 (MeOH/DCM, 5:95, v/v). The reaction mixture was diluted with DCM (1 mL) and washed with water (1 mL). After drying over MgSO$_4$, the organic layer was concentrated under reduced pressure. The crude product was purified by silica gel column chromatography. Elution was performed with MeOH/DCM, (0:100 → 6.5:93.5, v/v). Yield 18 mg (97%). − ^{1}H NMR ([D$_6$]DMSO): δ = 1.27, 1.39, 1.49 (6 H, m, 3 × CH$_2$ hexanoyl), 2.10 (2 H, t, CH$_2$ hexanoyl), 2.97 (2 H, m, CH$_2$ hexanoyl) 3.39 (1 H, m, $J_{4,5}$ = 7.7 Hz, $J_{5,6}$ = 4.0 Hz, $J_{5,6'}$ = 3.3 Hz, H-5), 3.55 (1 H, dd, $J_{5,6}$ = 4.0 Hz, $J_{6,6'}$ = 9.9 Hz, H-6), 3.59 (1 H, dd, $J_{5,6'}$ = 3.3 Hz, $J_{6,6'}$ = 9.9 Hz, H-6′), 3.79 (1 H, t, $J_{3,4}$ = 8.9 Hz, $J_{4,5}$ = 7.7 Hz, H-4), 3.87 (1 H, t, $J_{2,3}$ = 9.5 Hz, $J_{3,4}$ = 8.9 Hz, H-3), 4.13 (1 H, t, $J_{2,3}$ = 9.5 Hz, $J_{2,NH}$ = 8.7 Hz, H-2), 4.44−4.76 (6 H, m, 3 × CH$_2$ Bn), 5.01 (2 H, s, CH$_2$Z), 7.19 (1 H, t, NH aminohexanoyl), 7.26−7.38 (20 H, m, CH-arom Bn/*Z*), 7.68 (1 H, s, NH lactam), 8.19 (1 H, d, NH amide). − ^{13}C{^{1}H} NMR (CDCl$_3$): δ = 24.84, 26.05, 29.50, 35.90, 40.81 (5 × CH$_2$ hexanoyl), 54.32 (C-5), 55.23 (C-2), 66.56 (CH$_2$Z), 70.26 (C-6), 73.39, 74.59, 74.69 (3 × CH$_2$ Bn), 77.46 (C-4), 80.11 (C-3), 127.76−128.72 (CH-arom Bn/*Z*), 136.88, 137.43, 137.60, 138.16 (Cq Bn/*Z*), 156.53 (C=O *Z*), 168.79 (C=O lactam), 173.47 (C=O amide). − ES-MS: *m/z*: 694.3, [M + H]$^+$; monoisotopic MW calculated for $C_{41}H_{47}N_3O_7$ = 693.3.

3,4,6-*Tri*-*O*-benzyl-2-[(*R*)-3-(6-benzyloxycarbonylamino)-hexanoyloxytetradecanoylamino]-2-deoxy-D-glucono-δ-lactam (14): Compound **12** (11 mg, 22 μmol) was coupled with compound **8** (10 mg, 22 μmol) as described for the preparation of compound **13**. Yield 15.7 mg (76%). R_f = 0.51 (MeOH/DCM, 5:95, v/v). − ^{1}H NMR ([D$_5$]pyridine): δ = 0.86 (3 H, t, CH$_3$ acyloxyacyl), 1.29, 1.56, 1.65, 1.87, 2.37, 2.90, 3.33 (35 H, m, CH$_2$ acyloxyacyl), 3.68 (1 H, dd, $J_{5,6}$ = 5.7 Hz, $J_{6,6'}$ = 9.7 Hz, H-6), 3.82 (1 H, dd, $J_{5,6'}$ = 3.2 Hz, $J_{6,6'}$ = 9.7 Hz, H-6′), 3.90 (1 H, br. s, H-5), 4.06 (1 H, t, $J_{4,5}$ = 9.0 Hz, $J_{3,4}$ = 8.2 Hz, H-4), 4.55 (1 H, t, $J_{2,3}$ = 8.8 Hz, $J_{3,4}$ = 9.0 Hz, H-3), 4.82 (1 H, t, $J_{2,3}$ = 8.8 Hz, $J_{2,NH}$ = 8.0 Hz, H-2), 4.47−4.51 (6 H, m, 3 × CH$_2$ Bn), 5.33 (2 H, s, CH$_2$Z), 5.81 (1 H, m, CHO acyloxyacyl), 7.31−7.38 (20 H, m, CH-arom Bn/*Z*), 7.85 (1 H, bt, NH aminohexanoyl), 8.72 (1 H, s, NH lactam), 9.63 (1 H, d, $J_{2,NH}$ = 8.0 Hz, NH amide). − ^{13}C{^{1}H} NMR ([D$_5$]pyridine): δ = 13.57 (CH$_3$ acyloxyacyl), 22.23−40.82 (CH$_2$ acyloxyacyl), 54.51 (C-5), 55.24 (C-2), 65.42 (CH$_2$Z), 69.74 (C-6), 71.02 (CHO acyloxyacyl), 72.66, 73.76, 73.95 (3 × CH$_2$ Bn), 77.49 (C-4), 81.04 (C-3), 127.26−128.10 (CH-arom Bn/*Z*), 137.55, 137.91, 138.28, 138.72 (4 × Cq Bn/*Z*), 168.78 (C=O amide), 170.11 (C=O lactam), 172.43 (C=O ester). − ES-MS; *m/z*: 920.6, [M + H]$^+$; monoisotopic MW calculated for $C_{55}H_{73}N_3O_9$ = 919.5.

3,4,6-*Tri*-*O*-benzyl-2-[(6-benzyloxycarbonyl)aminohexanoylamino]-1,5-[(*tert*-butyloxycarbonyl)imino]-1,2,5-trideoxy-D-glucitol (15): Compound **11** (4.7 mg, 19 μmol) was coupled with compound **10** (10 mg, 19 μmol) as described for the preparation of compound **13**. Yield 14.2 mg (97%). R_f = 0.88 (MeOH/DCM, 5:95, v/v). − ^{1}H NMR ([D$_5$]pyridine): δ = 1.36 (2 H, m, CH$_2$ hexanoyl), 1.51 (9 H, s, *t*Bu), 1.55 (2 H, m, CH$_2$ hexanoyl), 1.63 (2 H, m, CH$_2$ hexanoyl), 2.14 (2 H, t, CH$_2$ hexanoyl), 3.35 (2 H, m, CH$_2$ hexanoyl), 3.46 (1 H, bd, H-1ax), 3.95 (2 H, m, H$_2$−6), 3.97 (1 H, t, H-3), 4.13 (1 H, br. s, H-4), 4.42 (1 H, br. s, H-1eq), 4.51−4.83 (6 H, m, 3 × CH$_2$ Bn), 4.58 (1 H, br. s, H-2), 7.22−7.53 (20 H, m, CH-arom Bn/*Z*), 7.91 (1 H, br. s, NH aminohexanoyl). − ^{13}C{^{1}H} NMR ([D$_5$]pyridine): δ = 24.92, 26.18, 29.43, 36.18, 40.61 (5 × CH$_2$ hexanoyl), 27.70 (CH$_3$*t*Bu), 39.2 (C-1), 45.83 (C-2), 52.5 (C-5), 65.46 (CH$_2$Z), 67.12 (C-6), 70.91, 71.38, 72.28 (3 × CH$_2$ Bn), 73.43 (C-4), 74.72 (C-3), 78.96 (Cq *t*Bu), 127.19−128.22 (CH-arom Bn), 137.55, 137.73, 137.91, 138.47 (Cq Bn/*Z*), 155.43, 156.52 (C=O Boc/*Z*), 171.15 (C=O amide). − ES-MS; *m/z*: 780.4, [M + H]$^+$; monoisotopic MW calculated for $C_{46}H_{57}N_3O_8$ = 779.4.

3,4,6-*Tri*-*O*-benzyl-2-[(*R*)-3-(6-benzyloxycarbonylamino)-hexanoyloxytetradecanoylamino]-1,5-[(*tert*-butyloxycarbonyl)imino]-1,2,5-trideoxy-D-glucitol (16): Compound **12** (9.2 mg, 19 μmol) was coupled with compound **10** (10 mg, 19 μmol) as described for the preparation of compound **13**. Yield 17.6 mg (93%). R_f = 0.63 (MeOH/DCM, 2:98, v/v). − ^{1}H NMR ([D$_5$]pyridine): δ = 0.87 (3 H, t, CH$_3$ acyloxyacyl), 1.24 (20 H, br. s, CH$_2$ acyloxyacyl), 1.52 (9 H, s, tBu), 1.67, 2.32−2.66, 3.37 (12 H, m, 6 × CH$_2$ acyloxyacyl), 3.50 (1 H, d, H-1ax), 3.93 (2 H, m, H$_2$−6), 4.00 (1 H, s, H-3), 4.14 (1 H, s, H-4), 4.42 (1 H, br. s, H-1eq), 4.50−4.84 (6 H, m, 3 × CH$_2$ Bn), 4.65 (1 H, t, H-2), 5.33 (2 H, br. s, CH$_2$Z), 5.63 (1 H, m, CHO acyloxyacyl), 7.32−7.50 (20 H, m, CH-arom Bn/*Z*), 7.65 (1 H, dd, NH amide), 7.89 (1 H, s, NH aminohexanoyl). − ^{13}C{^{1}H} NMR (CDCl$_3$): δ = 13.80 (CH$_3$ acyloxyacyl), 22.10−40.96 (CH$_2$ acyloxyacyl), 27.72 (CH$_3$*t*Bu), 39 (C-1), 46.05 (C-2), 53 (C-5), 65.48 (CH$_2$Z), 67.19 (C-6), 70.65 (CHO acyloxyacyl), 70.90, 71.43, 72.31 (3 × CH$_2$ Bn), 73.34 (C-4), 74.77 (C-3), 79.07 (Cq *t*Bu), 127.20−128.25 (CH-arom Bn/*Z*), 137.56, 137.86, 137.92, 138.47 (Cq Bn/*Z*), 155.45, 156.55 (C=O Boc/*Z*), 168.40, 172.32 (C=O amide/ester). − ES-MS; *m/z*: 1006.6, [M + H]$^+$; monoisotopic MW calculated for $C_{60}H_{83}N_3O_{10}$ = 1005.6.

2-[(6-Aminohexanoyl)amino]-2-deoxy-D-glucono-δ-lactam (17): Formic acid (20 μL) and Pd/C (10%, 20 mg) were added to a mixture of **13** (13 mg, 18 μmol) in isopropyl alcohol/EtOAc/H_2O (1.5 mL, 2/3/1, v/v/v). Hydrogen was passed through the stirred mixture for 2 h. After filtrating of the mixture over a PTFE filter, the filtrate was concentrated under reduced pressure. Yield 4.6 mg (88%). − ^{1}H NMR ([D$_4$]MeOH): δ = 1.46 (2 H, t, CH_2 hexanoyl), 1.69 (4 H, m, 2 × CH_2 hexanoyl), 2.31 (2 H, t, CH_2 hexanoyl), 2.93 (2 H, t, CH_2 hexanoyl), 3.25 (1 H, m, H-5), 3.58 (1 H, t, $J_{4,5}$ = 9.3 Hz, $J_{3,4}$ = 9.4 Hz, H-4), 3.63 (1 H, dd, $J_{6,6'}$ = 11.4 Hz, $J_{5,6}$ = 5.8 Hz, H-6), 3.82 (1 H, t, $J_{2,3}$ = $J_{3,4}$ = 9.8 Hz, H-3), 3.84 (1 H, dd, $J_{6,6'}$ = 11.4 Hz, $J_{5,6'}$ = 2.8 Hz, H-6′), 4.02 (1 H, d, $J_{2,3}$ = 10 Hz, H-2), 4.82 (3 H, br. s, 3 × OH), 8.58 (2 H, br. s, NH_2). − ^{13}C{^{1}H} NMR ([D$_4$]MeOH): δ = 25.69, 26.55, 28.03, 36.40, 43.43 (5 × CH_2 hexanoyl), 56.76 (C-2), 58.46 (C-5), 62.69 (C-6), 70.76 (C-4), 73.33 (C-3), 176.40 (C=O). − ES-MS; *m/z*: 290.2, [M + H]$^+$; monoisotopic MW calculated for $C_{12}H_{23}N_3O_5$ = 289.2.

2-[(*R*)-3-(6-Amino)hexanoyloxytetradecanoylamino]-2-deoxy-D-glucono-δ-lactam (18): Compound **14** (7.8 mg, 8.5 μmol) was treated as described for the preparation of compound **17**. Yield 5.0 mg (quantitative). − ^{1}H NMR ([D$_4$]MeOH): δ = 0.89 (3 H, t, CH_3 acyl), 1.28, 1.42, 1.65, 2.35, 2.50 (31 H, m, CH_2 acyloxyacyl), 2.77 (1 H, br. s, H-1ax), 2.93 (2 H, t, CH_2 acyloxyacyl), 2.96 (1 H, br. s, H-5), 3.28 (1 H, br. s, H-1eq), 3.48 (2 H, br. s, H-3, H-4), 3.85 (2 H, dd, H_2−6), 3.90 (1 H, br. d, H-2), 5.23 (1 H, m, CHO acyloxyacyl). − ^{13}C{^{1}H} NMR ([D$_4$]MeOH): δ = 14.37 (CH_3 acyl), 23.68−42.00 (CH_2 acyloxyacyl), 46.47 (C-1), 50.90 (C-2), 59.87 (C-6), 62.15 (C-5), 71.20 (C-4), 72.70 (CHO acyloxyacyl), 75.60 (C-3), 174.68 (C=O). − ES-MS; *m/z*: 516.5, [M + H]$^+$; monoisotopic MW calculated for $C_{26}H_{49}N_3O_7$ = 515.4.

2-(6-Aminohexanoyl)amino-1,2,5-trideoxy-1,5-imino-D-glucitol (19): To a cooled (0°C) and stirred solution of **15** (7.1 mg, 9.1 μmol) in DCM (0.5 mL) was added TFA (0.5 mL). After 30 min, TLC analysis showed complete conversion of the starting material into a compound with R_f = 0.56 (DCM/MeOH, 92/8, v/v). The mixture was concentrated in vacuo. Subsequently, the residue was treated as described for the preparation of compound **17**. Yield 5.5 mg (quantitative). − ^{1}H NMR ([D$_4$]MeOH): δ = 1.42, 1.67, 2.28 (8 H, m, 4 × CH_2 hexanoyl), 2.87 (1 H, t, $J_{1ax,1eq}$ = 12.7 Hz, $J_{1ax,2}$ = 12.3 Hz, H-1ax), 2.92 (2 H, m, CH_2 hexanoyl), 3.05 (1 H, m, $J_{4,5}$ = 9.1 Hz, $J_{5,6}$ = 5.3 Hz, $J_{5,6'}$ = 3.1 Hz, H-5), 3.38 (1 H, dd, $J_{1eq,2}$ = 5.1 Hz, $J_{1ax,1eq}$ = 12.7 Hz, H-1eq), 3.52 (2 H, m, $J_{2,3}$ = 9.6 Hz, $J_{4,5}$ = 9.1 Hz, H-3, H-4), 3.83 (1 H, dd, $J_{5,6}$ = 5.3 Hz, $J_{6,6'}$ = 11.8 Hz, H-6), 3.90 (1 H, dd, $J_{5,6'}$ = 3.1 Hz, $J_{6,6'}$ = 11.8 Hz, H-6′), 3.99 (1 H, m, $J_{1eq,2}$ = 5.1 Hz, $J_{2,3}$ = 9.6 Hz, H-2). − ^{13}C{^{1}H} NMR ([D$_4$]MeOH): δ = 25.99, 26.88, 28.24, 36.47, 40.56 (5 × CH_2 hexanoyl), 45.85 (C-1), 50.01 (C-2), 59.17 (C-6), 62.03 (C-5), 70.56 (C-4), 75.11 (C-3). − ES-MS; *m/z*: 276.3, [M + H]$^+$; monoisotopic MW calculated for $C_{12}H_{25}N_3O_4$ = 275.2.

2-[(*R*)-3-(6-Amino)hexanoyloxytetradecanoylamino]-1,2,5-trideoxy-1,5-imino-D-glucitol (20): Compound **16** (8.8 mg, 8.7 μmol) was treated as described for the preparation of compound **19**. Yield 3.2 mg (73%). − ^{1}H NMR ([D$_4$]MeOH): δ = 0.89 (3 H, t, CH_3 acyloxyacyl), 1.28, 1.42, 1.65 (27 H, m, CH_2 acyloxyacyl), 2.35 (2 H, t, CH_2 acyloxyacyl), 2.50 (2 H, d, CH_2 acyloxyacyl), 2.77 (1 H, br. s, H-1ax), 2.93 (2 H, t, CH_2 acyloxyacyl), 2.96 (1 H, br. s, H-5), 3.28 (1 H, br. s, H-1eq), 3.48 (2 H, br. s, H-3, H-4), 3.85 (2 H, m, H_2−6), 3.90 (1 H, br. d, H-2), 5.23 (1 H, m, CHO acyloxyacyl). − ^{13}C{^{1}H} NMR ([D$_4$]MeOH): δ = 14.37 (CH_3), 23.68−42.00 (CH_2 acyloxyacyl), 46.47 (C-1), 50.90 (C-2), 59.87 (C-6), 62.15 (C-5), 71.20 (C-4), 72.70 (CHO acyloxyacyl), 75.60 (C-3), 174.68 (C=O). − ES-MS; *m/z*: 502.6, [M + H]$^+$; monoisotopic MW calculated for $C_{26}H_{51}N_3O_6$ = 501.4.

Compound 22: To a stirred and cooled (0°C) solution of 5,5′-dithiobis(2-nitrobenzoic acid, succinimidyl ester) **(21)** (3.76 mg, 6.37 μmol) in DMF (0.40 mL) was added dropwise a solution of **17** (0.92 mg, 3.18 μmol) in DMF (0.1 mL). The reaction mixture was stirred for 16 h at 4°C. The crude product was purified by FPLC (PepRPC™ HR 10/10 column) to give pure **22** (1.0 mg, 1.3 μmol, 41%). − ES-MS; *m/z*: 765.0, [M + H]$^+$; monoisotopic MW calculated for $C_{30}H_{32}N_6O_{14}S_2$ = 764.1.

BSA Conjugate 23: To a stirred and cooled (0°C) solution of BSA (5 mg) in PBS (0.6 mL) was added dropwise a mixture of **22** (1.0 mg, 1.3 μmol) in DMF/PBS (0.1 mL, 7:3, v/v). The reaction mixture was stirred for 30 h at 4°C and subsequently applied to a centrifuge ultrafilter (Centrex UF-2, MW cutoff 10 kD). After filtration (1 h, 5000 g), the retentate containing BSA conjugate **23** was washed with PBS (2 × 1 mL), followed by filtration (2 × 1 h, 5000 g). Hapten density (6:1, hapten/BSA) was determined by measuring the release of thionitrophenolate ion (absorption at 412 nm) after reduction of the BSA conjugate **23** with TCEP.

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