Inverse electron demand Diels-Alder pyridazine elimination: synthetic tools for chemical immunology

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Chemical activation of iNKT-cells: design and synthesis of caged α-galactosylceramide derivatives

M.M.E. Isendoorn contributed to the work described in this Chapter.

5.1 Introduction

Natural killer T (NKT) cells act as immunomodulators upon recognition of endogenous and foreign (glyco)lipid antigens presented by major histocompatibility complex type-1 (MHC-I)-like CD1d proteins.[1] These T-cells combine properties of natural killer (NK) cells with CD1d-restricted αβ T-cell receptors (TCRs),[2] and whilst they constitute less than 1% of total T-cells present in blood,[3,4] their activation triggers rapid release of cytokines without relying on clonal expansion, making them key mediators in many branches of the immune response.[5,6]

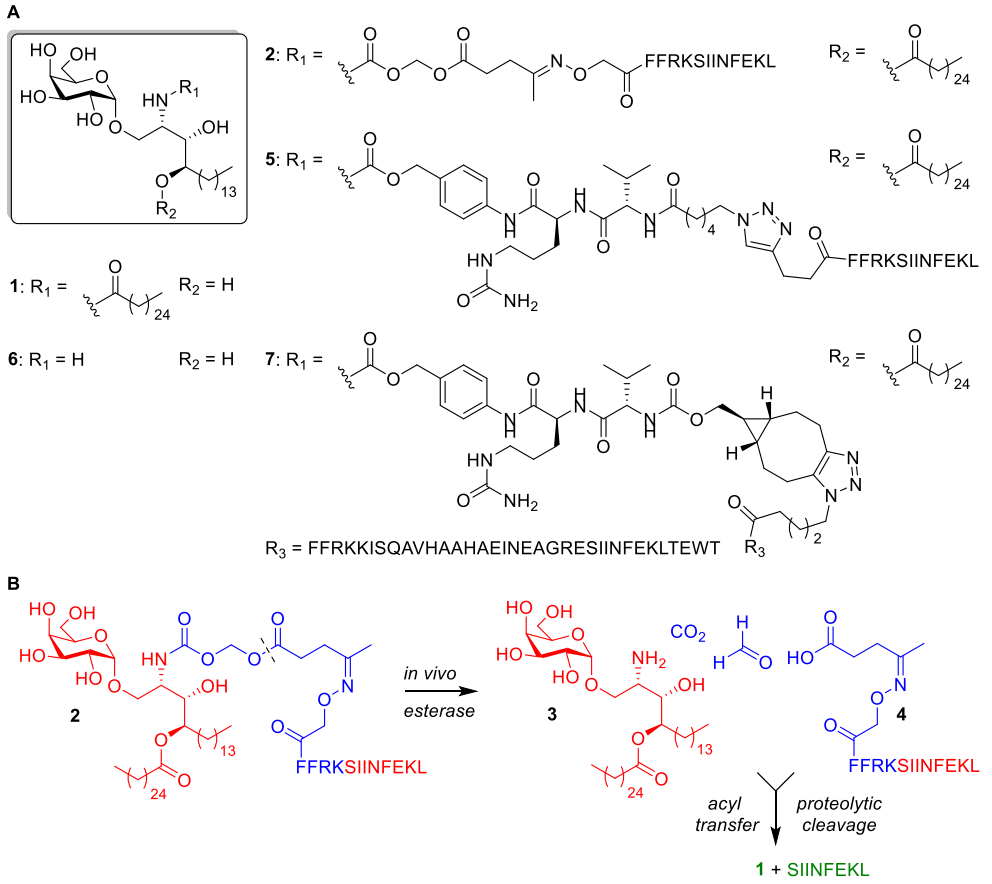


Figure 1 A) Synthetic glycolipid-peptide conjugates (**2**, **5**, **7**) developed as vaccines by Painter, Hermans and co-workers.[32, 36-40] The glycolipid portion of these vaccines are based on (a rearranged structure of) αGalCer (**1**). B) Mode of action of glycolipid vaccine **2**.[32] Upon uptake by a DC, esterases cleave the acyloxymethyl carbamate linker to give **3** and peptide fragment **4**. Intramolecular rearrangement of **3** provides **1** and proteolytic cleavage of **4**, which is accelerated by the N-terminal FFRK sequence,[34] affords the MHC-I epitope SIINFEKEL. The combined MHC-I and CD1d activation triggers DC priming and results in a potent $CD8^+$ T-cell response.

Invariant NKT (iNKT) cells, or type I NKT cells, account for 80% of NKT cells[3] and express a highly conserved TCR α-chain in conjunction with a limited scope of β-chains.[2–5] Isolation of agelasphins, α-linked galactosylceramides with antitumor properties, from the marine sponge *Agelas mauritianus*[7] and subsequent structure-activity relationship (SAR) studies[8] identified αGalCer (KRN7000, **1**, Figure 1A)[8] as a potent model antigen for iNKT cells.[2,9] Rigid binding of the acyl- and phytosphingosine lipid tails of **1** in the respective A'- and F'-pockets of CD1d enables surface exposure of the α-galactosyl moiety for recognition by type I NKT TCRs.[1,10] Presentation of **1** by dendritic cells (DCs) and subsequent activation of iNKT cells triggers secretion of both

pro-inflammatory T_H1 (for instance IFN-γ, TNF and IL-2) and immunomodulatory T_H2 (for instance IL-4, IL-10 and IL-13) cytokines,[6] thereby stimulating DC maturation. This iNKT-DC interaction, which operates via IL-12 signaling, subsequently triggers NK cell transactivation,[11] resulting in high levels of IFN-γ release, and stimulates both antigen cross presentation and T-cell activation.[4,12,13] Additionally, iNKT cells promote B-cell, macrophage and neutrophil activity.[4,12]

Following its discovery, compound **1** was initially considered as a stand-alone drug in cancer immunotherapy.[8] However, the majority of clinical trials conducted in this context have shown that compound **1** falls short in this;[14–16] predominantly because the effect of IFN-γ, as induced by compound **1**, is limited due to a mixed T_H1/T_H2 response. The induction of iNKT cell anergy[17,18] and hepatotoxicity[19] further limits its use. Ongoing studies to identify and develop novel αGalCer derivatives which elicit skewed T_H1 or T_H2 responses[20,21] are supported by novel approaches, such as the discovery of CD1d ligands which display covalent binding.[22]

Co-administration of **1** with peptide vaccines to enhance $CD8^+$ and $CD4^+$ T-cell responses has previously been established.[23–27] It was shown that this stimulatory effect requires presentation of both the specific peptide antigen and **1** by the same DC,[26,28] which emphasizes the targeted delivery of both components *in vivo*. In this regard, a particularly promising development is the employment of **1** as a covalent adjuvant[29] to stimulate the effectiveness of synthetic carbohydrate[30,31] and peptide[32] vaccines. Notably, Painter, Hermans and co-workers[32] reported a self-adjuvanting strategy, where an inactive pro-adjuvant (**2**) rearranges into **1** upon esterase activity: cleavage of the acyloxymethyl carbamate moiety[33] enables intramolecular oxygen-to-nitrogen acyl transfer from **3** to afford **1** (Figure 1B).[32] Additionally, proteolytic cleavage of the N-terminal FFRK sequence[34] afforded the MHC-I antigen SIINFEKL ($OVA_{257-264}$; OT-I)[35] from the aminooxy linked peptide fragment (**4**, Figure 1B).[32] Vaccine conjugate **2** was able to elicit a potent and specific $CD8^+$ T-cell response: effective release of IFN-γ was observed, owing to transactivation of NK cells, whilst reduced levels of IL-4 were detected and fewer NKT cells were activated compared to co-administration of **1** and the peptide construct.[32]

An additional advantage to these conjugate glycolipid-peptide vaccines, which induce iNKT-assisted priming of DCs to obtain potent $CD8^+$ T-cell responses, is their cost-effective synthesis: advanced intermediates can be stored and subsequently conjugated to the desired epitope regions in a single step. Consequently, the versatility of the self-adjuvanting approach[32] was explored for *in vivo* treatment of tumors,[36–38] influenza,[39] and malaria.[40] These studies also introduced protease-sensitive valine-citrulline-*para*-amino-benzyl (VC-PAB) linkers[41] for enhanced *in vivo* stability and

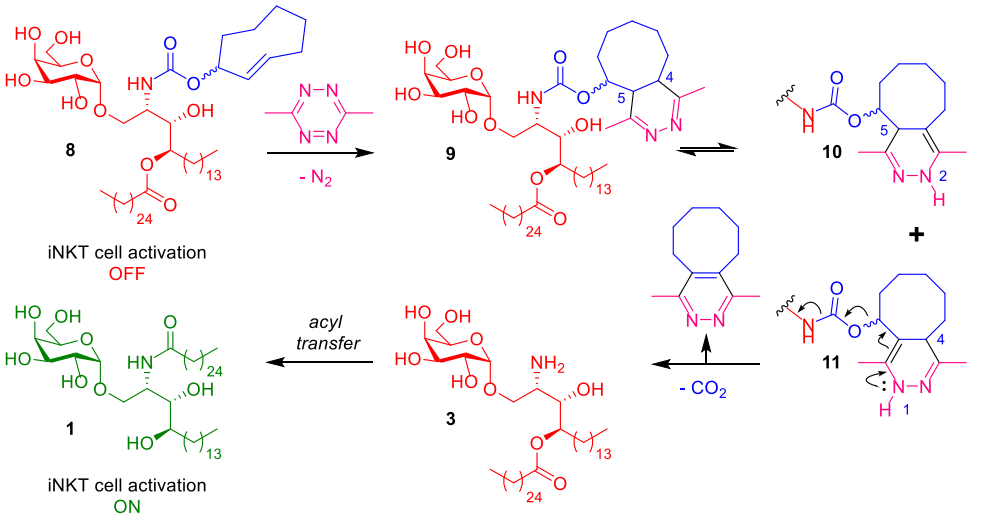
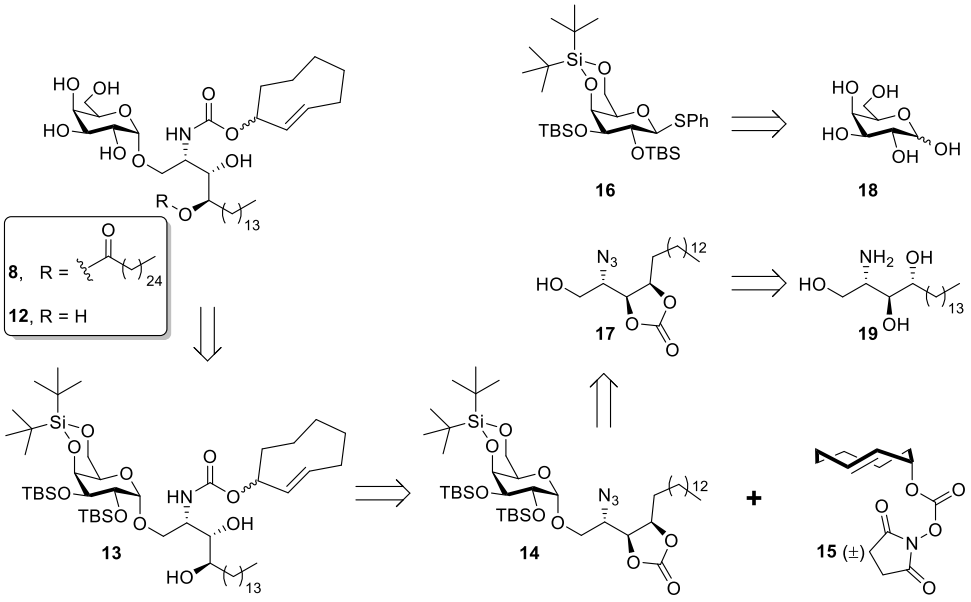


Figure 2 Design of TCO caged αGalCer **7**, which is unable to elicit iNKT activation via CD1d recognition. Upon ligation to a tetrazine, the 4,5-dihydropyridazine (**9**) can tautomerize to form the 2,5- and 1,4-tautomers (**10** and **11**, respectively). Elimination of CO_2 and the pyridazine adduct from **11** affords **3**, which can then undergo intramolecular acyl transfer to afford **1**, which induces iNKT activation.

both copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) and strain-promoted alkyne-azide cycloaddition (SPAAC) as alternative conjugation strategies (see for example Figure 1A, **5**).[36,39] The identification and application of α-galactosylphytosphingosine (αGalPhs, Figure 1A, **6**)[38] as partial agonist towards iNKTs enables further fine-tuning of the conjugate vaccines, for instance to reduce *in vivo* hepatotoxicity.[38] Another development is the use of synthetic long peptides (SLPs), containing both $CD4^+$ and $CD8^+$ T-cell epitopes, to encompass large immunogenic regions of target proteins (see for example, Figure 1A, **7**).[37–40]

It was hypothesized that a chemical trigger to activate covalent glycolipid-peptide vaccines would provide enhanced control over the priming of DCs, whilst retaining the favorable delivery observed for these conjugates.[32,36–38,40] In this regard, Trauner and co-workers recently demonstrated photochemical control over cytokine secretion with azobenzene-functionalized αGalCer derivatives.[42] The inverse electron demand Diels-Alder (IEDDA) pyridazine elimination,[43] a dissociative bioorthogonal reaction,[44–46] constitutes another attractive option for this approach. This "click-to-release" technique has demonstrated particular promise towards the (tetrazine mediated) activation of antibody-drug-conjugates (ADCs, Chapter 2), as shown by Robillard[47,48] as well as Royzen and Oneto,[49] MHC-I antigens (Chapter 4),[50] TLR ligands,[51] and even protein active sites.[52] It was therefore reasoned that protection of the amine

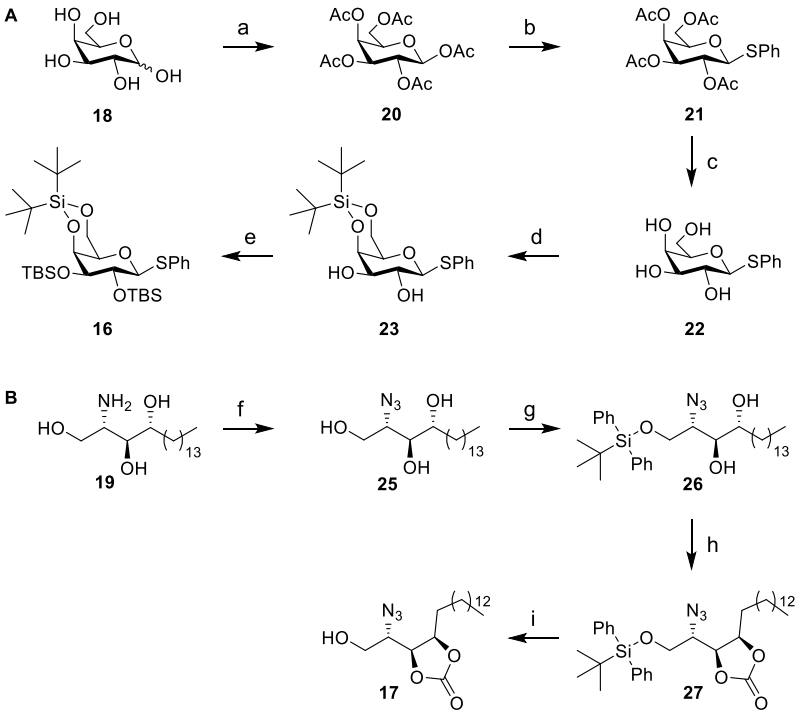


Scheme 1 Retrosynthetic design for caged αGalCer **8** and caged αGalPhs **12** from a shared intermediate (**13**), which can be synthesized from glycosylation partners **16** and **17**.

functionality of pro-adjuvant **3** with an allylic, substituted *trans*-cyclooctene (TCO) modality would render rearrangement of the resulting caged α-GalCer (**8**) under tetrazine control (Figure 2). In this scenario, tetrazine ligation of **8** with 3,6-dimethyltetrazine results in 4,5-dihydropyridazine **9**. Compound **9** tautomerizes to form 2,5-dihydropyridazine **10** and 1,4-dihydropyridazine **11**, the latter of which is able to eliminate **3** for subsequent acyl transfer to obtain **1**.

5.2 Results and discussion

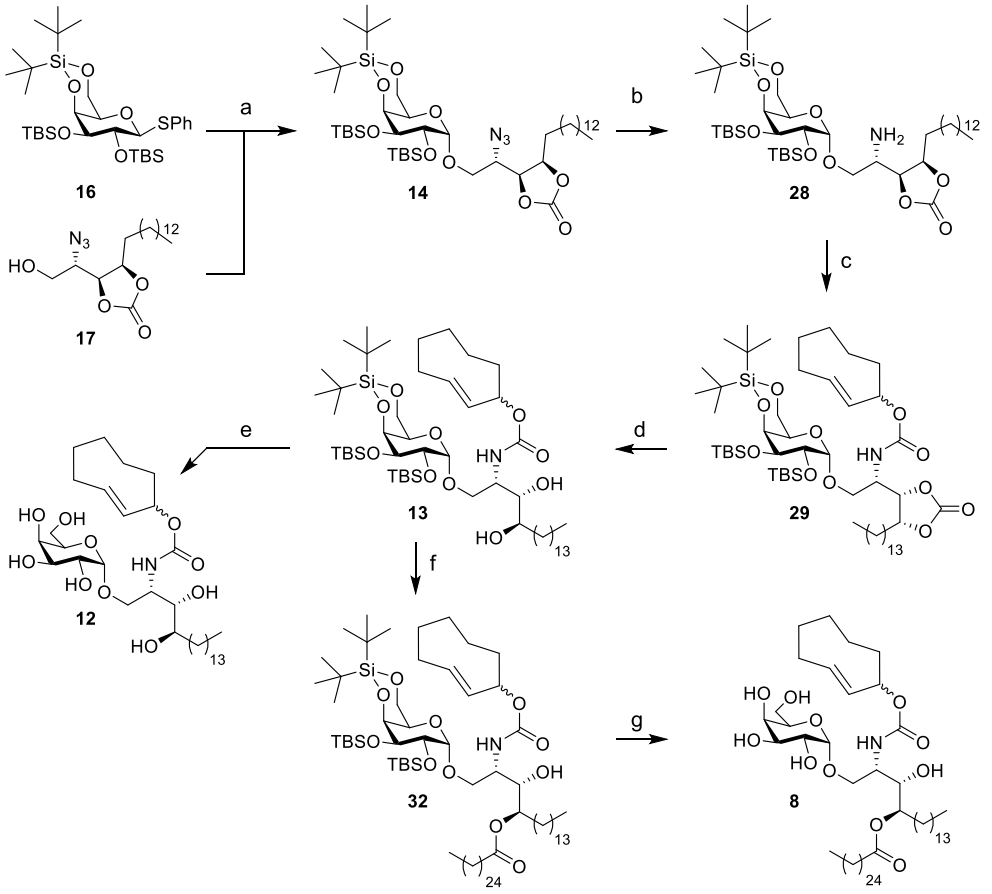
With the aim to evaluate chemical control over CD1d-mediated glycolipid recognition by means of click-to-release IEDDA chemistry, this Chapter describes the synthesis of caged lipids **8** and **12**, based on (pro)αGalCer (**3**) and αGalPhs (**6**), respectively (Scheme 1). Both compounds were synthesized from TCO-protected intermediate **13**, which in turn was obtained from the α-galactosylated intermediate **14** and axial TCO carbonate **15** (Chapter 3) in three steps. Late-stage (global) deprotection of (*para*-methoxy) benzyl protecting groups by means of hydrogenolysis or acid, as is often the case for αGalCer (**1**) syntheses reported in literature,[20] was ruled out with regard to preservation of the TCO moiety. Formation of **14** was envisaged by combining 4,6-di-*tert*-butylsilylene (DTBS)-directed α-galactosylation[53–55] with an azide protected phytosphingosine acceptor, as reported by Veerapen *et al*.[56] However, instead of protecting the remaining alcohol functionalities as benzoyl esters, 2,3-TBS-4,6-DTBS



Scheme 2 Synthesis of galactose donor **16** (A) and phytosphingosine acceptor **17** (B). Reagents/conditions: (a) Ac_2O, NaOAc, reflux, 52%; (b) PhSH, $BF_3 \cdot OEt_2$, DCM, 0°C to rt, 95%; (c) NaOMe, MeOH, rt, 95%; (d) DTBS$(OTf)_2$, pyridine, DMF, -40°C, 83%; (e) TBS-OTf, DMAP, pyridine, 0°C to rt, 95%; (f) imidazole-1-sulfonyl azide hydrogen sulfate (**24**), K_2CO_3, Cu(II) $\cdot$ 5 H_2O, MeOH, DCM, rt; (g) TBDPS-Cl, Et_3N, DMAP, DCM, rt, 83% over two steps; (h) CDI, DCM, rt, 79%; (i) HF $\cdot$ pyridine, pyridine, rt, 92%.

protected donor **16** and 2-azido-3,4-cyclic carbonate acceptor **17** were selected, as reported by Gold *et al*.[57] and Panza *et al*.[58], respectively. This approach would enable selective deprotection of the cyclic carbonate moiety after glycosylation, in addition to a mild desilylation as the final deprotection step. Additionally, if required, the reactivity of donor could be enhanced by transforming **16** into a more reactive imidate donor.[57] Therefore, donor **16** was synthesized from D-galactose (**18**) and acceptor **17** was synthesized from D-*ribo*-phytosphingosine (**19**).

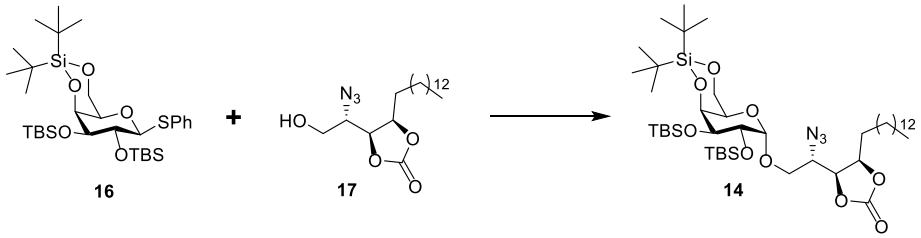
Peracetylation of **18** by refluxing in a mixture Ac_2O and NaOAc afforded **20** after crystallization in 52% yield. Anchimerically assisted installation of the anomeric thiophenol modality was achieved in the presence of $BF_3 \cdot OEt_2$ to obtain **21**, which gave **22** after Zemplén deacetylation. The 4,6-DTBS protecting group[59] was installed by treating **22** with DTBS$(OTf)_2$, and subsequent treatment with pyridine, to obtain **23** in 85% isolated yield after chromatographic purification. Finally, the 2-OH and 3-OH



Scheme 3 Synthesis of caged glycolipids **8** and **12** from galactose donor **16** and phytosphingosine acceptor **17**. Reagents/conditions: (a) NIS, TMS-OTf, DCM, -40°C, 67%; (b) PtO_2, H_2 (g), THF, rt; (c) TCO-NHS (**15**), DIPEA, DMAP, DMF, rt, 89% over two steps; (d) LiOH, THF, H_2O, rt, quant.; (e) $Et_3N \cdot 3HF$, THF, rt, 84%; (f) hexacosonoic acid (**30**), EDC · HCl, DIPEA, DMAP, DCM, rt, 31-34%; (g) $Et_3N \cdot 3HF$, THF, rt, 23%.

positions of **23** were protected as TBS esters using TBS-OTf in the presence of 4-dimethylaminopyridine (DMAP) and pyridine to give donor **16** in 95% yield. D-*ribo*-phytosphingosine (**19**) was protected by diazotransfer with imidazole-1-sulfonyl azide hydrogen sulfate (**24**)[60,61] in the presence of K_2CO_3 and Cu(II) · 5 H_2O to obtain azide **25**, followed by silylation of the primary alcohol to obtain *tert*-butyldiphenylsilyl (TBDPS) ester (**26**) in 83% over two steps. The 3,4-diol functionality was protected as the cyclic carbonate using 1,1'-carbonyldiimidazole (CDI) to obtain **27** in 79% yield. Desilylation in the presence of HF · pyridine afforded acceptor **17** in 92% yield. Direct formation of **17** from **25** using diphosgene, as reported by Panza *et al.*[58], did not provide reproducible results when moving beyond small scale preparations.

Table 2 Glycosylation of galactose donor **16** and phytosphingosine acceptor **17** to form **14**.



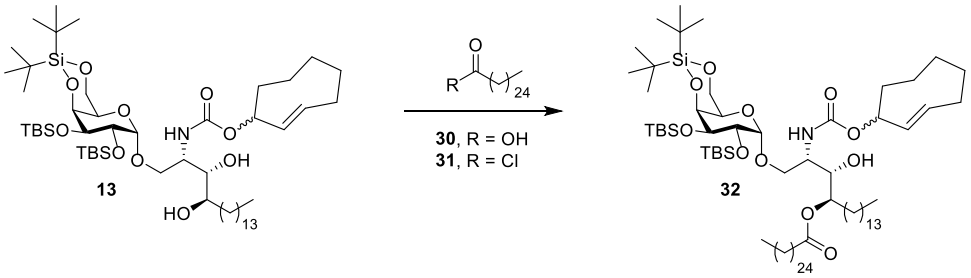
Entry	Scale (mmol)	Donor 16 (equiv)	Promotor system (equiv)	Solvent (M)	Temp. (°C)	Time (min)	Yield (%)[a]
1	0.1	1.5	IDCP (3.0)	DCM (0.2)	0 → rt	-	-
2	0.15	1.5	NIS (1.5), AgOTf (0.3)	DCM (0.2)	0 → rt	-	-
3	0.1	1.5	NIS (1.5), TfOH (0.2)	DCM (0.1)	-40	15	63
4	0.4	1.5	NIS (1.5), TfOH (0.2)	DCM (0.1)	-40	60	52
5	0.6	1.5	NIS (1.5), TfOH (0.1)	DCM (0.1)	-40	60	34
6	0.2	1.5	NIS (1.5), TMS-OTf (0.2)	DCM (0.1)	-40	15	85
7	2.4	1.2	NIS (1.5), TMS-OTf (0.2)	DCM (0.1)	-40	180	59
8	5.3	1.5	NIS (1.5), TMS-OTf (0.2)	DCM (0.1)	-40	300	67

[a]Isolated yield after aqueous workup and chromatographic purification.

Glycosylation of donor **16** and acceptor **17** was investigated next (Scheme 3; Table 1). Iodoniumdicollidine perchlorate (IDCP) proved unable to activate donor **16** (Table 1, entry 1). Instead, promotor systems based on N-iodosuccinimide (NIS) were evaluated. Activation of donor **16** with NIS/AgOTf resulted in a complex mixture of products (Table 1, entry 2). However, employing a mixture of NIS and catalytic TfOH at -40°C, as reported by Veraapen *et al*.[56] for a similar glycosylation, resulted in rapid α-selective glycosylation using donor **16** and 1.5 equivalents of acceptor **17** to obtain **14** in 63% yield (Table 1, entry 3). Additional experiments on small scale (≤ 1 mmol **16**) confirmed these findings (Table 1, entries 4 and 5) and also identified TMS-OTf as a more effective activator when used in combination with NIS (Table 1, entry 6). Glycosylation at 5 mmol scale, although requiring a prolonged reaction time, resulted in a yield of 67% (Table 1, entry 8).

Hydrogenation of the α-galactosylated product (**14**) in the presence of Adam's catalyst afforded amine **28**. Subsequently, axial TCO carbonate **15** (Chapter 3) was employed as a reagent to install the TCO carbamate moiety on **28**, in the presence of DIPEA and DMAP, to obtain **29** in 89% over two steps after chromatographic purification. Saponification of the cyclic carbonate functionality was performed with LiOH in a

Table 2 Esterification of **13** to obtain **32**.



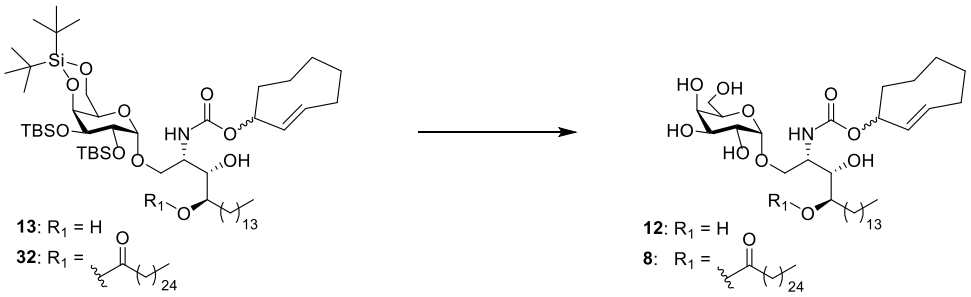
Entry	Scale (mmol)	Reagent (equiv)	Coupling conditions (equiv)	Solvent (M)	Temp. (°C)	Time (min/h/d)	Yield (%)[a]
1	0.08	**30** (1.5)	PyBOB (1.5) DIPEA (3.0)	DCM (0.04)	rt	5 d	-
2	0.09	**30** (1.3)	EEDQ (2.0)	EtOH (0.06)	0 → rt → 50	2 d	-
3	0.10	**31** (1.3)	DIPEA (4.0)	DCM (0.02)	-20 → rt	30 min	-
4	0.10	**30** (1.5)	EDC · HCl (1.5), DMAP (6.0) DIPEA (3.0)	DCM (0.03)	0 → rt	20 h	31
5	0.2	**30** (1.5)	EDC · HCl (1.5), DMAP (6.0) DIPEA (3.0)	DCM (0.05)	0 → rt	3 d	32
6	0.08	**30** (1.5)	TCBC (6.0), DMAP (6.0) Et_3N (6.0)	DCM (0.04)	rt	3 d	34

[a]Isolated yield after aqueous workup and chromatographic purification.

mixture of THF and H_2O to obtain **13** as a crude product which could be directly used for subsequent steps. An alternative three step reaction sequence for the conversion of **14** to **13** was initially investigated by subjugating **13** to saponification of the cyclic carbonate functionality, followed by Staudinger reduction in the presence of trimethylphosphine and NaOH and installation of the TCO carbamate as the final step. While this reaction sequence showed promising results on small scale, it resulted in a complicated purification procedure for **13** and generally resulted in lowered yields and increased reaction time.

Acylation with hexacosonoic acid (**30**) was investigated for **13** (Table 2). Esterification in the presence of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) or N-ethoxycarbonyl-2-ethoxy-1,2-dihydro quinoline (EEDQ)[62] proved ineffective, despite prolonged reaction times (entries 1 and 2). Reaction of **13** with hexacosanoyl chloride (**31**) resulted in a complex reaction mixture. Instead, Steglich esterification[63] of **13** and **30** in the presence of 1-ethyl-3-(3 -dimethylaminopropyl) carbodiimide hydrochloride (EDC · HCl), DMAP and DIPEA afforded **32** in 31% yield (entry 4). Extending the reaction time for this procedure gave similar results (entry 5). Yamaguchi esterification[64–67] in the presence of 2,4,6-trichlorobenzoyl chloride (TCBC), DMAP and Et_3N also afforded **32** in a comparable yield.

Table 3 Silyl deprotection of **13** and **32** to obtain **12** and **8**.



Entry	Compound (mmol)	Deprotection conditions (equiv)	Solvent (M)	Temp. (°C)	Time (h)	Yield (%)[a]
1	**13** (0.08)	HF · pyridine (excess)	-	rt	16	-
2	**13** (0.06)	HF · pyridine (10)	THF (0.03)	0	16	-
3	**13** (0.03)	HF · pyridine (10)	Pyridine (0.03)	rt	72	-
4	**13** (0.10)	TBAF (15)	THF (0.1)	rt	16	-
5	**13** (0.10)	TBAF (15), AcOH (4)	THF (0.1)	rt	16	-
6	**13** (0.10)	$Et_3N \cdot 3$ HF (6)	THF (0.1)	0 → rt	16	**12** (28)
7	**13** (0.56)	$Et_3N \cdot 3$ HF (6)	THF (0.1)	0 → rt	96	**12** (84)
8	**32** (0.30)	$Et_3N \cdot 3$ HF (10)	THF (0.1)	0 → rt	27	**8** (23)

[a]Isolated yield after aqueous workup and chromatographic purification.

Simultaneous deprotection of the cyclic DTBS protecting group and two TBS groups on the galactose moiety was evaluated for both **13** and **32** to obtain **12** and **8**, respectively (Table 3). Initial attempts relied on HF · pyridine and tetra-*n*-butylammoniumfluoride (TBAF), as individual reports on αGalCer derivatives have shown both of these reagents to be effective for 4,6-DTBS deprotection.[56,68,69] Treatment of **13** with HF · pyridine (neat) resulted in a complex mixture of products (entry 1) , which could not be circumvented by performing the deprotection at low temperature in THF (entry 2). Diluting the reaction mixture with pyridine resulted in a lack of conversion, despite prolonged incubation (entry 3). Deprotection of **13** in the presence of TBAF resulted in partial deprotection of silyl esters and TCO carbamate hydrolysis (entry 4). Addition of AcOH to the deprotection with TBAF also resulted in a complex mixture of reaction products (entry 5).

As an alternative, global deprotection of **13** with $Et_3N \cdot 3HF$ was investigated in THF, resulting in an isolated yield of 28% (**12**, entry 6) after 16 hours. Prolonging the incubation time for this deprotection resulted in an increased yield of 84% (**12**, entry 7). Finally, $Et_3N \cdot 3HF$ mediated deprotection conditions also enabled conversion of **32** to **8** in 23% yield without observing hydrolysis of the ester bond (entry 8).

NMR analysis for both **32** and **8** indicated the presence of a regioisomeric byproduct, implying the ester bond was installed without complete regioselectivity. Furthermore,

migration of the ester moiety was not observed during the deprotection of **32** to **8**. Additionally, LC-MS experiments with a non-releasing tetrazine (Chapter 3 and 4) confirmed the *trans* configuration of the double bond for **12** and **8**. Taken together, while further optimization for the esterification and deprotection steps is warranted for **8** specifically, the results described confirm the compatibility of the deprotection conditions towards the envisioned synthetic strategy.

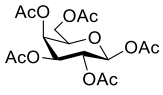
5.3 Conclusions

In conclusion, the synthesis of two TCO caged derivatives (**8** and **12**) of pro-αGalCer (**3**) and αGalPhs (**6**) is reported. α-Selective glycosylation of a 2,3-TBS-4,6-DTBS protected thiogalactoside (**16**) with a 2-azido-3,4-cyclic carbonate protected phytosphingosine (**17**) afforded key intermediate **14**, which was converted in three steps - hydrogenation, TCO carbamate formation and saponification - to obtain TCO protected intermediate **13**. Direct desilylation afforded **12**, whilst esterification and concomitant deprotection gave **8**.

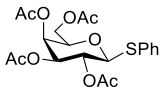
Looking ahead to future research, TCO protected glycolipids **8** and **12** are to be evaluated for the envisaged *in vivo* chemical control over iNKT cell activation. Initial *in vitro* experiments should compare the cytokine release profiles of **8** with **1** and **12** with **6**, respectively. These conditions can subsequently be compared to ones where a tetrazine trigger is additionally present. The detection of IFN-γ and IL-4 secreted by an NKT cell line, such as the DN32-D3 NKT hybridoma or isolated human iNKT cells, can establish whether chemical control over iNKT cell activation is offered by **8** and/or **12**, and will aid in designing *in vivo* experiments and also more advanced constructs which also incorporate a peptide antigen.

5.4 Experimental procedures

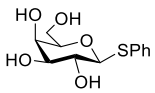
General methods: Commercially available reagents and solvents were used as received. Moisture and oxygen sensitive reactions were performed under N_2 atmosphere (balloon). DCM, toluene, THF, dioxane and Et_2O were stored over (flame-dried) 4 Å molecular sieves (8-12 mesh). Methanol was stored over (flame-dried) 3 Å molecular sieves. Pyridine, DIPEA and Et_3N were stored over KOH pellets. TLC analysis was performed using aluminum sheets, pre-coated with silica gel (Merck, TLC Silica gel 60 F_{254}). Compounds were visualized by UV absorption (λ = 254 nm), by spraying with either a solution of $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in H_2O, a solution of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4 \cdot 2H_2O$ (10 g/L) in 10% H_2SO_4, 20% H_2SO_4 in EtOH, or phosphomolybdic acid in EtOH (150 g/L), where appropriate, followed by charring at ca. 150°C. Column chromatography was performed on Screening Devices b.v. Silica Gel (particle size 40-63 µm, pore diameter 60 Å). Celite Hyflo Supercel (Merck) was used to impregnate the reaction mixture prior to silica gel chromatography when indicated. ^1H, ^{13}C APT, ^1H COSY, HSQC and HMBC spectra were recorded with a Bruker AV-400 (400/100 MHz), AV-500 (500/125 MHz) or AV-600 (600/150 MHz) spectrometer. Chemical shifts are reported as δ values (ppm) and were referenced to tetramethylsilane (δ = 0.00 ppm) or the residual solvent peak as internal standard. *J* couplings are reported in Hz. High resolution mass spectra were recorded by direct injection (2 µL of a 1 µM solution in H_2O/MeCN 1:1 and 0.1% formic acid) on a mass spectrometer (Q Exactive HF Hybrid Quadrupole-Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275°C) with resolution R = 240,000 at m/z 400 (mass range m/z = 160-2,000) and an external lock mass. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). The synthesis of TCO carbonate **15** is described in Chapter 3.



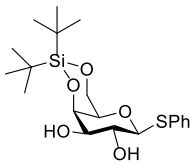
Peracetylated β-D-galactopyranoside 20: Synthesis was performed according to a modified procedure.[70] A suspension of sodium acetate (25.0 g, 305 mmol, 1.1 equiv) in acetic anhydride (350 mL, 3.71 mol, 13.4 equiv) was stirred in a three-neck, round-bottom flask and heated towards reflux in an oil bath set at 160°C. When the suspension was fully refluxing, the flask was removed from the oil bath and D-galactose (**18**, 50.0 g, 278 mmol, 1.0 equiv) was slowly added in portions to the mixture. The reaction mixture turned into a clear, yellow solution and was stirred for a further 5-10 min before pouring it into ice water (2 L). The aqueous mixture was stirred for 1 h at room temperature. DCM (600 mL) was added and the organic layer was washed with H_2O (1.5 L), $NaHCO_3$ (satd., 1.5 L), brine (1 L), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was obtained as a light yellow solid and purified by recrystallization in EtOH to obtain **20** (56.4 g, 144 mmol, 52%) as white crystals: R_f = 0.4 (30% EtOAc in pentane); ^1H NMR (400 MHz, $CDCl_3$) δ 1H NMR (400 MHz, CDCl3) δ 5.71 (d, J = 8.3 Hz, 1H), 5.43 (dd, J = 3.4, 1.1 Hz, 1H), 5.34 (dd, J = 10.4, 8.3 Hz, 1H), 5.09 (dd, J = 10.4, 3.4 Hz, 1H), 4.21 – 4.03 (m, 3H), 2.17 (s, 3H), 2.13 (s, 3H), 2.05 (2 s, 6H), 2.00 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.4, 170.2, 170.0, 169.5, 169.1, 92.2, 71.8, 70.9, 67.9, 66.9, 61.1, 20.9, 20.7, 20.7, 20.6; HRMS: calculated for $C_{16}H_{22}O_{11}Na$ 413.10543 $[M+Na]^+$; found 413.10521. Spectroscopic data was in agreement with literature.[70]



Thiogalactoside 21: Synthesis was performed according to a modified procedure.[70] β-D-galactose pentaacetate (**20**, 32.8 g, 84.0 mmol, 1.0 equiv) was dissolved in anhydrous DCM (~600 mL) under N_2. The solution was cooled down to 0°C before slowly adding thiophenol (12.9 mL, 126 mmol, 1.5 equiv) and boron trifluoride etherate (15.5 mL, 126 mmol, 1.5 equiv). The reaction mixture was stirred for 24 h and allowed to warm to room temperature. The reaction mixture was cooled to 0°C and quenched by adding Et_3N (20 mL, 143 mmol, 1.7 equiv) and subsequently washed with $NaHCO_3$ (satd., 1 L) and back-extracted with DCM (500 mL). The combined organic layers were washed with NaOH (5 % w/w, 1 L), dried over $MgSO_4$, filtered and concentrated *in vacuo.* The crude product was purified by silica gel chromatography (20% EtOAc in pentane → 30% EtOAc in pentane) to obtain **21** (35.2 g, 79.9 mmol, 95%) as a colorless waxy solid: R_f = 0.7 (50% EtOAc in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55 – 7.48 (m, 2H), 7.35 – 7.29 (m, 3H), 5.42 (d, *J* = 2.7 Hz, 1H), 5.24 (t, *J* = 10.0 Hz, 1H), 5.05 (dd, *J* = 9.9, 3.3 Hz, 1H), 4.72 (d, *J* = 10.0 Hz, 1H), 4.20 (dd, *J* = 11.3, 7.0 Hz, 1H), 4.12 (dd, *J* = 11.3, 6.2 Hz, 1H), 3.94 (t, *J* = 6.6 Hz, 1H), 2.13 (s, 3H), 2.10 (s, 3H), 2.05 (s, 3H), 1.98 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5, 170.3, 170.2, 169.6, 132.7 (x2), 132.6, 129.0 (x2), 128.3, 86.8, 74.6, 72.1, 67.4, 67.3, 61.8, 21.0, 20.8, 20.8, 20.7; HRMS: calculated for $C_{20}H_{24}O_9SNa$ 463.10332 $[M+Na]^+$; found 463.10277. Spectroscopic data was in agreement with literature.[70]

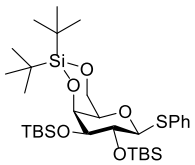


Thiogalactoside 22: Compound **21** (35.1 g, 79.8 mmol, 1.0 equiv) was dissolved in anhydrous MeOH (250 mL) under N_2. The pH of the reaction mixture was adjusted to > 10 by adding slowly adding sodium whilst stirring. The resulting reaction mixture was stirred overnight and subsequently neutralized by adding Amberlyst® (H^+ form, washed 3 x with MeOH prior to usage) in small portions, gently swirling the flask and monitoring the pH until neutral. The neutralized solution was filtered and concentrated *in vacuo* to obtain **22** (20.6 g, 75.6 mmol, 95%) as a colorless oil: R_f = 0.4 (20% MeOH in DCM); ^{1}H NMR (400 MHz, MeOD) δ 7.58 – 7.52 (m, 2H), 7.32 – 7.25 (m, 2H), 7.25 – 7.19 (m, 1H), 4.60 (d, *J* = 9.7 Hz, 1H), 3.91 (d, *J* = 3.2 Hz, 1H), 3.77 (dd, *J* = 11.4, 6.9 Hz, 1H), 3.71 (dd, *J* = 11.5, 5.2 Hz, 1H), 3.62 (t, *J* = 9.4 Hz, 1H), 3.57 (t, *J* = 6.1 Hz, 1H), 3.51 (dd, *J* = 9.2, 3.3 Hz, 1H); ^{13}C NMR (101 MHz, MeOD) δ 136.0, 132.0 (x2), 129.8 (x2), 127.9, 90.2, 80.5, 76.2, 70.9, 70.3, 62.5; HRMS: calculated for $C_{12}H_{16}O_5SNa$ 295.06107 $[M+Na]^+$; found 295.06106. Spectroscopic data was in agreement with literature.[71,72]



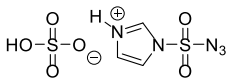
Thiogalactoside 23: compound **22** (15.8 g, 57.9 mmol, 1.0 equiv) was co-evaporated with anhydrous DMF (150 mL) in a 1 L round-bottom flask before dissolving the starting material in anhydrous DMF (240 mL) under N_2. The solution was cooled to -40°C before slowly adding di-*tert*-butylsilyl bis(trifluoromethanesulfonate) (24.2 g, 55.0 mmol, 0.95 equiv). The reaction mixture was stirred at -40°C for 30 min before adding anhydrous pyridine (14.1 mL, 174 mmol, 3.0 equiv). The reaction mixture was stirred for 45 min and was subsequently diluted with Et_2O (1 L), washed with H_2O (4 x 500 mL), brine (750 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (5% acetone in DCM, isocratic) to obtain the silylated product **23** (19.8 g, 48.0 mmol, 83%) as a clear viscous oil which crystallized under reduced pressure: R_f = 0.4 (5% acetone in DCM); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.58 – 7.52 (m, 2H), 7.33 – 7.25 (m, 3H), 4.56 (d, *J* = 9.8 Hz, 1H), 4.44 (d, *J* = 3.4 Hz, 1H),

4.29 – 4.22 (m, 2H), 3.75 (t, *J* = 9.3 Hz, 1H), 3.58 – 3.50 (m, 1H), 3.47 (s, 1H), 2.86 (br s, 2OH), 1.05 (s, 9H), 1.03 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 133.2, 132.7 (x2), 129.0 (x2), 128.0, 89.1, 75.3, 75.2, 72.6, 70.7, 67.2, 27.6 (x3), 27.5 (x3), 23.4, 20.7; HRMS: calculated for $C_{20}H_{32}O_5SSiNa$ 435.16319 $[M+Na]^+$; found 435.16279. Spectroscopic data was in agreement with literature.[57]

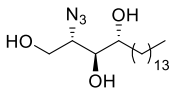


Thiogalactoside donor 16: Compound **23** (2.06 g, 5.0 mmol, 1.0 equiv) and DMAP (61 mg, 0.5 mmol, 0.1 equiv) were dissolved in anhydrous pyridine (20 mL) under N_2. The solution was cooled to 0°C before slowly adding TBS-OTf (4.59 mL, 20.0 mmol, 4.0 equiv). The reaction mixture was stirred for 16 h and allowed to warm to room temperature. The reaction mixture was concentrated *in vacuo*, diluted with 100 mL EtOAc, washed with HCl (1 M, 100 mL), $NaHCO_3$ (satd., 100 mL) and brine (100 mL). The aqueous layers were back-extracted with EtOAc (50 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (pentane → 20% DCM in pentane → 40% DCM in pentane) to obtain **23** (3.03 g, 4.73 mmol, 95%) as a clear oil: R_f = 0.3 (40% DCM in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.51 – 7.45 (m, 2H), 7.29 – 7.19 (m, 4H), 4.56 (d, *J* = 9.4 Hz, 1H), 4.32 (dd, *J* = 3.0, 1.0 Hz, 1H), 4.19 (dd, *J* = 12.2, 1.6 Hz, 1H), 4.15 (dd, *J* = 12.1, 1.7 Hz, 1H), 4.01 (t, *J* = 9.0 Hz, 1H), 3.52 (dd, *J* = 8.6, 2.8 Hz, 1H), 3.36 – 3.31 (m, 1H), 1.12 (s, 9H), 1.04 (s, 9H), 0.96 (s, 9H), 0.95 (s, 9H), 0.26 (s, 3H), 0.15 (s, 3H), 0.12 (s, 3H), 0.10 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 136.0, 131.8 (x2), 128.8 (x2), 127.1, 90.6, 78.0, 74.8, 74.7, 70.4, 67.4, 27.9 (x3), 27.5 (x3), 26.7 (x3), 26.6 (x3), 23.6, 20.9, 18.4, 18.4, -1.9, -3.2, -3.3, -3.6; HRMS: calculated for $C_{36}H_{64}O_5SSi_3N$ 658.38075 $[M+NH_4]^+$; found 658.38031. Spectroscopic data was in agreement with literature.[57]

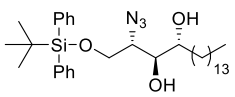
**Note:* this *procedure could also be performed at 10 gram scale (24 mmol) to obtain similar results.*



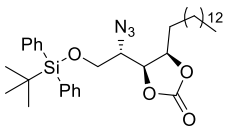
Imidazole-1-sulfonyl azide hydrogen sulfate (24): Synthesis was performed according to literature precedence.[61] Sodium azide (7.50 g, 115 mmol, 1.0 equiv) was placed in a 500 mL round-bottom flask and subsequently dissolved in anhydrous ethyl acetate (120 mL) under N_2. The suspension was cooled to 0°C before slowly adding sulfuryl chloride (9.38 mL, 115 mmol, 1.0 equiv) over 10 min. The yellow reaction mixture was stirred for 19 h and allowed to warm to room temperature. Subsequently, the reaction mixture was cooled to 0°C before slowly adding imidazole (14.9 g, 219 mmol, 1.9 equiv) over 5 min whilst maintaining an inert atmosphere. The reaction mixture was stirred for 3 h at 0°C before slowly adding $NaHCO_3$ (satd., 225 mL) to basify the reaction mixture. The organic layer was washed with H_2O (225 mL) and dried over $MgSO_4$. The dried organic phase was filtered, cooled to 0°C and placed under a continuous stream of N_2 before slowly adding sulfuric acid (6.15 mL, 115 mmol, 1.0 equiv) over 5 min. The acidified solution was stirred for 30 min and allowed to warm to room temperature. A colorless precipitate formed, which was collected by filtration to obtain **24** (22.5 g, 83.0 mmol, 72%) as a white solid. Spectroscopic data was in agreement with literature.[61]



Phytosphingosine 25: D-*ribo*-phytosphingosine (**19**, 10.0 g, 31.5 mmol, 1.0 equiv) was suspended in a mixture of MeOH (300 mL) and DCM (100 mL) under N_2. K_2CO_3 (10.5 g, 76.0 mmol, 2.4 equiv) and Cu(II) · 5 H_2O (79 mg, 0.32 mmol, 1.0 mol%) were dissolved in H_2O (100 mL) and the resulting aqueous solution was added to the suspension to give a foamy reaction mixture. After 5 min, imidazole-1-sulfonyl azide hydrogen sulfate (**24**, 10.3 g, 37.8 mmol, 1.2 equiv) was added and the reaction mixture was stirred for 20 h at room temperature. The reaction mixture was partially concentrated *in vacuo* (≥ 100 mbar, 40°C) before adding HCl (1 M, 250 mL). The aqueous phase was extracted with EtOAc (3 x 350 mL, 40°C). The combined organic layers were washed with $NaHCO_3$ (satd., 250 mL), brine (250 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* to obtain **25** (10.8 g, 31.5 mmol, 100%) as a solid which was used in the next step without further purification: R_f = 0.5 (10% MeOH in DCM); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.00 (dd, *J* = 11.7, 5.5 Hz, 1H), 3.89 (dd, *J* = 11.7, 4.5 Hz, 1H), 3.84 – 3.74 (m, 2H), 3.66 (q, *J* = 4.9 Hz, 1H), 1.65 – 1.44 (m, 3H), 1.38 – 1.21 (m, 23H), 0.88 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 74.7, 72.7, 63.2, 61.8, 32.1, 32.0, 29.8 (x2), 29.8, 29.8 (x2), 29.7, 29.7 (x2), 29.5, 25.9, 22.8, 14.3; HRMS: calculated for $C_{18}H_{38}N_3O_3$ 344.29077 $[M+H]^+$; found 344.29020. Spectroscopic data was in agreement with literature.[73,74]



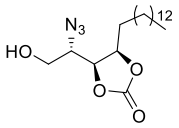
Phytosphingosine 26: Synthesis was performed according to a modified procedure.[75] Crude 2-azido-phytosphingosine (**25**, 10.8 g, 31.5 mmol, 1.0 equiv) was dissolved in anhydrous DCM (155 mL) and anhydrous DMF (35 mL) under N_2. The solution was cooled to 0°C before adding Et_3N (11.0 mL, 79.0 mmol, 2.5 equiv), DMAP (192 mg, 1.58 mmol, 0.1 equiv) and *tert*-butyldiphenylchlorosilane (TBDPS-Cl, 9.83 mL, 37.8 mmol, 1.2 equiv). The reaction mixture was stirred for 25 h and allowed to warm to room temperature. The reaction mixture was quenched with MeOH (1.53 mL, 37.8 mmol, 1.2 equiv) and diluted with EtOAc (1 L). The organic phase was washed with brine (2 x 600 mL) and the combined aqueous layers were back-extracted with EtOAc (500 mL). The combined organic layers were dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (pentane → 2.5% acetone in pentane → 10% acetone in pentane) to obtain **26** (15.1 g, 26.0 mmol, 83% over 2 steps) as an oil: R_f = 0.2 (5% acetone in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 – 7.64 (m, 4H), 7.51 – 7.35 (m, 6H), 4.03 (dd, J = 10.9, 4.2 Hz, 1H), 3.91 (dd, J = 11.0, 5.7 Hz, 1H), 3.72 – 3.64 (m, 2H), 3.59 – 3.53 (m, 1H), 2.52 (d, J = 4.1 Hz, 1OH), 2.00 (br s, 1OH), 1.57 – 1.37 (m, 3H), 1.37 – 1.20 (m, 23H), 1.08 (s, 9H), 0.88 (t, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 135.8 (x2), 135.7 (x2), 132.7, 132.6, 130.2 (x2), 128.1 (x2), 128.0 (x2), 74.3, 72.5, 64.3, 63.5, 32.1, 32.0, 29.8, 29.8, 29.8, 29.8 (x2), 29.8, 29.7, 29.7, 29.5, 26.9 (x3), 25.8, 22.8, 19.2, 14.3; HRMS: calculated for $C_{34}H_{55}N_3O_3SiNa$ 604.39049 $[M+Na]^+$; found 604.39029. Spectroscopic data was in agreement with literature.[75]



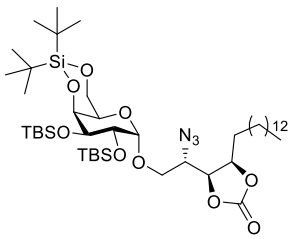
Phytosphingosine 27: Phytosphingosine **26** (8.40 g, 14.4 mmol, 1.0 equiv) was dissolved in anhydrous DCM (100 mL) under N_2. 1,1'-Carbonyldiimidazole (CDI, 7.02 g, 43.3 mmol, 3.0 equiv) was added and the reaction mixture was stirred for 72 h at room temperature. The reaction mixture was concentrated *in vacuo* and the resulting crude product was purified by silica gel chromatography (pentane → 5% Et_2O in pentane) to obtain **27** (6.95 g, 11.4 mmol, 79%) as a

white solid: R_f = 0.2 (5% Et_2O in pentane); ^1H NMR (400 MHz, $CDCl_3$) δ 7.71 – 7.65 (m, 4H), 7.50 – 7.39 (m, 6H), 4.69 (ddd, *J* = 10.4, 7.2, 2.7 Hz, 1H), 4.54 (dd, *J* = 10.1, 7.2 Hz, 1H), 4.03 (dd, *J* = 11.1, 2.7 Hz, 1H), 3.88 (dd, *J* = 11.1, 6.1 Hz, 1H), 3.61 (ddd, *J* = 9.7, 6.1, 2.7 Hz, 1H), 1.80 – 1.68 (m, 1H), 1.68 – 1.55 (m, 2H), 1.48 – 1.19 (m, 23H), 1.09 (s, 9H), 0.88 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 135.7 (x2), 135.7 (x2), 132.5, 132.3, 130.3, 130.2, 128.1 (x2), 128.1 (x2), 79.6, 75.6, 64.3, 60.0, 32.1, 29.8, 29.8, 29.8 (x2), 29.7, 29.6, 29.5, 29.5, 29.3, 29.0, 26.9 (x3), 25.6, 22.8, 19.3, 14.3.

*Note: the ^{13}C signal for the carbonate protecting group (C=O) was not reported due to a lack of resolution on the spectrum of **27**.*

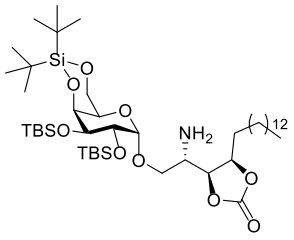


Phytosphingosine acceptor 17: Phytosphingosine **27** (6.95 g, 11.4 mmol, 1.0 equiv) was dissolved in HF · pyridine (10.3 mL, 114 mmol, 10 equiv) in a plastic tube under N_2. The reaction mixture was stirred for 22 h at room temperature. The reaction mixture was slowly added to $NaHCO_3$ (satd., 50 mL) and the resulting mixture was extracted with DCM (3 x 50 mL). The combined organic layers were washed with $CuSO_4$ (1 M, 3 x 30 mL), H_2O (30 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (20% EtOAc in pentane, isocratic) to obtain acceptor **17** (3.88 g, 10.5 mmol, 92%) as a white solid: R_f = 0.3 (20% EtOAc in pentane); ^1H NMR (400 MHz, $CDCl_3$) δ 4.76 (ddd, *J* = 10.4, 7.3, 2.9 Hz, 1H), 4.62 (dd, *J* = 9.9, 7.3 Hz, 1H), 4.08 (dd, *J* = 11.9, 2.4 Hz, 1H), 3.91 (dd, *J* = 11.9, 5.5 Hz, 1H), 3.70 (ddd, *J* = 9.8, 5.4, 2.7 Hz, 1H), 3.38 (br s, 1OH), 1.84 – 1.65 (m, 2H), 1.64 – 1.53 (m, 1H), 1.49 – 1.18 (m, 23H), 0.88 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.2, 79.9, 75.8, 62.3, 59.8, 32.0, 29.7, 29.7, 29.7 (x2), 29.6, 29.6, 29.4, 29.4, 29.2, 28.9, 25.6, 22.7, 14.1; HRMS: calculated for $C_{19}H_{36}N_3O_4$ 370.27003 $[M+H]^+$; found 370.26988. Spectroscopic data was in agreement with literature.[58]

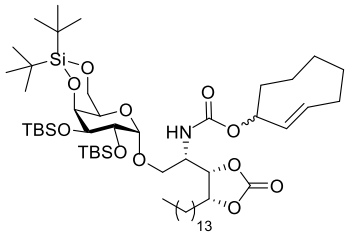


Compound 14: Galactose donor **16** (5.07 g, 7.91 mmol, 1.5 equiv) and phytosphingosine acceptor **17** (1.95 g, 5.27 mmol, 1.0 equiv) were co-evaporated with toluene (3 x 3 mL) before dissolving the reactants in anhydrous DCM (40 mL) in the presence of flame-dried molecular sieves (3Å) under N_2. After 15 min, the reaction mixture was cooled to -40°C before adding *N*-iodosuccinimide (NIS, 1.78 g, 7.91 mmol, 1.5 equiv) and trimethylsilyl trifluoromethanesulfonate (TMS-OTf, 191 µL, 1.05 mmol, 0.2 equiv). The reaction mixture was stirred for 5 h at -40°C and subsequently quenched by adding Et_3N (7.35 mL, 52.7 mmol, 10 equiv). The crude mixture was diluted with EtOAc (250 mL), washed with $NaHCO_3$ (satd.,150 mL), $Na_2S_2O_3$ (satd., 150 mL) and brine (150 mL), dried over $MgSO_4$, filtered, impregnated with Celite and concentrated *in vacuo*. The impregnated crude product was purified by silica gel chromatography (pentane → 2% EtOAc in pentane → 5% EtOAc in pentane) to obtain the glycosylated product **14** (3.19 g, 3.54 mmol, 67%) as a yellow oil: R_f = 0.3 (5% EtOAc in pentane); ^1H NMR (400 MHz, $CDCl_3$) δ 4.83 (d, *J* = 3.4 Hz, 1H), 4.77 – 4.70 (m, 2H), 4.32 (d, *J* = 2.2 Hz, 1H), 4.26 (dd, *J* = 12.5, 1.8 Hz, 1H), 4.18 – 4.12 (m, 3H), 3.87 (dd, *J* = 9.6, 2.9 Hz, 1H), 3.72 – 3.65 (m, 2H), 3.62 – 3.54 (m, 1H), 1.86 – 1.74 (m, 1H), 1.73 – 1.55 (m, 2H), 1.47 – 1.19 (m, 23H), 1.04 (s, 9H), 1.04 (s, 9H), 0.93 (s, 9H), 0.91 (s, 9H), 0.88 (t, *J* = 6.7 Hz, 3H), 0.09 (s, 3H), 0.09 (s, 3H), 0.07 (s, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 153.5, 101.4, 79.5, 75.3, 74.9, 70.9, 69.2, 68.4, 68.3, 67.3, 57.9,

32.1, 29.8, 29.8, 29.8 (x2), 29.7, 29.6, 29.5, 29.5, 29.3, 29.0, 27.6 (x3), 27.5 (x3), 26.2 (x3), 26.1 (x3), 25.6, 23.6, 22.8, 20.8, 18.2, 18.2, 14.3, -4.0, -4.1, -4.3, -4.5; HRMS: calculated for $C_{45}H_{93}N_4O_9Si_3$ 917.62449 $[M+NH_4]^+$; found 917.62451.

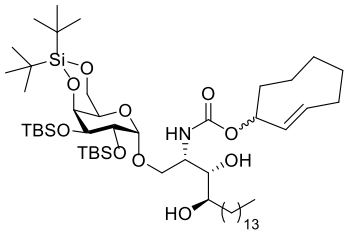


Compound 28: Azide **14** (1.34 g, 1.49 mmol, 1 equiv) was dissolved anhydrous THF (30 mL) under N_2. N_2 was purged through the stirring solution for 15 min (flow) before adding PtO_2 (101 mg, 0.45 mmol, 0.3 equiv) and purging N_2 through the stirred suspension for 15 min (flow). The reaction mixture was purged with H_2 (balloon) whilst stirring and was subsequently left to stir under H_2 (balloon) for 24 h. The reaction mixture was purged with N_2 (flow), filtered over a pad of Celite and concentrated *in vacuo* to obtain the crude amine **28** (1.31 g) as a yellow oil which was used in the next step without further purification: R_f = 0.2 (15% EtOAc in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.80 (d, *J* = 3.4 Hz, 1H), 4.75 – 4.68 (m, 1H), 4.50 (dd, *J* = 9.8, 7.2 Hz, 1H), 4.31 (d, *J* = 2.4 Hz, 1H), 4.23 (dd, *J* = 12.4, 1.6 Hz, 1H), 4.15 – 4.09 (m, 2H), 3.87 – 3.81 (m, 2H), 3.61 (br s, 1H), 3.38 (dd, *J* = 10.0, 5.8 Hz, 1H), 3.16 (ddd, *J* = 9.2, 5.7, 3.1 Hz, 1H), 1.91 – 1.81 (m, 1H), 1.72 – 1.54 (m, 2H), 1.41 – 1.19 (m, 23H), 1.03 (s, 9H), 1.03 (s, 9H), 0.93 (s, 9H), 0.89 (s, 9H), 0.87 (t, *J* = 7.0 Hz, 3H), 0.09 (s, 6H), 0.08 (s, 3H), 0.07 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.4, 101.2, 80.2, 78.9, 74.9, 71.2, 70.9, 69.5, 68.0, 67.3, 49.7, 32.0, 29.8, 29.8, 29.8 (x2), 29.7, 29.6, 29.6, 29.5, 29.4, 28.8, 27.6 (x3), 27.4 (x3), 26.2 (x3), 26.1 (x3), 25.6, 23.5, 22.8, 20.8, 18.2, 18.2, 14.2, -3.9, -4.2, -4.2, -4.5; HRMS: calculated for $C_{45}H_{92}NO_9Si_3$ 874.60744 $[M+H]^+$; found 874.60676.

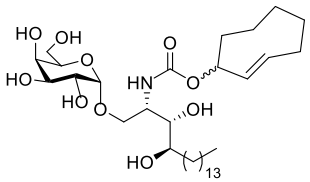


Compound 29: The crude amine **28** (1.31 g) obtained in the previous hydrogenation step and axial TCO carbonate **15** (481 mg, 1.80 mmol, 1.2 equiv) were dissolved in anhydrous DMF (15 mL) under N_2. DIPEA (0.39 mL, 2.25 mmol, 1.5 equiv) and DMAP (37 mg, 0.30 mmol, 0.2 equiv) were added and the reaction mixture was stirred for 21 h at room temperature. Subsequently, EtOAc (100 mL) was added and the organic phase was washed with HCl (1 M, 80 mL), $NaHCO_3$ (satd., 3 x 80 mL), brine (80 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (7% EtOAc in pentane, isocratic) to obtain the diastereomeric mixture **29** (**29$_A$** : **29$_B$**, ~ **1** : **1**, 1.36 g, 1.32 mmol, 89% over two steps) as a yellow oil: R_f = 0.3 (10% EtOAc in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.81 – 5.67 (m, 1H, **29$_A$** + **29$_B$**), 5.54 (dd, *J* = 16.6, 1.9 Hz, 1H, **29$_A$** + **29$_B$**), 5.41 – 5.20 (m, 1H + 1NH, **29$_A$** + **29$_B$**), 4.82 (d, *J* = 3.5 Hz, 1H, **29$_A$** + **29$_B$**), 4.81 – 4.76 (m, 1H, **29$_A$** + **29$_B$**), 4.75 – 4.67 (m, 1H, **29$_A$** + **29$_B$**), 4.32 (br s, 1H, **29$_A$** + **29$_B$**), 4.21 (d, *J* = 12.6 Hz, 1H, **29$_A$** + **29$_B$**), 4.17 – 4.04 (m, 3H, **29$_A$** + **29$_B$**), 3.85 (d, *J* = 9.4 Hz, 1H, **29$_A$** + **29$_B$**), 3.81 – 3.73 (m, 1H, **29$_A$** + **29$_B$**), 3.68 (dd, *J* = 10.4, 2.8 Hz, 1H, **29$_A$** + **29$_B$**), 3.59 (br s, 1H, **29$_A$** + **29$_B$**), 2.52 – 2.39 (m, 1H, **29$_A$** + **29$_B$**), 2.08 – 1.95 (m, 3H, **29$_A$** + **29$_B$**), 1.94 – 1.82 (m, 1H, **29$_A$** + **29$_B$**), 1.81 – 1.20 (m, 30H, **29$_A$** + **29$_B$**), 1.04 (s, 18H, **29$_A$** + **29$_B$**), 0.95 (s, 9H, **29$_A$**), 0.94 (s, 9H, **29$_B$**), 0.91 (s, 9H, **29$_A$** + **29$_B$**), 0.88 (t, *J* = 6.9 Hz, 3H, **29$_A$** + **29$_B$**), 0.84 – 0.73 (m, 1H, **29$_A$** + **29$_B$**), 0.13 (s, 3H, **29$_A$**), 0.12 (s, 3H, **29$_A$** + **29$_B$**), 0.11 (s, 3H, **29$_B$**), 0.10 (s, 3H, **29$_A$** + **29$_B$**), 0.09 (s, 3H, **29$_A$** + **29$_B$**); ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.0, 155.0, 153.9 (x2), 131.9 (x2), 131.4, 131.2,

101.4, 101.3, 79.9, 79.8, 77.7, 77.3, 74.8, 74.8, 74.6, 74.6, 71.2, 71.1, 69.4, 69.4, 68.2, 68.1, 67.2, 67.2, 67.1, 67.1, 49.4 (x2), 40.8, 40.7, 36.0, 36.0, 36.0, 35.9, 32.0 (x2), 29.8 (x2), 29.8 (x2), 29.8 (x4), 29.7 (x2), 29.7 (x2), 29.6 (x2), 29.5 (x2), 29.5 (x2), 29.2 (x2), 29.1, 29.0, 28.6, 28.6, 27.5 (x6), 27.4 (x6), 26.2 (x6), 26.1 (x3), 26.1 (x3), 25.7, 25.6, 24.2, 23.5 (x2), 22.8 (x2), 20.8 (x2), 18.2, 18.2 (x2), 14.2 (x2), -3.8, -3.9, -4.1 (x2), -4.2 (x2), -4.6 (x2); HRMS: calculated for $C_{54}H_{104}NO_{11}Si_3$ 1026.69117 $[M+H]^+$; found 1026.69013.

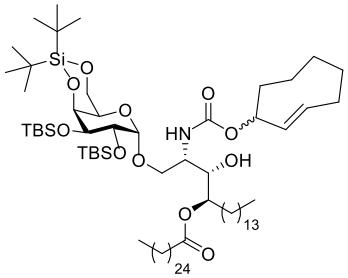


Compound 13: Carbonate **29** (1.36 g, 1.32 mmol, 1.0 equiv) was dissolved in a mixture of THF (7.5 mL) and H_2O (2.5 mL) under N_2. The solution was cooled to 0°C before adding LiOH (253 mg, 10.6 mmol, 8.0 equiv). The reaction mixture was stirred for 24 h and allowed to warm to room temperature. The pH of the reaction mixture was neutralized by adding dry ice. Subsequently, the reaction mixture was concentrated *in vacuo* to obtain the crude diol **13** (**13$_A$** : **13$_B$**, ~ **1** : **1**, 1.32 g, 1.32 mmol, quant.) as an oil which was used for the next step without further purification: R_f = 0.4 (10% EtOAc in pentane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.86 – 5.65 (m, 1H, **13$_A$** + **13$_B$**), 5.59 – 5.40 (m, 1H + 1NH, **13$_A$** + **13$_B$**), 5.39 – 5.26 (m, 1H, **13$_A$** + **13$_B$**), 4.89 – 4.81 (m, 1H, **13$_A$** + **13$_B$**), 4.30 (d, *J* = 2.0 Hz, 1H, **13$_A$** + **13$_B$**), 4.24 – 4.05 (m, 4H, **13$_A$** + **13$_B$**), 4.02 – 3.87 (m, 1H, **13$_A$** + **13$_B$**), 3.79 (td, *J* = 9.5, 8.5, 3.0 Hz, 1H, **13$_A$** + **13$_B$**), 3.68 (d, *J* = 10.2 Hz, 1H, **13$_A$** + **13$_B$**), 3.62 – 3.46 (m, 3H, **13$_A$** + **13$_B$**), 2.49 – 2.40 (m, 1H, **13$_A$** + **13$_B$**), 2.08 – 1.93 (m, 3H, **13$_A$** + **13$_B$**), 1.92 – 1.21 (m, 31H, **13$_A$** + **13$_B$**), 1.03 (s, 18H, **13$_A$** + **13$_B$**), 0.95 (s, 9H, **13$_A$**), 0.94 (s, 9H, **13$_B$**), 0.92 (s, 9H, **13$_A$** + **13$_B$**), 0.88 (t, *J* = 6.8 Hz, 3H, **13$_A$** + **13$_B$**), 0.85 – 0.72 (m, 1H, **13$_A$** + **13$_B$**), 0.12 (s, 9H, **13$_A$** + **13$_B$**), 0.10 (s, 3H, **13$_A$** + **13$_B$**); ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.5, 155.3, 132.0, 132.0, 131.6, 131.4, 100.4, 100.1, 77.0, 76.9, 74.8, 74.8, 74.2, 73.9, 73.2 (x2), 71.6, 71.5, 69.4, 69.3, 68.3, 68.2, 67.2, 67.1, 66.7 (x2), 50.7, 50.5, 40.9, 40.9, 36.1, 36.1, 36.0 (x2), 34.3, 34.2, 32.1 (x2), 29.9 (x2), 29.8 (x10), 29.8 (x4), 29.8 (x2), 29.5 (x2), 29.2, 29.1, 27.6 (x6), 27.4 (x6), 26.3 (x6), 26.2 (x6), 24.2 (x2), 23.5, 23.5, 22.8 (x2), 20.8 (x2), 18.4, 18.2, 14.3 (x2), -3.8, -3.8, -4.1 (x2), -4.3, -4.3, -4.5 (x2); HRMS: calculated for $C_{53}H_{106}NO_{10}Si_3$ 1000.71190 $[M+H]^+$; found 1000.71102.



TCO Caged αGalPhs (12): The crude diol **13** (562 mg, 0.56 mmol, 1.0 equiv) was dissolved in anhydrous THF (5.6 mL) under N_2. The solution was cooled to 0°C before adding $Et_3N \cdot 3HF$ (0.55 mL, 3.40 mmol, 6.0 equiv). The reaction mixture was stirred for 96 h and allowed to warm to room temperature. Subsequently, the reaction mixture was concentrated *in vacuo*, redissolved in distilled EtOAc (150 mL), washed with H_2O (2 x 100 mL), brine (100 mL), dried over $MgSO_4$, filtered, impregnated with Celite and concentrated *in vacuo*. The impregnated crude product was purified by silica gel chromatography (1% MeOH in DCM → 2.5% MeOH in DCM → 5% MeOH in DCM → 10% MeOH in DCM) to obtain caged αGalPhs **12** (**12$_A$** : **12$_B$**, ~ **1** : **1**, 297 mg, 0.47 mmol, 84%) as a crystalline solid: R_f = 0.25 (10% MeOH in DCM); ^{1}H NMR (600 MHz, Pyridine-d_5) δ 7.94 (d, *J* = 9.0 Hz, 1NH, **12$_A$**), 7.89 (d, *J* = 8.8 Hz, 1NH, **12$_B$**), 7.11 – 5.95 (m, 6OH, **12$_A$** + **12$_B$**), 5.88 (ddd, *J* = 15.1, 11.5, 3.2 Hz, 1H, **12$_A$**), 5.82 (ddd, *J* = 15.2, 11.5, 3.2 Hz, 1H, **12$_B$**), 5.61 (br s, 1H, **12$_A$** + **12$_B$**), 5.59 (d, *J* = 14.2 Hz, 1H, **12$_A$**), 5.53 (d, *J* = 16.2 Hz, 1H, **12$_B$**), 5.48 (d, *J* =

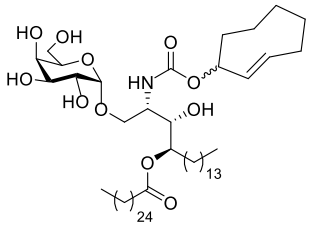
3.4 Hz, 1H, **12A** + **12B**), 4.94 – 4.85 (m, 1H, **12A** + **12B**), 4.66 – 4.60 (m, 1H, **12A** + **12B**), 4.59 – 4.55 (m, 1H, **12A** + **12B**), 4.54 – 4.46 (m, 1H, **12A** + **12B**), 4.43 – 4.18 (m, 7H, **12A** + **12B**), 2.37 – 2.28 (m, 1H, **12A** + **12B**), 2.27 – 2.18 (m, 1H, **12A** + **12B**), 2.16 – 2.09 (m, 1H, **12A** + **12B**), 1.99 – 1.79 (m, 4H, **12A** + **12B**), 1.77 – 1.68 (m, 1H, **12A** + **12B**), 1.68 – 1.58 (m, 2H, **12A** + **12B**), 1.56 – 1.48 (m, 1H, **12A** + **12B**), 1.48 – 1.15 (m, 23H, **12A** + **12B**), 1.13 – 1.01 (m, 1H, **12A** + **12B**), 0.92 (t, *J* = 7.1 Hz, 3H, **12A** + **12B**), 0.76 – 0.66 (m, 1H, **12A** + **12B**); ^{13}C NMR (151 MHz, Pyridine-d_5) δ 157.1, 157.1, 133.3, 133.0, 132.4, 132.0, 101.8, 101.7, 77.1, 77.1, 74.4, 74.4, 73.3, 73.3, 73.0, 72.9, 72.0 (x2), 71.5, 71.5, 70.7, 70.7, 68.8 (x2), 63.1, 63.1, 53.5 (x2), 41.6, 41.6, 36.8 (x2), 36.7, 36.6, 34.9, 34.8, 32.8 (x2), 31.0, 31.0, 30.8, 30.8, 30.7 (x2), 30.7 (x8), 30.6 (x2), 30.3 (x2), 29.8, 29.8, 27.1, 27.1, 25.0, 25.0, 23.6 (x2), 15.0 (x2); ^{1}H NMR (500 MHz, Dioxane-d_8) δ 6.16 – 5.96 (m, 1NH, **12A** + **12B**), 5.86 – 5.69 (m, 1H, **12A** + **12B**), 5.53 (d, *J* = 16.4 Hz, 1H, **12A** + **12B**), 5.27 (br s, 1H, **12A** + **12B**), 4.84 (d, *J* = 4.2 Hz, 1H, **12A**), 4.83 (d, *J* = 3.9 Hz, 1H, **12B**), 4.04 – 3.94 (m, 1H, **12A** + **12B**), 3.92 – 3.70 (m, 4H, **12A** + **12B**), 3.68 – 3.57 (m, 4H, **12A** + **12B**), 3.49 – 3.37 (m, 2H, **12A** + **12B**), 2.51 – 2.38 (m, 1H, **12A** + **12B**), 2.08 – 1.90 (m, 3H, **12A** + **12B**), 1.89 – 1.78 (m, 1H, **12A** + **12B**), 1.72 – 1.56 (m, 3H, **12A** + **12B**), 1.56 – 1.42 (m, 2H, **12A** + **12B**), 1.41 – 1.19 (m, 24H, **12A** + **12B**), 1.13 – 1.01 (m, 1H, **12A** + **12B**), 0.88 (t, *J* = 6.9 Hz, 3H, **12A** + **12B**), 0.86 – 0.79 (m, 1H, **12A** + **12B**); ^{13}C NMR (126 MHz, Dioxane-d_8) δ 156.0 (x2), 133.3, 133.1, 131.8, 131.5, 100.7 (x2), 76.7, 76.6, 74.3, 74.3, 72.5, 72.5, 72.3, 72.2, 71.5 (x2), 70.6, 70.5, 70.2 (x2), 68.2, 68.1, 62.6, 62.6, 52.5 (x2), 41.2, 41.2, 36.8 (x2), 36.4, 36.4, 34.2, 34.2, 32.7 (x2), 30.6, 30.6, 30.6, 30.5 (x5), 30.5 (x6), 30.4 (x2), 30.1 (x2), 29.8, 29.7, 26.6 (x2), 25.1, 25.0, 23.4 (x2), 14.4 (x2); HRMS: calculated for $C_{33}H_{62}NO_{10}$ 632.43682 $[M+H]^+$; found 632.43640. Compound **12** was redissolved in dioxane and lyophilized in small quantities for immunology experiments.



Compound 32: Hexacosanoic acid (60 mg, 0.15 mmol, 1.5 equiv), EDC · HCl (29 mg, 0.15 mmol, 1.5 equiv) and DMAP (73 mg, 0.60 mmol, 6 equiv) were dissolved in anhydrous DCM (1.0 mL) under N_2. The suspension was cooled to 0°C and stirred for 45 min. A solution of compound **13** (100 mg, 100 µmol, 1.0 equiv) in anhydrous DCM (2.0 mL) under N_2 was subsequently added to the reaction mixture. DIPEA (52 µL, 0.30 mmol, 3.0 equiv) was added and the reaction mixture was stirred for 20 h and allowed to warm to room temperature. The reaction mixture was diluted with EtOAc (30 mL), washed with HCl (1 M, 20 mL), $NaHCO_3$ (satd., 20 mL), brine (20 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (3% EtOAc in pentane, isocratic) to obtain compound **32** (**32A** : **32B**, ~ **1** : **1**, 42.3 mg, 31.0 µmol, 31%) as a yellow oil: R_f = 0.7 (10% EtOAc in pentane); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.86 – 5.68 (m, 1H, **32A** + **32B**), 5.55 – 5.46 (m, 1H, **32A** + **32B**), 5.42 (d, *J* = 8.2 Hz, 1NH, **32A**), 5.37 (d, *J* = 8.1 Hz, 1NH, **32B**), 5.34 – 5.26 (m, 1H, **32A** + **32B**), 4.95 – 4.85 (m, 2H, **32A** + **32B**), 4.31 (d, *J* = 1.9 Hz, 1H, **32A** + **32B**), 4.28 – 4.20 (m, 2H, **32A** + **32B**), 4.18 – 4.12 (m, 2H, **32A** + **32B**), 3.80 – 3.71 (m, 2H, **32A** + **32B**), 3.70 – 3.61 (m, 3H, **32A** + **32B**), 2.51 – 2.41 (m, 1H, **32A** + **32B**), 2.38 (t, *J* = 7.4 Hz, 2H, **32A** + **32B**), 2.36 – 2.26 (m, 3H, **32A** + **32B**), 2.08 – 1.92 (m, 3H, **32A** + **32B**), 1.90 – 1.81 (m, 1H, **32A** + **32B**), 1.73 – 1.20 (m, 73H, **32A** + **32B**), 1.05 – 1.01 (m, 18H, **32A** + **32B**), 0.95 – 0.93 (m, 9H, **32A** + **32B**), 0.93 – 0.91 (m, 9H, **32A** + **32B**), 0.88 (t, *J* = 7.0 Hz, 6H, **32A** + **32B**), 0.83 – 0.73 (m, 1H, **32A** + **32B**), 0.14 (s, 3H, **32A** + **32B**), 0.12 (s,

3H, **32A** + **32B**), 0.11 (s, 3H, **32A**), 0.11 (s, 3H, **32B**), 0.10 (s, 3H, **32A**), 0.10 (s, 3H, **32B**); 177.4 (x2),* 174.1 (x2), 155.5 (x2), 155.2 (x2),* 132.1 (x2), 131.9 (x2),* 131.6 (x2),* 131.3 (x2), 101.3 (x2), 78.0, 78.0, 75.0 (x2),* 74.8 (x2), 74.3, 74.2, 73.9*, 74.8,* 71.5 (x2), 70.9, 70.8, 69.5 (x2), 69.2 (x2)* 68.3 (x2), 67.5, 67.2 (x3), 51.6, 51.4, 43.0 (x2), 40.8 (x2), 36.0 (x4), 34.6 (x2), 34.5, 34.3, 34.3, 33.8, 32.1 (x2), 29.9 (x 50), 29.7 (x2), 29.6 (x2), 29.5 (x2), 29.5 (x2), 29.4 (x2), 29.3 (x2), 29.2, 29.2, 27.6 (x6), 27.5 (x6), 26.3 (x6), 26.2 (x6), 25.1 (x3), 24.9, 24.2, 24.0, 23.6, 22.8 (x2), 20.8, 18.5, 18.2, 14.3 (x4), -3.9 (x2), -4.0 (x2), -4.3 (x2), -4.6 (x2).

*Note: Additional ^{13}C signals encountered which indicate the presence of an additional regioisomer are denoted.**



TCO caged αGalCer produg (8): Compound **32** (41.0 mg, 30.0 µmol, 1.0 equiv) was dissolved in anhydrous THF (300 µL) under N_2. The solution was cooled to 0°C before adding $Et_3N \cdot 3HF$ (48 µL, 297 µmol, 10.0 equiv). The reaction mixture was stirred for 27 h and allowed to warm to room temperature. The reaction mixture was concentrated *in vacuo*, redissolved in distilled EtOAc (20 mL), washed with H_2O (2 x 10 mL), brine (10 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by silica gel chromatography (100% distilled EtOAc, isocratic) to obtain caged αGalCer **8** (**8A** : **8B**, ~ **1** : **1**, 7.0 mg, 6.93 µmol, 23%) as a crystalline solid: R_f = 0.2 (100% EtOAc); ^1H NMR (600 MHz, Dioxane-d_8) δ 6.27 (d, *J* = 9.3 Hz, 1NH)*, 6.04 (d, *J* = 9.1 Hz, 1NH, **8A**), 6.01 (d, *J* = 8.8 Hz, 1NH, **8B**), 5.85 – 5.66 (m, 1H, **8A** + **8B**), 5.52 (d, *J* = 16.4 Hz, 1H, **8A** + **8B**), 5.24 (d, *J* = 13.7 Hz, 1H, **8A** + **8B**), 4.99 – 4.85 (m, 1H, **8A** + **8B**), 4.81 – 4.68 (m, 1H, **8A** + **8B**), 4.21 – 4.09 (m, 1H, **8A** + **8B**), 3.98 – 3.38 (m, 9H, **8A** + **8B**), 2.51 – 2.39 (m, 1H, **8A** + **8B**), 2.35 – 2.26 (m, 2H, **8A** + **8B**), 2.22 (t, *J* = 7.4 Hz, 1H)*, 2.09 – 1.90 (m, 3H, **8A** + **8B**), 1.88 – 1.77 (m, 1H, **8A** + **8B**), 1.73 – 1.13 (m, 75H, **8A** + **8B**), 1.12 – 0.99 (m, 1H, **8A** + **8B**), 0.88 (t, *J* = 6.9 Hz, 6H, **8A** + **8B**); ^{13}C NMR (151 MHz, Dioxane-d_8) δ 174.8 (x2),* 173.8 (x2), 156.0 (x2), 133.3,* 133.2, 133.1, 133.1,* 131.8, 131.5, 101.5,* 101.3,* 100.5, 100.4, 77.0 (x2), 74.5 (x2), 72.4 (x2), 71.5 (x2), 70.7, 70.6, 70.4 (x2), 70.3 (x2), 67.9,* 67.8,* 67.5, 67.4, 62.6, 62.5, 52.9 (x2),* 52.0 (x2), 42.9,* 41.2 (x2), 36.8 (x2), 36.4 (x2), 34.9, 34.8, 34.0,* 33.9,* 32.7 (x4), 30.4 (x 50), 30.1 (x6), 30.0 (x2), 29.7 (x2), 26.4, 26.1, 25.9, 25.7, 25.6, 25.5, 25.1, 25.1, 24.5,* 24.1,* 23.4 (x4), 14.5 (x4); HRMS: calculated for $C_{59}H_{111}NO_{11}$ 1010.82299 $[M+H]^+$; found 1010.82277. Compound **8** was redissolved in dioxane and lyophilized in small quantities for immunology experiments.

*Note: Additional ^1H and ^{13}C signals encountered which indicate the presence of an additional regioisomer are denoted.**

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