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Towards the development of synthetic vaccines against tuberculosis

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Design and immunological evaluation of peptide-conjugates containing Mincle ligands

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Abstract

Mycobacterium tuberculosis (*Mtb*), the etiological agent of tuberculosis (TB), is one of the most successful pathogens on earth with an estimated 10 million newly infected people and 1.4 million deaths worldwide in 2019 only. Subunit vaccines containing proteins or peptide-epitopes combined with well-defined adjuvants can be attractive tools in the fight against *Mtb*. The glycolipids glucose monomycolate (GMM) and trehalose dimycolate (TDM) are promising immune adjuvants showing affinity for the murine and human C-type lectin receptor Mincle. In this Chapter the synthesis of four simplified GMM and TDM analogues is described and their ability to stimulate murine and human immune cells is tested. During the synthesis, the analogues were successfully equipped with a ligation handle to allow for further conjugation to an *Mtb*-derived peptide epitope. After verifying the ability of the novel synthetic glycolipids to interact with Mincle two TDM analogues were conjugated to an HLA-DR3 presented *Mtb* peptide antigen. An HLA-DR3 transgenic mouse model was then used to investigate the vaccine potential of one of these constructs. Although there was an absence of detectable CD4⁺ T-cell responses, the self-adjuvanting peptide induced significant systemic humoral immune responses. Interestingly immunisation with this construct induced a reduction of the bacterial load in the spleen of intranasally *Mtb* challenged mice in the absence of detectable T-cell responses in the circulation. Further *in vitro* experiments to translate the findings to human DCs suggested that this compound was not able to activate these cells. A second self-adjuvanting peptide, carrying a very similar TDM analogue, however, was able to do so. The present study provides the first example of self-adjuvanting peptides capable of interacting with the C-type lectin receptor Mincle, provides insights in their immune-stimulatory potential in the context of *Mtb* infection, and shows that protection against *Mtb* challenge can be obtained in the presence of humoral responses.

Introduction

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*), is responsible for the death of approximately 1.4 million people worldwide in 2019 alone.¹ It is estimated that one quarter to one third of the population is latently infected with *Mtb* and 5-10% of these will develop active disease during their lifetime.² In most cases, TB can be treated with antibiotic combinations, but multidrug-resistant strains are emerging, which limit treatment options.³ In countries with a high incidence of active TB, the World Health Organization (WHO) recommends vaccination of infants with *Mycobacterium bovis* Bacille Calmette-Guérin (BCG), which is the only commercially available vaccine against TB.¹

The BCG vaccine is an attenuated live mycobacterium derived from *M. bovis*. It can be formulated using different protocols and using (six) different original bacterial inocula-derived strains, with reports indicating that strain variability may influence efficacy.⁴ Although there is evidence supporting the utility of this vaccine, especially when administered in the first months of a human's life, epidemiological studies indicate inadequate protection against pulmonary TB in adults and the occurrence of disseminating BCG infection in immunocompromised infants.^{5,6} A novel prevention strategy is required to reduce TB burden and possibly eradicate the pathogen.^{1,7}

An alternative to attenuated bacteria is the use of subunit vaccines, where antigenic fragments of the pathogen are presented together with molecular adjuvants to increase immunogenicity. Subunit vaccines present a better safety profile compared to whole-cell mycobacteria as they are usually more stable and can be modified at the molecular level to achieve the desired effect.⁸ The efficacy of subunit vaccines against *Mtb* infection is suggested through the exemplary case of the M72/AS01 vaccine, consisting of two proteins, Rv1196 (PPE18) and Rv0125 (serine protease, pepA), in liposome and used as a booster to BCG in latently infected individuals. The results of a phase 2b clinical trial indicated 49.7% protection in immunized individuals at 36 months after vaccination.^{9,10} However, at the moment, a lack of detailed understanding of the molecular mechanisms governing a successful immune response and clearance of *Mtb*, and the consequent lack of definitive immune markers to protection, hinders the further development of synthetic subunit vaccines from reaching their full potential. In the context of *Mtb* and the use of subunit vaccines, significant effort is placed in exploiting pathogen-associated molecular patterns (PAMPs) to increase the immunogenicity of protein and peptide antigens.¹¹

The work reported in this Chapter describes the generation of a prototype synthetic *Mtb*-vaccine, formed by linking a PAMP glycolipid adjuvant and a *Mtb*-derived peptide antigen. Chemically linking an antigen and an adjuvant to generate a

conjugate is a method employed to ensure co-delivery of these two essential components of subunit vaccines. Co-delivery through a single-molecule vaccine usually results in the induction of strong immune-responses.¹² For example, Huang *et al.* have previously reported that co-delivery of the Ag85B-HspX fusion protein with arabinogalactan and the TLR3 ligand Poly(I:C) through chemical conjugation successfully induced humoral and cellular responses against *Mtb in vivo*, while the antigenic protein alone induced only marginal levels of inflammation.¹³

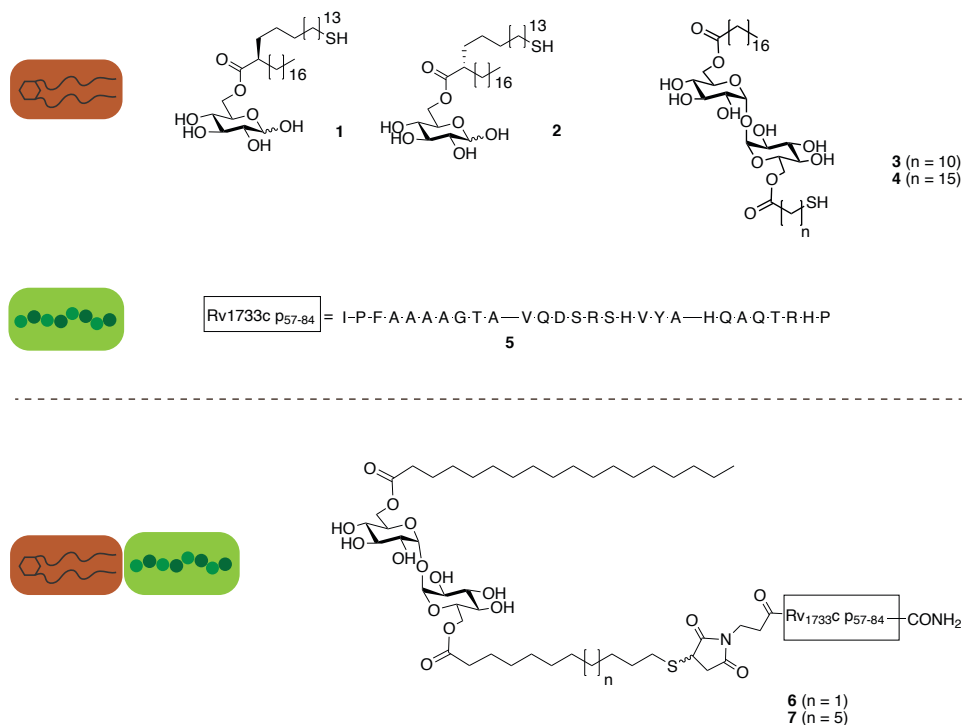


Figure 1 – Synthetic compounds designed and described in this Chapter. GMM analogues (compounds 1 and 2) TDM analogues (compounds 3 and 4) equipped with a thiol ligation handle for further conjugation synthetic long peptide p57 (compound 5). Self-adjuvanting peptides 6 and 7 were synthesized from maleimide functionalized peptide 5.

A suitable candidate for the generation of the self-adjuvanting peptides described in this Chapter is the Rv1733c peptide 5, depicted in Figure 1. This peptide, derived from a latency protein, was previously shown by Coppola *et al.* to have antigenic properties and to be presented through the human HLA-DR3 molecule to T-cells *in vivo* using a transgenic HLA-DR3/Ab⁰ murine model.¹⁴

With the idea of chemically coupling this peptide antigen to a relevant synthetic adjuvant, a series of four glycolipids designed to bind to the macrophage inducible C-type lectin (Mincle) receptor was generated (see Figure 1 for their chemical structure). To date, no reports have been published on synthetic conjugates containing ligands for Mincle, although there is ample evidence that this receptor performs important roles in immunity against pathogens: recognition of mycobacterial glycolipids and production of pro-inflammatory cytokines through a signalling cascade that results in activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B).¹⁵ Finally, it has been shown that Mincle activation enhances the transcription of inflammatory genes, while it also inhibits the late-stage NLRP3-inflammasome activation, preventing excessive inflammation.¹⁶ Because of these desirable characteristics, Mincle represents an interesting target to exploit for the development of vaccines against TB.

Mincle is a transmembrane pattern recognition receptor (PRR) expressed on macrophages, monocytes and dendritic cells, that is able to recognize glycolipids from fungi and mycobacteria. The first ligands for Mincle, trehalose dimycolate (TDM) (pictured in Figure 2a) and its analogue trehalose dibehenate (TDB), were identified in 2009 by Ishikawa *et al.*¹⁷ Recognition of TDB and TDM by Mincle is mediated through its carbohydrate recognition domain in the extracellular region.¹⁸ Measurement of the direct interaction of TDM and its analogues is limited by their poor solubility limiting crystallography studies to analogues containing short alkyl chains. However, a general pattern has been suggested that binding affinity increases for longer alkyl chains.¹⁵ Binding of a ligand to Mincle has been shown to activate the adapter protein Fc receptor γ -chain (FcR γ) which is required for the recruitment of spleen tyrosine kinase (SYK). SYK recruitment is followed by a cascade of signaling events which culminate in NF- κ B activation.¹⁵

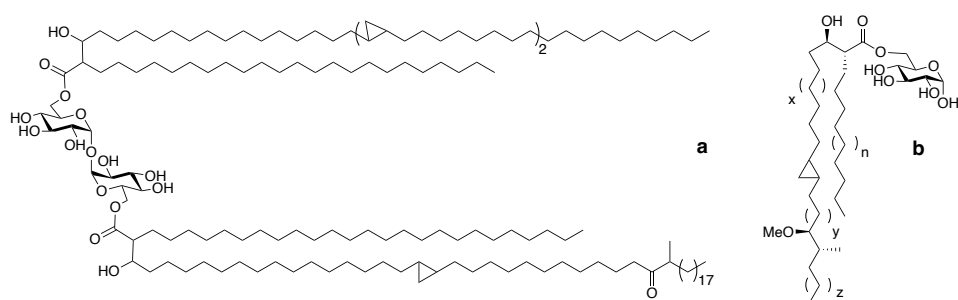


Figure 2 – Chemical structures of natural glycolipids that have been shown to interact with the Mincle receptor. a) Trehalose dimycolate (TDM); b) glucose monomycolate (GMM).

Since 2009, the year of first discovery of TDM/Mincle interaction, several groups have designed simplified synthetic analogues of known Mincle ligands to study their immune-stimulatory potential and reduce toxicity to cells. For example, a series of synthetic TDM analogues was designed by Kallerup *et al.* and Huber *et al.*, and their results indicated that the compounds are able to bind to Mincle and induce G-CSF and NO production in murine macrophages, suggesting that these compounds might also function as adjuvants.^{19,20} Other ligands belonging to glycolipid families other than TDM have been discovered and shown to bind to Mincle. Glucose monomycolate (GMM) is one such ligand (chemical structure of GMM shown in Figure 2b).

The present study describes the synthesis of two TDM and two GMM simplified analogues, equipped with simple saturated linear lipid chains and a ligation handle with the ultimate goal being the incorporation of a Mincle ligand in a self-adjuvanting peptide construct. At a later stage this construct could be further expanded to include an additional adjuvant.

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The design of the synthetic route of the two TDM analogues described in this Chapter was inspired by the research of Toubiana *et al.*, Johnson, Datta *et al.*,^{21–23} using a TMS-protecting group based strategy, advantageous in comparison to the benzyl protecting group strategy used by Nishizawa *et al.* in the ease of scaling up the final deprotection reaction.²⁴ The choice of lipids for coupling to the carbohydrate core was based on: 1) structural studies suggesting increased binding interaction of trehalose 6-OH mono-substituted compounds with increasing alkyl chain length and the requirement for interaction between the 3-OH and 4-OH positions of trehalose to the carbohydrate recognition domain on the receptor;^{25,26} 2) experimental evidence that biological activity in symmetrically substituted trehaloses increased with increasing chain length, with C18 showing little to no difference as compared to C22 (length of alkyl chain in the well-studied analogue TDB);^{19,27,28} 3) no evidence was found suggesting the requirement of the two alkyl chains having the same number of carbon atoms. Based on these considerations, the new TDM analogues were designed to contain: a C18 alkyl chain on the 6-OH position of the trehalose and a C11 or C16 alkyl chain on the 6'-OH position, with this latter lipid chemically modified to present a thiol ligation handle for further conjugation.

The design of the two GMM analogues was inspired by a publication by Decout *et al.* reporting of a synthetic GMM analogue, produced as racemic mixture, and named GlcC14C18. This compound exhibited a stronger adjuvant effect than TDB, when formulated in liposomes and it induced protective immunity in a mouse model of *Mtb* infection.²⁹ Separately, it was shown for multiple TLR2 ligands that different stereoisomers can elicit different biological responses.^{30,31} Based on these results, the two GMM analogues containing the enantiomerically pure C14C18 lipids equipped with a thiol ligation handle were designed and synthesised using a similar TMS-protecting group approach as the one selected for the synthesis of the TDM analogues.

To probe the ability of the four synthetic glycolipids to interact with the C-type lectin receptor Mincle, *in vitro* assays using the human embryonic kidney 293 (HEK-293) expressing murine Mincle reporter cell line were performed. The glycolipids showing the best binding affinity for Mincle were conjugated to the *Mtb*-derived peptide. These were evaluated *in vitro* in murine and human cell systems for their immunomodulatory activity. An HLA-DR3/Ab⁰ transgenic mouse model was used to investigate the vaccine potential of one of these constructs *in vivo* and their effect on the bacterial load when challenged with live *Mtb*.

Results

Synthesis of GMM and TDM analogues

The synthetic strategy for the generation of the four glycolipids depicted in Figure 1 is based on a protecting group strategy using trimethyl silyl ethers (TMS) and a Steglich esterification approach to introduce the lipid chains. The enantiomeric fatty acids contained in the two GMM analogues, compounds **1** and **2**, were synthesized starting from the same pseudoephedrine derivative **9** (see Figure 3A) via two stereo-divergent approaches.

Starting from compound **9**, a Myers alkylation with diisopropyl amine (DIPA) and allyl bromide delivered the *R*-isomer of α -allyl stearic acid **10**. The *S*-configured lipid **14** was obtained by an O-allylation with sodium hydride and allyl bromide followed by a Claisen rearrangement using triflic anhydride and 2-fluoropyridine.¹ A cross metathesis with *S*-(dodec-11-en-1-yl) ethanethioate then delivered the enantiomeric branched acids **12** and **15**, which were obtained in 46% and 38% yield over 5 steps starting from stearic acid, respectively. The two GMM analogues **1** and **2** were obtained from α -D-glucose, as shown in Figure 3B. α -D-Glucose **16** was first protected as the TMS ethers using TMS chloride, hexamethyldisilazane (HMDS) and pyridine which was followed by deprotection of the primary alcohol with ammonium acetate. The lipids **12** and **15** were then installed by esterification utilizing 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and DMAP. Afterwards, a simultaneous reduction of the alkyl double bond and thioester with *p*-nitrosulfonyl hydrazide was carried out. Finally, removal of the TMS-protecting groups with amberlite resin and treatment with tris(2-carboxyethyl)phosphine (TCEP) to reduce the disulfide resulted in compounds **1** and **2** with 24% and 14% yield over 6 steps, respectively.

Next, the attention was shifted to the synthesis of TDM analogues **3** and **4** (see Figure 4). Lipid thioester **21**, required for the generation of the conjugation ready TDM analogue **3**, was synthesized in one step via nucleophilic substitution of

¹ A schematic depiction of the suggested mechanism of the reaction and intermediates can be found in the supporting information (Figure S5).

commercially available 11-bromoundecanoic acid with potassium ethanethioate in 91% yield.

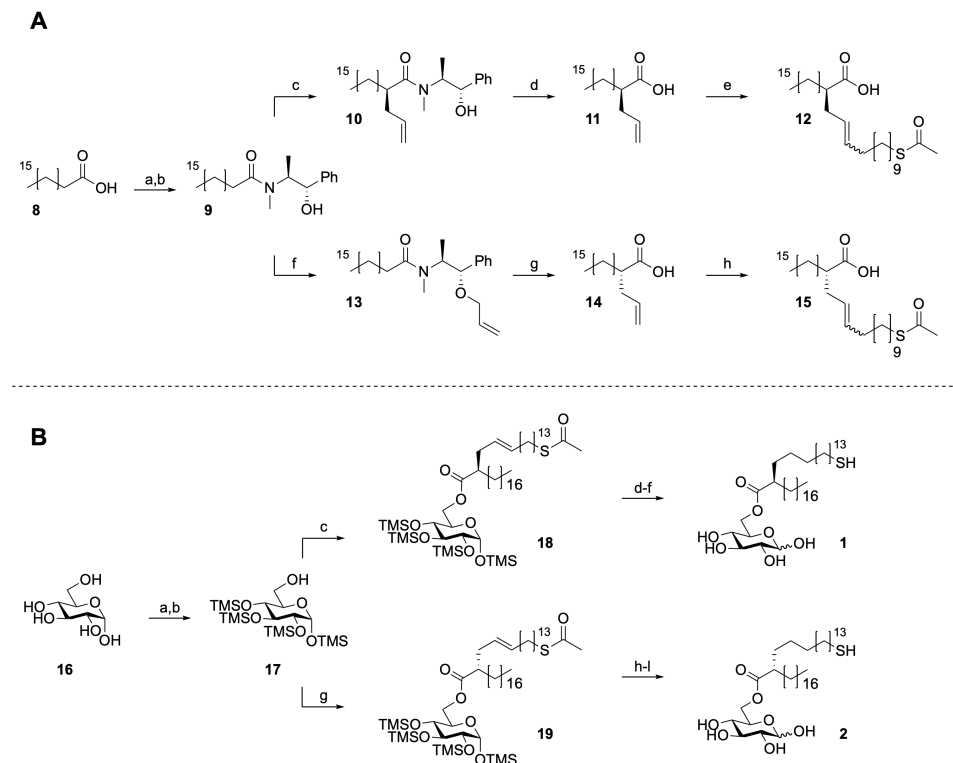


Figure 3 - Synthetic route optimized to generate GMM analogues and enantiomeric lipids. (A) Reaction scheme for the synthesis of lipids. a) oxalyl chloride, DMF, toluene, y: 99%, b) pseudoephedrine, Et₃N, DCM, y: 99%, c) DIPA, LiCl, allyl bromide, THF, y: 84%, d) H₂SO₄ (5N)/dioxane 1:1, y: 73%, e) S-(dodec-11-en-1-yl) ethanethioate, Grubbs 1st generation, DCM, reflux, y: 76%, f) NaH, allyl bromide, DCM, y: 86%, g) i. Tf₂O, 2-fluoropyridine, DCM, 0°C, ii. H₂SO₄ (2.5 N)/dioxane 1:1, 100°C, y: 60%, h) S-(dodec-11-en-1-yl) ethanethioate, Grubbs 1st generation, DCM, reflux, y: 76%. **(B)** Reaction scheme for the synthesis of glycolipids. a) TMSCl, HMDS, pyridine, y: quant. b) NH₄OAc, DCM/MeOH 1:1, y: 73%, c) lipid, EDCl, DMAP, y: 52%, d) p-nitrosulfonyl hydrazide, Et₃N, DCM, e) amberlite H⁺, DCM/MeOH 1:1, f) TCEP.HCl, PBS, MeOH/CH₃CN 1:1, y: 62% over 3 steps, g) lipid, EDCl, DMAP, y: 50%, h) p-nitrosulfonyl hydrazide, Et₃N, DCM, i) amberlite H⁺, DCM/MeOH 1:1, l) TCEP.HCl, PBS, MeOH/CH₃CN 1:1, y: 73% over three steps.

For the synthesis of thioester **24**, the chosen reaction sequence consisted of the opening of cyclohexadecanolide with NaOMe, subsequent bromination to replace the hydroxyl moiety, saponification of the ester and nucleophilic substitution of the bromide with potassium thioacetate to obtain lipid **24** in 43% overall yield. In line

with the assembly of **1** and **2**, the alcohols of trehalose were protected as TMS-ethers except this time the reaction was carried out using *N,O*-bis(trimethylsilyl)acetamide and TBAF. After the primary hydroxyl groups were deprotected with potassium carbonate in methanol, stearic acid was installed with EDCI and DMAP to provide the monoesters (**Figure 4B**). Next, a condensation with lipid thioesters **21** and **24** provided the fully protected TDM analogues **27** and **28**. Removal of the silyl ethers under acidic conditions and conversion of thioester to free thiol with hydrazine then provided compounds **3** and **4** in 26% and 27% yield, respectively, over 5 steps starting from trehalose.

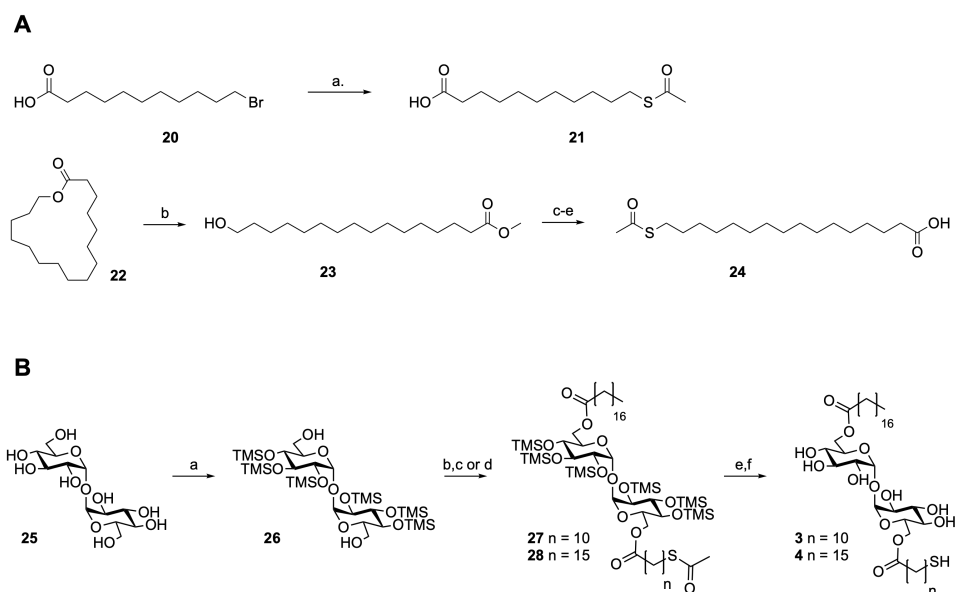


Figure 4 - Synthetic route optimized to generate TDM analogues and lipids with different chain length. (A) Reaction scheme for the synthesis of lipids. a) potassium thioacetate, DMF, y: 91%, b) NaOMe, MeOH, y: 85%, c) PPh_3 , NaHCO_3 , NBS, y: 88%, d) LiOH, THF, y: 91%, e) potassium thioacetate, DMF, y: 63%. **(B)** Reaction scheme for the synthesis of glycolipids. a) i. *N,O*-bis(trimethylsilyl)acetamide, TBAF, DMF, ii. K_2CO_3 , MeOH, y: 79%, b) stearic acid, EDCI, DMAP, toluene, y: 47%, c) 11-(acetylthio)undecanoic acid, EDCI, DMAP, toluene, y: 70%, d) amberlite H^+ , DCM/MeOH 1:1, y: quant., e) $\text{NH}_2\text{NH}_2\cdot\text{CH}_3\cdot\text{COOH}$, DCM/MeOH 1:1, y: quant., f) 11-(acetylthio)hexadecanoic acid, EDCI, DMAP, toluene, y: 85%, g) amberlite H^+ , DCM/MeOH 1:1, y: 87%, h) $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$, DCM/MeOH 1:1, y: quant. (isolated as mixture of oxidized and reduced thiol).

In vitro evaluation of Mincle ligand-peptide conjugates

With the simplified GMM and TDM analogues in hand, the HEK-Blue mMincle reporter cell line was used to probe the binding of these constructs. HEK-Blue mMincle is a commercially available cell line transfected to overexpress murine

Mincle. Functional binding of a ligand to Mincle in this cellular system results in release of secreted alkaline phosphatase (SEAP) in the cells supernatant, which induces a colour change in the QUANTI-blue medium proportional to the amount of secreted SEAP. The colour change is detectable by eye and can be measured quantitatively using a spectrophotometer at 620-655 nm. The synthetic TLR2 ligand UPam, known immune stimulator for human and murine antigen presenting cells (APCs),³⁰ was used as negative control to validate the assay's specificity.

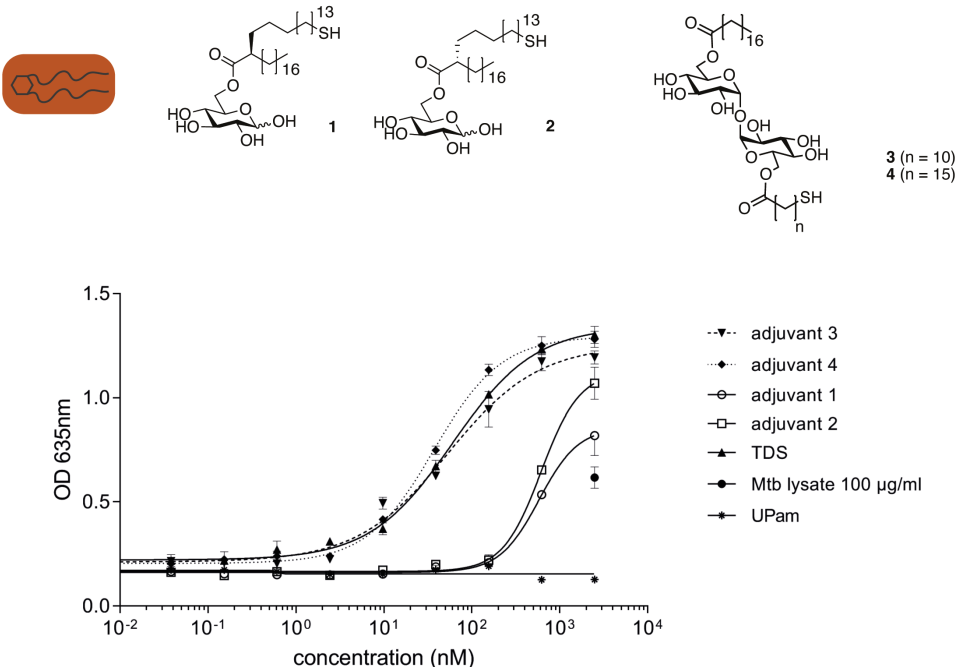
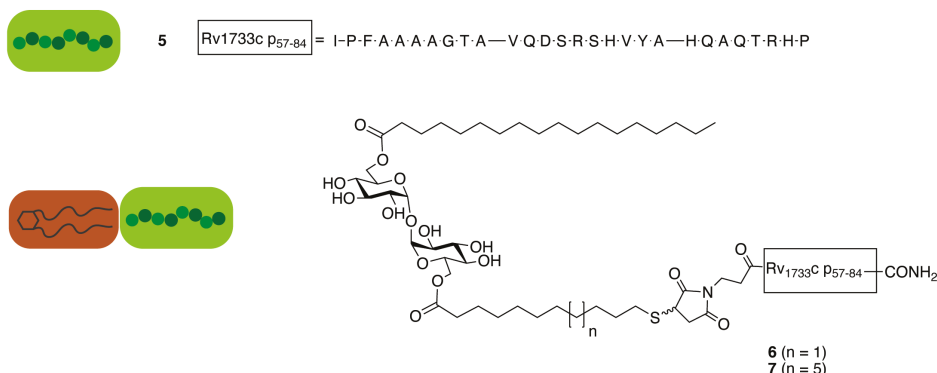


Figure 5 - Dose-response curves for HEK-293 mMincle activation. HEK-293 cells expressing murine Mincle and a NF- κ B-inducible reporter system were stimulated with plate-bound glycolipids for 16h. NF- κ B activation was determined by measuring secreted embryonic alkaline phosphatase (SEAP) activity and reading O.D. at 635 nm after mixing of 20 μ l of the culture supernatant with 180 μ l of Quanti-BlueTM (InvivoGen). TDS and Mtb lysate (expected to contain TDM) were used as positive controls. UPam, a TLR2 ligand, was used as negative control. Dots represent mean + SEM of duplicates from one experiment. Curves were interpolated using a non-linear regression model with 4 parameters as calculated using GraphPad Prism software.

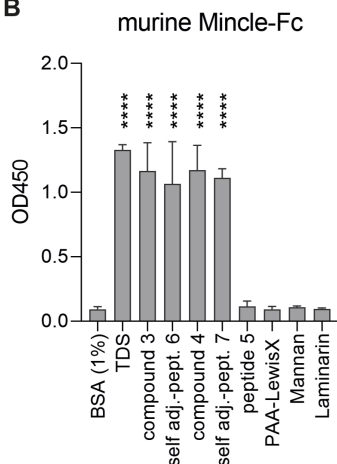
Cells stimulated with each analogue responded to the stimulation by releasing SEAP in the cell supernatant, indicating that all four analogues are able to bind to and activate the murine Mincle receptor (see Figure 5). The two TDM analogues,

compounds **3** and **4**, bound the receptor with higher potency than the two GMM analogues, compounds **1** and **2**, and were therefore chosen for the generation of the two self-advantaging peptides described in the next section.

A



B



C

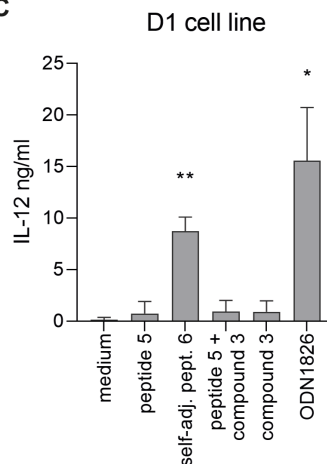


Figure 6 - Binding experiment using soluble murine Mincle (B) and stimulation experiment using murine D1 dendritic cells (C). (A) Chemical structures of synthetic long peptide p57 (compound **5**) and self-advantaging peptides **6** and **7**. **(B)** ELISA experiment was performed using plate-bound glycolipids (5 nmol/well), self-advantaged peptides (5 nmol/well) and control compounds. TDS = Trehalose distearate (5 nmol/well) was used as positive control. PAA-LewisX (10 $\mu\text{g/ml}$), Mannan (10 $\mu\text{g/ml}$) and Laminarin (3 $\mu\text{g/ml}$) known to bind other CLRs, and the reference peptide p57 (5 nmol/well) were chosen as negative controls. Soluble recombinant murine Mincle Fc chimera protein was used for assaying binding interaction. **(C)** IL-12p40 production by murine D1 DC cells stimulated for 20 hours using synthetic compounds (50 μM) or ODN1826 control (1 $\mu\text{g/ml}$), as measured by ELISA. Bars represent mean + SD of duplicates from two independent experiments. Statistical significance with reference to the negative control group, BSA (1%) in buffer (plot a) and DMSO (0.1%) in medium (plot b), was determined by one-way ANOVA with Tukey's multiple comparisons test (****p < 0.0001, **p < 0.01, *p < 0.05).

Peptide **5** (amino acid sequence shown in Figure 6A) was functionalized with an N-terminal maleimide ligation handle for conjugation to the thiol-functionalized TDM analogues. It was synthesized using an automatic solid phase synthesizer and obtained in 6.7% yield after purification. The conjugation of the glycolipids to the synthetic long peptide was performed via thiol-maleimide addition. Self-adjuvanting peptides **6** and **7** (chemical structures shown in Figure 6A) were obtained with, respectively, 73% and 25% yield after HPLC purification. Partial precipitation and low solubility of self-adjuvanting peptide **7** in many organic solvents rendered isolation and purification of the product difficult resulting in a lower yield.

The two self-adjuvanting peptides were tested for their binding to murine Mincle. An ELISA assay was performed using the commercially available Mincle-Fc-chimera recombinant soluble receptor. This assay, as shown in Figure 6B, indicated that the two self-adjuvanting peptides had a fully preserved ability to interact with this receptor. As expected, no binding between soluble Mincle receptor and peptide **5** was observed. This result confirmed that the interaction between the self-adjuvanting peptides and Mincle is mediated by the glycolipid moiety. As additional negative controls, known CLRs ligands, such as Lewis X polyacrylamide conjugate (PAA-LewisX), Mannan and Laminarin,³² were selected and none of them bound to Mincle. The synthetic compound TDS was used as positive control in all binding assays.²

Next, the functional effect of self-adjuvanting peptide **6** was studied using the well-defined, immature murine D1 dendritic cell line. As shown in Figure 6C, stimulation using self-adjuvanting peptide **6** resulted in production of significant amounts of IL-12p40, although no increase in CD40 or CD86 activation markers could be observed (data not shown). Somewhat unexpectedly, this was not the case for the mixture of separate peptide **5** and compound **3**, where neither activation nor production of the pro-inflammatory cytokine IL-12p40 was observed. ODN1826, potent immune-stimulatory agent able to interact with dendritic cells via TLR9, was used as positive control.³³

To functionally characterise the synthetic TDM analogues and derived self-adjuvanting peptides, human dendritic cells and macrophages were generated starting from monocytes. Changes in activation or T cell co-stimulatory markers and released pro-inflammatory/anti-inflammatory cytokines were used as a measure of immunogenicity of novel constructs. Immature monocyte-derived dendritic cells (moDCs) and macrophages were differentiated as described in the Materials and Methods section of this Chapter, and their cell surface marker phenotype was characterised by CD1a, CD14, CD11b, CD163 expression.^{34,35} Flow cytometry analysis of the CD83 activation marker for dendritic cells showed that little or no activation

² Studies performed using an HEK-Blue mMincle reporter assay resulted in absence of detectable amounts of SEAP when stimulating cells with the two self-adjuvanting peptides.

was induced by treatment with the compounds containing the shorter alkyl chains, namely compound **3** and self-adjuvanting peptide **6**, while the compounds containing the longer alkyl chain, compound **4** and self-adjuvanting peptide **7**, did induce up-regulation of this activation marker to a comparable extent to that induced by LPS (Figure 7). In the case of the CD86 marker, compound **4** induced a 5-fold increase in marker expression as compared to the negative control. The self-adjuvanting peptide **7** was responsible for a more modest up-regulation of CD86, with a 3-fold increase, comparable to the increase induced by self-adjuvanting peptide **6**. Up-regulation of the CD80 marker in human moDCs was induced only by stimulation with self-adjuvanting peptide **7**. A two-fold increase in MHC class II expression as compared to unstimulated control is observed for moDCs treated with compounds **3** and **4**, peptide **5** and the self-adjuvanting peptides. Cell supernatants were analysed via ELISA to quantify production of IL-12p40 and IL-10 (see supporting Figure S6). The anti-inflammatory cytokine IL-10 was only detected in the LPS control. On the other hand, a ten-fold increase of IL-12p40 was detected in supernatants of cells treated with the self-adjuvanting peptide **7**. Luminex analysis of the same supernatants allowed for the identification of IL-6 and TNF- α as two additional pro-inflammatory cytokines released upon treatment with self-adjuvanting peptide **7**. However, the increase in cytokine production for this compound did not show statistical significance.

Macrophage differentiation by GM-CSF, which leads to pro-inflammatory M1-type cells, and M-CSF, leading to anti-inflammatory M2-type macrophages was studied next, and the results are summarised in Figure 8. The CD80 marker was significantly upregulated on M1 and M2 macrophages upon stimulation with self-adjuvanting peptides **6** and **7**. The latter was also shown to induce CD83 upregulation on both macrophage types. Interestingly, stimulation using the unconjugated TDM analogues **3** and **4** did not promote expression of CD80. However, analogue **4** caused upregulation of CD83 to a similar extent to self-adjuvanting peptide **7** and LPS control.

Analysis of cell supernatants via ELISA for the quantification of IL-12p40 and IL-10 indicates that IL-10 is released by M-CSF/M2 macrophages upon stimulation with both self-adjuvanting peptides (Figure 9). No IL-10 nor IL-12p40 was detected in supernatants of GM-CSF/M1 macrophages.

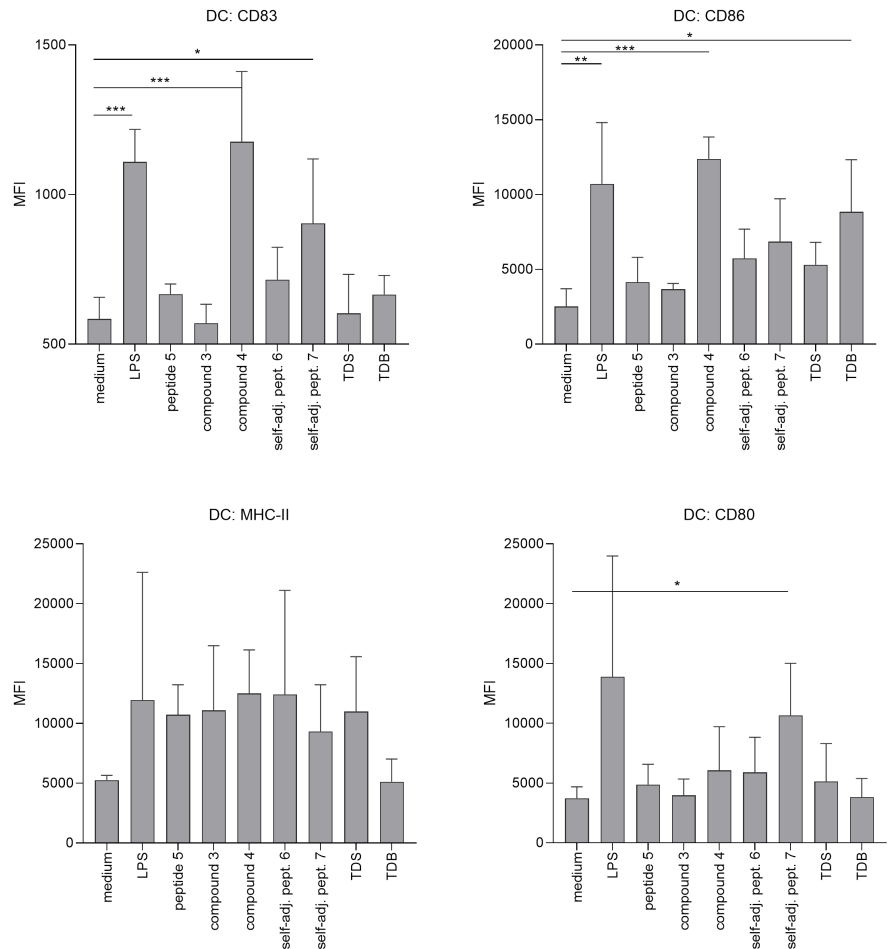


Figure 7 - Bar plots depicting the mean expression of selected surface markers on monocyte-derived dendritic cells from 3 different donors as measured by flow cytometry. All synthetic compounds were used at a final concentration of 20 μ M in medium. LPS (100 ng/ml) is used as a positive control. Statistical significance with reference to cells exposed to medium + DMSO was calculated through one-way ANOVA method (** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Bars indicate the mean value + SD of the MFI dataset for each condition as calculated using GraphPad Prism.

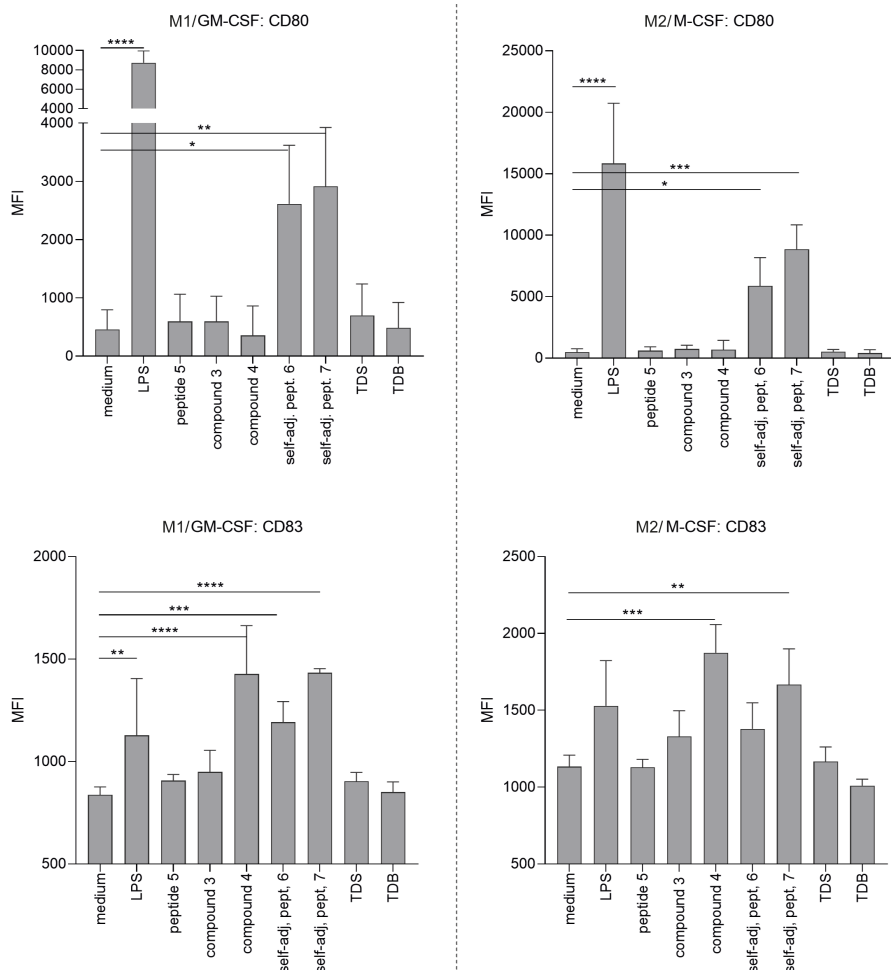


Figure 8 - Bar plots depicting the mean expression of CD80 and CD83 markers on GM-CSF/M1 and M-CSF/M2 macrophages cells from 3 different donors as measured by flow cytometry. All synthetic compounds were used at a final concentration of 20 μ M in medium. LPS (100 ng/ml) is used as a positive control. Statistical significance with reference to cells exposed to medium + DMSO (0.1%) was calculated through one-way ANOVA method (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). Bars indicate the mean value + SD of the MFI dataset for each condition as calculated using GraphPad Prism.

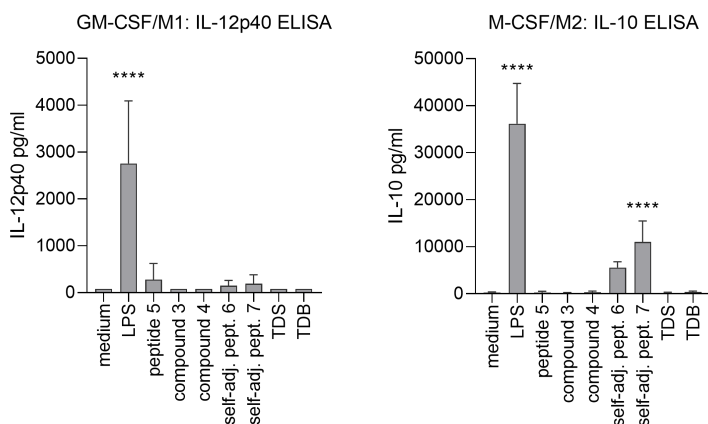


Figure 9 - Cytokine production profile of human macrophages stimulated for 20 hours using TDM analogues 3 and 4, and self-adjuvanting peptides 6 and 7, as measured by ELISA (IL-12p40 and IL-10) or Luminex (IL-6 and TNF- α). All synthetic compounds were used at a final concentration of 20 μ M in medium. LPS (100 ng/ml) is used as a positive control. Bars indicate the mean value + SD of duplicates from three donors as calculated using GraphPad Prism. Statistical significance with reference to cells exposed to medium + DMSO was calculated through one-way ANOVA method (** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

In vivo evaluation of Mincle ligand-peptide conjugate

An HLA-DR3 transgenic mouse model lacking murine MHC class II system (HLA-DR3/Ab⁰), previously shown to be suitable for the *in vivo* study of peptide p57 HLA-DR3 restricted T-cell immunity, ¹⁴ was used to evaluate the *in vivo* immunogenicity of self-adjuvanting peptide 6. In this model, mice were immunised subcutaneously three times with either peptide 5 in admixture with ODN1826 as positive control, peptide 5 in admixture with compound 3, self-adjuvanting peptide 6 or injected with PBS as negative control. Intracellular IFN- γ , TNF- α and IL-17 production by CD4⁺ T-cells was measured via flow-cytometry after *in vitro* stimulation of splenocytes with either the peptide antigen or recombinant protein. The control mixture of the peptide with ODN1826 induced strong Th1 responses, as previously reported, [28] a result which was confirmed also by analysis of the cell supernatants by IFN- γ ELISA (see supporting information S7). Immunisation with either the mixture of peptide 5 with 3 or with self-adjuvanting peptide 6 did not induce detectable CD4⁺ T-cell responses.

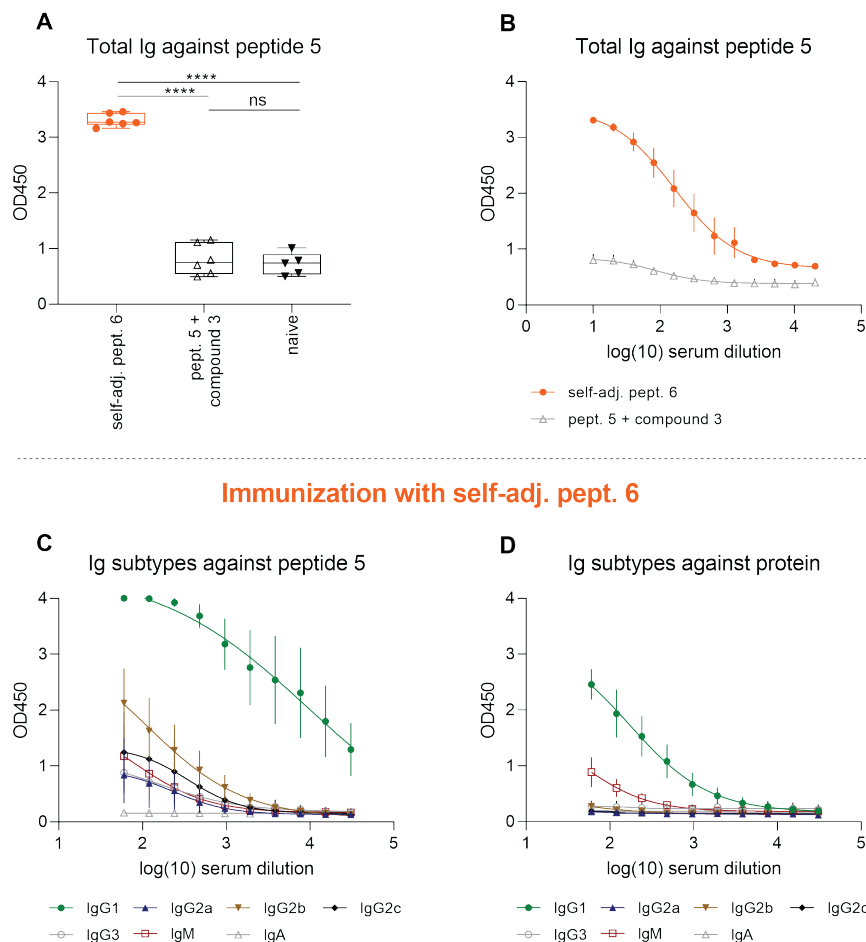


Figure 10 - Measurement of antigen-specific antibodies from sera of mice (n=6) immunized with self-adjuvanting peptide 6 (40 nmol), peptide 5 (40 nmol) in admixture with UPam adjuvant (40 nmol) or treated with PBS only (naïve group). Plate-bound peptide p57 antigen was used for the Total Ig antibodies assay. **(A)** Box plots representing absorbance (OD450) corresponding to amount of Total Ig antibodies and measured for sera diluted 10 times (n = 5 for naïve, n = 6 for the other groups). Statistical significance was calculated using one-way ANOVA as calculated with GraphPad Prism software (****p < 0.0001, ns > 0.05). **(B)** Dose-response dot plots representing mean + SEM of absorbance measurements from 5 or 6 mice as calculated using GraphPad Prism. **(C)** Measurement of antigen-specific antibody isotypes from sera of mice (n=6) immunised with self-adjuvanted peptide 6. Plate-bound peptide 5 antigen was used for the Ig subtypes antibodies assay. Dots represent mean + SEM of single measurements from 6 mice as calculated using GraphPad Prism. **(D)** Measurement of antigen-specific antibody isotypes from sera of mice (n=6) immunised with self-adjuvanted peptide 6. Plate-bound Rv1733c recombinant protein antigen was used for the Ig subtypes antibodies assay. Dots represent mean + SEM of single measurements from 6 mice as calculated using GraphPad Prism.

To verify if humoral responses were induced by the chosen treatments, sera were analysed for the presence of antigen-specific antibodies (see Figure 10). The mixture of antigen plus adjuvant did not stimulate production of antigen-specific antibodies. In striking contrast, self-adjuvanting peptide **6** consistently induced production of antibodies able to bind peptide **5**, indicating the importance of antigen-adjuvant co-delivery. Analysis of antibody subtypes lead to the identification of high titers of antigen-specific IgG1, associated with Th2 activation in mice, able to recognize both peptide **5** and its source, Rv1733c protein. In addition, IgG2b antibodies, associated with T-cell independent responses, that were able to recognize the peptide antigen were also found.^{36–38}

To further assess the vaccine potential of the self-adjuvanting peptide **6**, its prophylactic effect was evaluated using a live *Mtb* challenge model. Here enumeration of the colony forming units (CFU) in the lung and spleen of infected mice was used as a measure of protection.

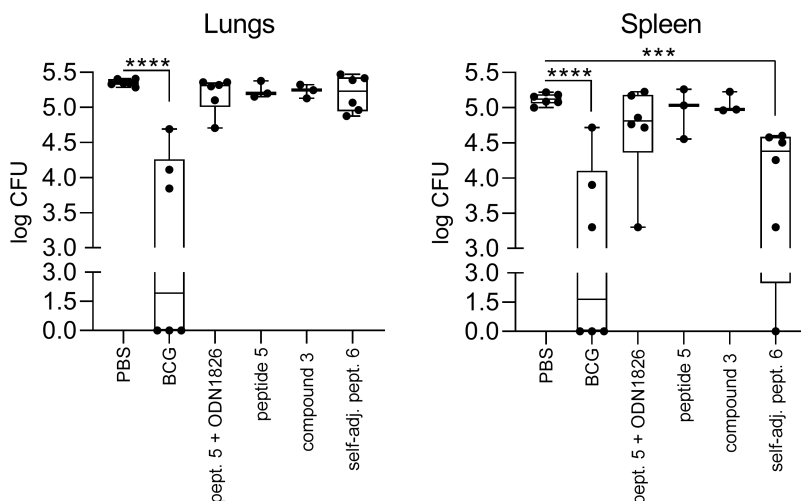


Figure 11 - Protective efficacy against *Mtb* infection in the spleen of mice that were immunised s.c. for 3 times with 2 weeks interval. Mice (n=6) were immunised with a mixture of peptide 5 (40 nmol) with ODN1826 (50 µg/ml) as positive control, with self-adjuvanted peptide 6 (40 nmol) or mice (n=3) were treated with TDM analogue 3 (40 nmol) or with peptide 5 (40 nmol). Six weeks later, the mice were challenged with intranasal *Mtb* H37Rv (10^5 CFU). Alternatively, the mice received 10^6 CFU BCG s.c. 10 weeks before challenge. Colonies in lungs and spleen were counted after 3 weeks of incubation at 37 °C. Statistical significance with reference to the naive group was calculated through one-way ANOVA method (****p < 0.0001, ***p < 0.001). Box plots were generated using GraphPad Prism.

The following groups were included in the study: mice immunised with a mixture of peptide **5** with ODN1826, mice immunised with the standard BCG1331 vaccine, mice immunised with peptide **5** only, mice immunised with TDM analogue **3** and mice immunised the self-adjuvanting peptide **6**. As shown in Figure 11, analysis of infection progression in the lungs of immunised mice indicated that only the BCG control was able to cause a reduction of the bacterial load locally in the lungs at the time of organ isolation. Nevertheless, signs of a protective systemic immune response were evident when analysing the spleen of mice immunised with the self-adjuvanting peptide **6**, as the latter significantly reduced the number of CFU in the spleen of vaccinated mice (Figure 11). This reduction is present only in the case of vaccination with the self-adjuvanting peptide, and not following vaccination with the unconjugated peptide or compound **3**. Vaccination with the self-adjuvanting peptide led to a lower bacterial count than vaccination with the mixture of the peptide with ODN1826.

Discussion

The study discussed in this Chapter shows that conjugation of the latency antigen Rv1733c p57 peptide to synthetic compound **3**, a novel Mincle ligand, induces stronger immune responses *in vivo* than the simple admixture of adjuvant-antigen. This study provides the first evidence that a self-adjuvanting peptide containing a TDM analogue can induce protection against TB in an *in vivo* model.

Considerations on the design rationale of the TDM and GMM analogues containing a ligation handle for conjugation to the Rv1733c p57 peptide antigen were detailed in the introduction section of this Chapter. Binding of TDM analogues **3** and **4** to the murine Mincle receptor did not seem to be affected by the introduction of a thiol ligation handle, which was purposefully located on the 6' O-alkyl chain. Comparison of the dose-response curves for compounds **3**, **4** and the structurally similar experimental control TDS, lacking the thiol ligation handle, indicated comparable activation of the HEK-Blue mMincle reporter cell line upon stimulation with the aforementioned compounds.

Using the same HEK-Blue mMincle reporter assay, it was observed that the two GMM synthetic adjuvants also activated this reporter cell line. A difference was, however, recognized when comparing the two TDM analogues to the two GMM analogues, with the GMM analogues inducing significantly lower activation.

For this reason, the TDM analogues were used to generate the two single molecule subunit vaccines. The synthesis of the trehalose conjugates was achieved by reacting the thiol-functionalised glycolipids with a maleimide functionalised peptide. This reaction required judicious solvent optimization, to meet the different solubility requirements of the two reagents. A mixture of DMF/chloroform/water 4:3:1 was

identified to allow for optimal solubilization of both reagents and by adjusting the pH of the mixture to 8 by addition of N-methyl-morpholine.

The conjugates, composed of the peptide antigen and TDM analogues, were assayed for their ability to activate HEK-Blue mMincle cells. Unexpectedly, the responses were very low for both self-adjuvanting peptides. Aggregation of the conjugates was observed experimentally at the highest concentrations, and this could explain the reduced binding of the conjugates in the HEK-Blue mMincle cells.³ Further research into the specific cause of the unexpected result would be required to shed light on the significance of these results. To verify if this result was connected to the model or it would indicate disrupted binding of the glycolipid moiety to the Mincle receptor, an additional model was selected to qualitatively assess the interaction to the murine receptor. Following the steps of Decout *et al.* and Chinthamani *et al.*,^{29,40} an ELISA assay was performed using the commercially available murine Mincle-Fc-chimera receptor. This assay indicated that the two self-adjuvanting peptides preserved the ability to interact with Mincle.⁴

3

The functional effect of the synthetic glycolipids and self-adjuvanting peptides was studied using human monocyte-derived dendritic cells and macrophages. Changes in expression of CD80, CD83 and CD86 molecules were monitored via flow cytometry as an indication of stimulatory activity of the synthetic compounds. CD80 and CD86 are type I glycoproteins expressed on the surface of APCs, with the ability to interact with CD28 and with cytotoxic T lymphocyte antigen-4 (CTLA-4) expressed on activated T cells.⁴¹ They are therefore conventionally considered co-stimulatory molecules for T cells, and their upregulation is associated with APC activation.⁴² CD83 is a member of the immunoglobulin family and is conventionally used as a marker for mature dendritic cells, although it is expressed on the cell surface of several APCs.⁴³

The results here described clearly indicate that human monocyte-derived dendritic cells respond differently to the two synthetic TDM analogues and that the response depends on the difference in alkyl chain length. Flow cytometry analysis of the CD83 and CD86 activation markers for dendritic cells showed that little or no activation is induced by treatment with the compounds containing the shorter C11-alkyl chain (compounds **3** and **6**), while the compounds containing the longer C16-alkyl chain (compounds **4** and **7**) induced up-regulation of these activation markers to an extent comparable to that induced by LPS. As previously mentioned, several reports have suggested that biological activity depends on the lipid chain length in trehalose mono- and di-esters. However, those studies focused on murine bone-marrow derived macrophages and employed plate-bound glycolipids. It is not possible to

³ An alternative hypothesis could be endoplasmic reticulum stress, which has been previously shown to cause critical interference in secreted protein-based reporter assays.³⁹

⁴ The use of human Mincle-Fc-chimera in a preliminary ELISA assay indicated that the novel adjuvants and self-adjuvanting peptides are also capable of binding to the human receptor (see Chapter 6).

directly compare the results obtained using dissolved/suspended compounds to those referring to experiments performed using plate-bound glycolipids.^{19,28} With the goal of incorporating a Mincle ligand in a self-adjuvanting peptide construct, and, at a later stage, further expand the self-adjuvanting construct to include an additional adjuvant, the use of plate-bound lipid was avoided in the evaluation of the *in vitro* functional studies reported in this Chapter. Furthermore, the supernatants from moDCs were analysed via ELISA and Luminex for the quantification of pro-inflammatory cytokines, and it was observed that only the self-adjuvanting peptide **7** induced release of IL-12p40, IL-6 and TNF- α . These cytokines were not released upon stimulation with the stand-alone trehalose adjuvant nor the peptide, suggesting either a synergistic effect of the two or, possibly, formation of supramolecular structures which results in improved immunogenicity for the self-adjuvanting peptide.

Analysis of the activation markers for GM-CSF/M1 and M-CSF/M2 macrophages further indicated that the self-adjuvanting peptides more strongly activated human antigen-presenting cells than the TDM analogues alone, as exemplified by expression of CD80 for M1 and M2 and cytokine production by M2 macrophages. However, activation of macrophages seems to be less sensitive to the alkyl chain length of the TDM analogue linked to the peptide. Further structure-activity relationship studies to identify how the chain length influences response in human monocyte-derived dendritic cells are warranted.

To compare the vaccine potential of the self-adjuvanting peptide **6** to that of a mixture of adjuvant **3** and peptide **5**, an *in vivo* experiment was performed to assess both cellular and humoral responses in class II-deficient mice, transgenic for HLA-DRA/B1*0301 (DR3) allele. The expected responses were found in the group of mice immunised subcutaneously with positive control, where IFN- γ TNF- α double positive CD4⁺ T-cell responses were detected. The IFN- γ production by restimulated splenocytes in the control group was confirmed by ELISA assay performed on splenocytes supernatant. On the contrary, no CD4⁺ T-cell responses were detected for either the mixture of antigen plus compound **3** nor self-adjuvanting peptide **6**. Splenocytes supernatants were also assayed using a Luminex kit for the identification of cytokine increase upon restimulation. Also in this case, no increase in quantity of cytokines was observed upon restimulation. To verify if humoral responses were induced by the self-adjuvanting peptide, sera were analysed for the presence of antigen-specific antibodies. The mixture of antigen plus compound **3** did not stimulate production of antigen-specific antibodies. On the contrary, high titers of IgG1 and IgG2b antibodies were induced by immunisation with the self-adjuvanting peptide **6**. Murine IgG1 antibodies are usually associated with a Th2 type response and their production is shown to increase in the presence of IL-4.^{36,37} Switching to IgG2b antibodies seems to be mediated by transforming-growth factor- β and it has been associated with a T-independent immune response.³⁸

The majority of TB vaccine candidates currently in clinical trials have been selected on the basis of pre-clinical studies focused on inducing a CD4⁺ Th1/Th17 cellular response.⁴⁴ The paradigm of protection that revolved around the induction of polyfunctional CD4⁺ T-cells in response to immunisation is only very recently shifting towards a broader interest in the interplay between innate, cellular and humoral immunity to tackle the challenge of developing an effective TB vaccine.^{44–46} To further assess if the self-adjuvanting peptide could effectively counteract *Mtb* infection, even though only humoral responses were detected, a challenge experiment with live *Mtb* was performed. Following the same immunisation protocol described above, and using BCG immunisation as positive control, it was determined that the self-adjuvanting peptide **6** was able to reduce hepatic bacterial load in vaccinated mice, although no effect was observed in the lungs of the same mice. This adds to the growing body of evidence that significant protective effects against mycobacterial infection can be observed in the presence of humoral responses.^{47,48}

Conclusion

3

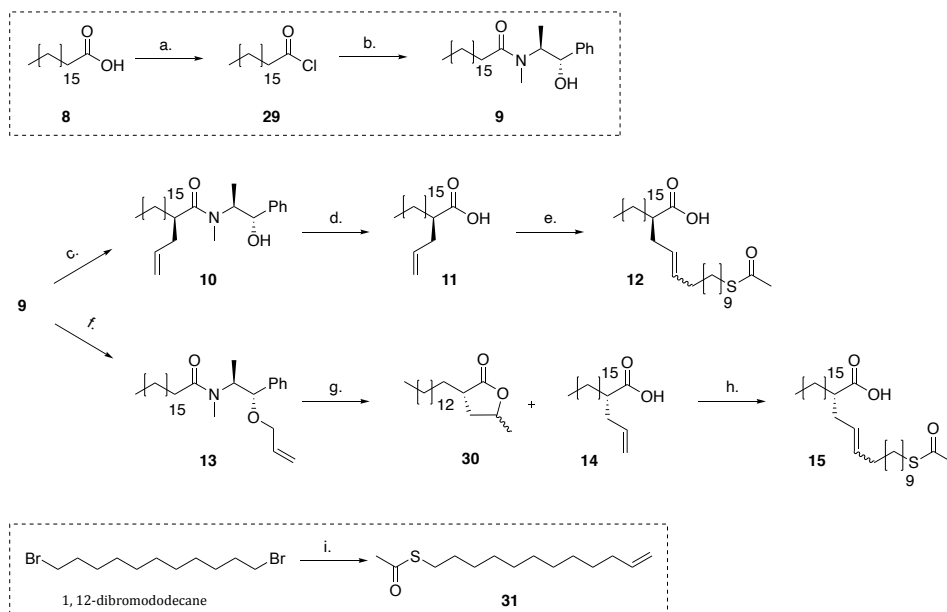
Subunit vaccines comprised of fully-synthetic antigens and adjuvants represent a promising strategy to overcome safety-related issues connected to BCG immunisation against *Mtb*, and co-delivery of antigen and adjuvant in single-molecule vaccines allows for the induction of strong immune-responses. The work reported in this Chapter describes the generation of a prototype synthetic *Mtb*-vaccine, formed by linking a glycolipid adjuvant and a *Mtb* derived peptide. For that purpose, four novel simplified analogues of immune adjuvants TDM and GMM were designed and successfully synthesized already equipped with a thiol ligation handle for conjugation to antigenic peptides. The TDM analogues, the most potent Mincle binders identified in the present study, were chosen for the generation of two self-adjuvanting peptides. This study provides a proof of principle that fully synthetic self-adjuvanting peptides containing a Mincle ligand are immunogenic and can induce protection against *Mtb* infection, in association with humoral (including IgG1 and IgG2b) but unexpectedly, no detectable cellular (CD4⁺ T cell) responses. The results from the murine experiments here described indicate the presence of antigen-specific IgG1 and IgG2b antibodies in the sera of mice immunised with self-adjuvanting peptide **6** and a reduced bacterial load in their spleen, which is indicative of a systemic and protective immune response induced by this construct. Further research is required to determine if the observed protection can be generalised to other antigens and whether this solely depends on humoral immune responses.

Materials and methods

General synthetic methods

General synthetic methods and description of analytical instrumentation are provided in the materials and methods section of Chapter 2.

Glucose monomycolate analogues



S1 Figure - Synthetic scheme for the generation of lipid moieties of glucose monomycolate analogues. a) oxalyl chloride, DMF, toluene, y: 99%, b) pseudoephedrine, Et₃N, DCM, y: 99%, c) DIPA, LiCl, allyl bromide, THF, y: 84%, d) H₂SO₄ (5N)/dioxane 1:1, y: 73%, e) S-(dodec-11-en-1-yl) ethanethioate, Grubbs 1st generation, DCM, reflux, y: 76%, f) NaH, allyl bromide, DCM, y: 86%, g) i. Tf₂O, 2-fluoropyridine, DCM, 0 °C, ii. H₂SO₄ (2.5 N)/dioxane 1:1, 100, y of **14**: 60%, h) S-(dodec-11-en-1-yl) ethanethioate, Grubbs 1st generation, DCM, reflux, y: 76%, i) i. Potassium tert-butoxide, THF/toluene 2:1, 5 °C, ii. Potassium thioacetate, DMF, 80 °C, y: 19%.

S-(dodec-11-en-1-yl) ethanethioate (31).

A solution of 1, 12-dibromododecane (39.4 g, 120 mmol, 1 eq) in THF/toluene 2:1 (80 ml) was cooled to 0 °C. Potassium tert-butoxide (20.2 g, 180 mmol, 1.5 eq) was added to the solution in portions of about 2 g over 30 minutes. After addition of the last portion of potassium tert-butoxide, the reaction mixture was stirred at 5 °C for 1 hour. The reaction was then quenched with water and HCl (1 M) and transferred to a separatory funnel. The water layer was extracted (3x) with toluene and the combined organic layers were washed (1x) with HCl (1 M) and (2x) with brine. The toluene layer was dried over MgSO₄, filtered and concentrated in vacuo. The crude from the elimination reaction was dissolved in DMF (800 ml) for the subsequent reaction. Potassium thioacetate (25 g, 219 mmol, 1.8 eq) was added to this solution and heated to 80 °C for 2 hours. The reaction was then quenched with water, diluted with Et₂O and transferred to a separatory funnel. The water layer was extracted (3x) with Et₂O, then the combined organic layers were washed (1x) with water and (1x) with brine. The organic layer was dried over MgSO₄, filtered and concentrated in vacuo. Compound **31** was obtained after silicagel chromatography (Pentane/Et₂O 95:5; DCM loading of crude) as a transparent oil (5.6 g, 23.1 mmol, 19% over two steps). ¹H NMR (300 MHz, CDCl₃) δ: 5.80 (ddt, *J* = 16.9, 10.2, 6.7 Hz, 1H, CH=), 5.07 – 4.80 (m, 2H, CH₂=), 2.86 (t, *J* = 7.3 Hz, 2H, CH₂-S), 2.31 (s, 3H, CH₃-thioacetyl), 2.10 – 1.94 (m, 2H, CH₂-allylic), 1.63 – 1.47 (m, 2H, CH₂-lipid), 1.44 – 1.12 (m, 16H, CH₂-lipid). ¹³C-APT NMR (75 MHz, CDCl₃) δ: 195.9 (S-C=O), 139.2 (CH=), 114.2 (CH₂=), 33.9 (CH₂-allylic), 30.6 (CH₃-thioacetyl), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.2 (CH₂-lipid), 29.1 (CH₂-lipid), 29.0 (CH₂-lipid), 28.9 (CH₂-lipid). HRMS [M+H]⁺: 243.17762 found, 243.17771 calculated.

Stearoyl chloride (29).

Stearic acid (17.71 g, 60 mmol, 1 eq) and DMF (0.6 ml, 7.8 mmol, 0.13 eq) were dissolved in toluene (600 ml) and cooled to 0 °C. Oxalyl chloride (21 ml, 240 mmol, 4 eq) was added dropwise to the solution via cannula over 15 minutes. The resulting solution was slowly allowed to heat up to room temperature. After stirring for 20 hours at RT, the solution was heated to reflux to complete the reaction. Solvents and volatiles were removed by rotatory evaporation under fume hood. Compound **29** was obtained as a white solid (18.02 g, 59.5 mmol, 99%) without any further purification. NMR analysis confirmed purity of the product, whose ¹H NMR and ¹³C NMR spectra were in agreement with published literature.⁴⁹

N-((1S,2S)-1-hydroxy-1-phenylpropan-2-yl)-N-methylstearamide (9).

Pseudoephedrine (3.3 g, 20 mmol, 1 eq) was dissolved in DCM (70 ml). Triethylamine (5.6 ml, 40 mmol) was added and this solution was cooled to 0 °C. A solution of compound **29** in DCM (30 ml) was added dropwise to the pseudoephedrine solution via cannula over 20 minutes. The reaction mixture was then heated up to room temperature and stirred overnight. The reaction mixture was quenched with a saturated solution of NH₄Cl (aq), diluted in EtOAc and transferred to a separatory funnel. The water layer was extracted (3x) with EtOAc and the combined organic layers were dried over MgSO₄, filtered and concentrated in vacuo. Compound **9** was obtained after silicagel chromatography (Pentane/EtOAc 5:1→1:1; DCM loading of crude; Et₃N neutralization of silica) as a white solid (8.57 g, 19.8 mmol, 99%). **Rotamer a:** ¹H NMR (400 MHz, CDCl₃) δ: 7.42 – 7.14 (m, 5H, H-arom), 4.73 – 4.51 (m, 2H, CH-Bn, OH), 4.51 – 4.34 (m, 1H, CH-N), 2.79 (s, 3H, CH₃-N), 2.33 – 2.17 (m, 2H, CH₂-C=O), 1.66 – 1.49 (m, 2H, CH₂-lipid), 1.36 – 1.16 (m, 28H, CH₂-lipid), 1.08 (d, *J* = 6.9 Hz, 3H, CH₃-lipid), 0.92

– 0.82 (m, 3H, CH₃-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 175.5 (C=O), 142.6 (C-arom), 128.3 (C-arom), 127.6 (C-arom), 126.4 (C-arom), 76.5 (CH-Bn), 58.4 (CH-N), 34.4 (CH₂-C=O), 32.9 (CH₃-N), 32.0 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.5 (CH₂-lipid), 29.5 (CH₂-lipid), 29.4 (CH₂-lipid), 25.1 (CH₂-lipid), 22.7 (CH₂-lipid), 14.5 (CH₃-lipid), 14.2 (CH₃-lipid). **Rotamer b:** ¹H NMR (400 MHz, CDCl₃) δ: 7.42 – 7.14 (m, 5H, H-arom), 4.73 – 4.51 (m, 1H, CH-Bn), 3.97 (dq, J = 8.7, 6.8 Hz, 1H, CH-N), 3.52 (s, 1H, OH), 2.88 (s, 3H, CH₃-N), 2.38 (t, J = 7.8 Hz, 2H, CH₂-C=O), 1.66 – 1.49 (m, 2H, CH₂-lipid), 1.36 – 1.16 (m, 28H, CH₂-lipid), 0.96 (d, J = 6.7 Hz, 3H, CH₃-lipid), 0.92 – 0.82 (m, 3H, CH₃-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 174.4 (C=O), 141.7 (C-arom), 128.6 (C-arom), 128.2 (C-arom), 127.0 (C-arom), 75.4 (CH-Bn), 58.4 (CH-N), 33.8 (CH₂-C=O), 32.0 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.5 (CH₂-lipid), 29.5 (CH₂-lipid), 29.5 (CH₂-lipid), 29.4 (CH₂-lipid), 26.8 (CH₃-N), 25.5 (CH₂-lipid), 22.7 (CH₂-lipid), 15.4 (CH₃-lipid), 14.2 (CH₃-lipid). HRMS [M+Na]⁺: 454.3652 found, 454.3655 calculated.

(R)-2-allyl-N-((1S,2S)-1-hydroxy-1-phenylpropan-2-yl)-N-methyloctadecanamide (10).

Diisopropylamine (1.39 ml, 9.9 mmol, 4.4 eq) and LiCl (1.14 g, 27 mmol, 12 eq) were suspended in dry THF (45 ml) and the suspension was cooled to –78 °C. A solution of *n*-BuLi (1.6 M in hexanes) (5.6 ml, 9 mmol, 4 eq) was added dropwise via syringe to the THF solution. The reaction mixture was quickly warmed to 0 °C, stirred at this temperature for 10 minutes, then cooled again to –78 °C. Compound **9** (1.94 g, 4.5 mmol, 2 eq) was co-evaporated (3x) with dry toluene, then dissolved in THF (10 ml) and slowly added to the freshly made LDA solution. The reaction mixture was stirred at –78 °C for 1 hour and 30 minutes before being heated up to 0 °C and stirred at this temperature for 15 minutes. The reaction mixture was then heated up further to RT for 5 minutes and cooled again at 0 °C for the addition of allyl bromide (195 μL, 2.25 mmol, 1 eq). After 2 hours, the reaction was quenched by the addition of a saturated solution of NH₄Cl (aq) (100 ml, 10 minutes stirring at 0 °C), diluted with EtOAc and transferred to a separatory funnel. The water layer was extracted (3x) with EtOAc and the combined organic layers were dried over MgSO₄, filtered and concentrated in vacuo. Compound **10** was obtained after silicagel chromatography (Pentane/EtOAc 8:1→4:1; DCM loading of crude; Et₃N neutralization of silica) as a white solid (0.90 g, 1.9 mmol, 84%). **Rotamer a:** ¹H NMR (400 MHz, CDCl₃) δ: 7.45 – 7.17 (m, 5H, H-arom), 5.64 (ddt, J = 17.2, 10.1, 7.0 Hz, 1H, CH=), 5.00 – 4.90 (m, 2H, CH₂=), 4.70 – 4.53 (m, 1H, CH-Bn), 4.41 (s, 1H, CH-N), 2.84 (bs, 3H, CH₃-N), 2.63 (tt, J = 8.4, 5.6 Hz, 1H, CH-C=O), 2.27 (ddt, J = 26.6, 13.6, 6.6 Hz, 1H, CH₂-allylic), 2.19 – 2.07 (m, 1H, CH₂-allylic), 1.70 – 1.52 (m, 1H, CH₂-lipid), 1.52 – 1.35 (m, 1H, CH₂-lipid), 1.34 – 1.17 (m, 28H, CH₂-lipid), 1.14 (d, J = 7.0 Hz, 3H, CH₃-lipid), 0.92 – 0.79 (m, 3H, CH₃-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 178.0 (C=O), 142.6 (C-arom), 136.1 (CH=), 128.4 (C-arom), 127.6 (C-arom), 126.4 (C-arom), 116.6 (CH₂=), 76.4 (CH-Bn), 58.4 (CH-N), 42.4 (CH-C=O), 37.2 (CH₂-allylic), 33.7 (CH₃-N), 32.7 (CH₂-lipid), 32.0 (CH₂-lipid), 29.9 (CH₂-lipid), 29.9 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 27.5 (CH₂-lipid), 27.1 (CH₂-lipid), 22.8 (CH₂-lipid), 14.7 (CH₃-lipid), 14.3 (CH₃-lipid). **Rotamer b:** ¹H NMR (400 MHz, CDCl₃) δ: 7.45 – 7.17 (m, 5H, H-arom), 5.95–5.71 (m, 1H, CH=), 5.19 – 5.00 (m, 2H, CH₂=), 4.70 – 4.53 (m, 1H, CH-Bn), 4.21 – 4.04 (m, 1H, CH-N), 2.91 (s, 3H, CH₃-N), 2.89 – 2.77 (m, 1H, CH-C=O), 2.53 – 2.42 (m, 1H, CH₂-allylic), 2.42 – 2.19 (m, 1H, CH₂-allylic), 1.70 – 1.52 (m, 1H, CH₂-lipid), 1.52 – 1.35 (m, 1H, CH₂-lipid), 1.34 – 1.17 (m, 28H, CH₂-lipid), 0.99 (d, J = 6.7 Hz, 3H, CH₃-lipid), 0.92 – 0.79 (m, 3H, CH₂-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 176.8 (C=O), 141.2 (C-arom), 137.0 (CH=), 128.8 (C-arom),

128.5 (C-arom), 127.1 (C-arom), 116.8 (CH₂=), 116.7 (CH₂=), 76.3 (CH-Bn), 75.5 (CH-Bn), 58.4 (CH-N), 41.5 (CH-C=O), 37.4 (CH₂-allylic), 32.9 (CH₂-lipid), 32.0 (CH₂-lipid), 29.9 (CH₂-lipid), 29.9 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 27.7 (CH₂-lipid), 27.1 (CH₃-N), 22.8 (CH₂-lipid), 15.7 (CH₃-lipid), 14.3 (CH₃-lipid). HRMS [M+Na]⁺: 494.3971 found, 494.3968 calculated.

(R)-2-allyl octadecanoic acid (11).

Compound **10** (192 mg, 0.41 mmol, 1 eq) was dissolved in dioxane (2 ml). A solution of H₂SO₄ in water (5 N, 2 ml) was added and the reaction mixture was stirred at reflux over the weekend. After cooling the reaction mixture to RT, water (40 ml) and DCM (20 ml) were added before transferring to a separatory funnel. The water layer was extracted (3x) with DCM. The combined organic layers were dried over MgSO₄, filtered and concentrated in vacuo. Compound **11** was obtained after silicagel chromatography (Pentane/EtOAc 8:1→1:1; Pentane/EtOAc 8:1 + a drop of DCM for loading of crude) as a white solid (103 mg, 0.3 mmol, 73%). ¹H NMR (400 MHz, CDCl₃) δ: 5.77 (ddt, J = 16.8, 10.1, 6.8 Hz, 1H, CH=), 5.19 – 4.93 (m, 2H, CH₂=), 2.51 – 2.32 (m, 2H, CH-C=O, CH₂-allylic), 2.32 – 2.18 (m, 1H, CH₂-allylic), 1.73 – 1.57 (m, 1H, CH₂-lipid), 1.57 – 1.45 (m, 1H, CH₂-lipid), 1.32 – 1.23 (m, 28H, CH₂-lipid), 0.93 – 0.83 (m, 3H, CH₃-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 182.6 (C=O), 135.4 (CH=), 117.1 (CH₂=), 45.4 (CH-C=O), 36.3 (CH₂-lipid), 32.1 (CH₂-lipid), 31.7 (CH₂-lipid), 29.9 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 27.3 (CH₂-lipid), 22.9 (CH₂-lipid), 14.3 (CH₃-lipid). HRMS [M+H]⁺: 325.3105 found, 325.3101 calculated.

(R)-2-(13-(acetylthio)tridec-2-en-1-yl)octadecanoic acid (12).

Compound **11** (227 mg, 0.7 mmol, 1 eq) was co-evaporated with toluene (2x) and dissolved in dry DCM (15 ml). Compound **31** (970 mg, 4 mmol, 5.7 eq) was also co-evaporated with toluene (2x) before being dissolved in DCM (5 ml) and added to the carboxylic acid solution. After addition of Grubbs 1st generation catalyst (41 mg, 0.05 mmol, 0.07 eq), the solution was heated to reflux overnight. Compound **12** was obtained after removal of volatiles followed by silicagel chromatography (Pentane/EtOAc 20:1→5:1; DCM for loading of crude) and size exclusion (LH-20, DCM/MeOH, 1/1, v/v) as a white solid (308 mg, 0.53 mmol, 76%). ¹H NMR (400 MHz, CDCl₃) δ: 5.47 (dt, J = 14.9, 6.6 Hz, 1H, CH=), 5.41 – 5.28 (m, 1H, CH=), 2.86 (t, J = 7.3 Hz, 2H, CH₂S), 2.51 – 2.09 (m, 6H, CH₃-thioacetyl, CH₂-allylic, CH-C=O), 1.99 (dq, J = 20.9, 7.6, 7.0 Hz, 2H, CH₂-allylic), 1.61 – 1.44 (m, 4H, CH₂-lipid), 1.36 – 1.19 (m, 42H, CH₂-lipid), 0.93 – 0.82 (m, 3H, CH₃-lipid). ¹³C NMR (101 MHz, CDCl₃) 196.2 (S-C=O), 182.5 (C=O), 133.5 (CH=), 126.5 (CH=), 45.7 (CH-C=O), 35.2 (CH₂-allylic), 32.7 (CH₂-allylic), 32.1 (CH₂-lipid), 31.6 (CH₂-lipid), 30.8 (CH₃-thioacetyl), 29.9 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.3 (CH₂-lipid), 29.3 (CH₂-lipid), 29.2 (CH₂-lipid), 29.0 (CH₂-lipid), 27.4 (CH₂-lipid), 22.8 (CH₂-lipid), 14.3 (CH₃-lipid). HRMS [M+Na]⁺: 561.4315 found, 561.4312 calculated.

N-((1S, 2S)-1-(allyloxy)-1-phenylpropan-2-yl)-N-methylstearamide (13).

Compound **9** (3.44 g, 7.98 mmol, 1 eq) was co-evaporated (2x) with toluene and dissolved in a mixture of dry DCM/DMF 1:1 (80 ml) and CH₃CN (1 ml). Allylbromide (1 ml, 11.97 mmol, 1.5 eq) was added and the reaction mixture was cooled to 0 °C. After the addition of NaH (478 mg,

11.97 mmol, 1.5 eq) the solution was slowly heated to RT and stirred overnight. The reaction was quenched with water on ice, diluted with EtOAc and transferred to a separatory funnel. The organic layer was washed (2x) with brine, dried over MgSO₄, filtered and concentrated in vacuo. Compound **13** was obtained as a white solid (3.22 g, 6.83 mmol, 86%) without the need for further purification. **Rotamer a:** ¹H NMR (400 MHz, CDCl₃) δ: 7.41 – 7.24 (m, 5H, H-arom), 5.81 (dddd, J = 16.8, 10.6, 6.1, 4.7 Hz, 1H, CH=), 5.25 – 5.06 (m, 2H, CH₂=), 4.20 (d, J = 8.3 Hz, 1H, CH-Bn), 4.09 – 3.99 (m, 1H, CH-N), 3.88 (dddd, J = 17.7, 13.0, 4.7, 1.6 Hz, 1H, CH₂-allylic), 3.65 (dddd, J = 14.5, 12.9, 6.1, 1.5 Hz, 1H, CH₂-allylic), 2.87 (s, 3H, CH₃-N), 2.34 (dd, J = 8.1, 7.6 Hz, 2H, CH₂-C=O), 1.71 – 1.48 (m, 2H, CH₂-lipid), 1.38 – 1.16 (m, 28H, CH₂-lipid), 0.99 (d, J = 6.6 Hz, 3H, CH₃-lipid), 0.88 (t, J = 6.8 Hz, 3H, CH₃-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 174.4 (C=O), 139.4 (C-arom), 134.5 (CH=), 128.8 (C-arom), 128.4 (C-arom), 127.6 (C-arom), 116.9 (CH₂=), 82.1 (CH-Bn), 69.5 (CH₂-allylic), 57.3 (CH-N), 33.6 (CH₂-C=O), 32.0 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.4 (CH₂-lipid), 29.3 (CH₂-lipid), 27.2 (CH₃-N), 25.5 (CH₂-lipid), 25.2 (CH₂-lipid), 25.0 (CH₂-lipid), 22.8 (CH₂-lipid), 14.3 (CH₃-lipid), 14.2 (CH₃-lipid). **Rotamer b:** ¹H NMR (400 MHz, CDCl₃) δ: 7.41 – 7.24 (m, 5H, H-arom), 5.81 (dddd, J = 16.8, 10.6, 6.1, 4.7 Hz, 1H, CH=), 5.25 – 5.06 (m, 2H, CH₂=), 5.03 – 4.83 (m, 1H, CH-N), 4.34 (bs, 1H, CH-Bn), 3.88 (dddd, J = 17.7, 13.0, 4.7, 1.6 Hz, 1H, CH₂-allylic), 3.65 (dddd, J = 14.5, 12.9, 6.1, 1.5 Hz, 1H, CH₂-allylic), 2.87 (s, 3H, CH₃-N), 2.25 (td, J = 7.3, 1.9 Hz, 1H, CH₂-C=O), 1.71 – 1.48 (m, 2H, CH₂-lipid), 1.38 – 1.16 (m, 28H, CH₂-lipid), 0.99 (d, J = 6.6 Hz, 3H, CH₃-lipid), 0.88 (t, J = 6.8 Hz, 3H, CH₃-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 173.8 (C=O), 139.6 (C-arom), 134.9 (CH=), 128.4 (C-arom), 128.0 (C-arom), 127.7 (C-arom), 116.5 (CH₂=), 82.3 (CH-Bn), 69.6 (CH₂-allylic), 57.3 (CH-N), 34.2 (CH₂-C=O), 32.0 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.4 (CH₂-lipid), 29.3 (CH₂-lipid), 27.2 (CH₃-N), 25.5 (CH₂-lipid), 25.2 (CH₂-lipid), 25.0 (CH₂-lipid), 22.8 (CH₂-lipid), 15.8 (CH₃-lipid), 14.2 (CH₃-lipid). HRMS [M+H]⁺: 472.4142 found, 472.4149 calculated.

(S)-2-allyloctadecanoic acid (**14**).

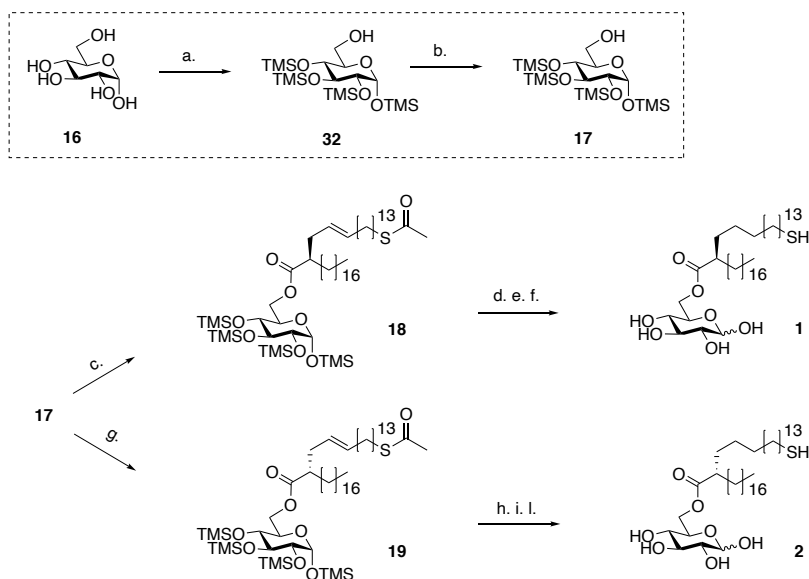
Compound **13** (377 mg, 0.8 mmol, 1 eq) was co-evaporated (3x) with toluene and dissolved in dry DCM (10 ml). 2-fluoropyridine (179 μL, 2.08 mmol, 2.6 eq) was added and the solution was cooled to 0 °C. Triflic anhydride (262 μL, 1.56 mmol, 1.95 eq) was added and the reaction mixture was stirred at 0 °C overnight. The day after the mixture was concentrated, dioxane (5 ml) and H₂SO₄ (2.5 N, 5 ml) were added and this reaction mixture was heated up to 100 °C and stirred overnight. The day after the reaction was diluted in water (50 ml) and DCM (25 ml) and transferred to a separatory funnel. The water layer was extracted (3x) with DCM and the combined organic layers were dried over MgSO₄, filtered and concentrated in vacuo. Compound **14** was obtained after silicagel chromatography (Petroleum ether/EtOAc 20:1→9:1; DCM for loading of crude) as a white solid (155 mg, 0.48 mmol, 60%). ¹H NMR (400 MHz, CDCl₃) δ: 11.80 (s, 1H, COOH), 5.76 (ddt, J = 16.8, 10.2, 6.8 Hz, 1H, CH=), 5.12 – 4.98 (m, 2H, CH₂=), 2.50 – 2.31 (m, 2H, CH-C=O, CH₂-allylic), 2.24 (dt, J = 13.4, 6.1 Hz, 1H, CH₂-allylic), 1.62 (ddd, J = 14.0, 8.6, 5.0 Hz, 1H, CH₂-lipid), 1.49 (ddd, J = 13.5, 8.3, 5.3 Hz, 1H, CH₂-lipid), 1.34 – 1.22 (m, 28H, CH₂-lipid), 0.88 (t, J = 6.8 Hz, 3H, CH₃-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 182.1 (C=O), 135.4 (CH=), 117.1 (CH₂=), 45.3 (CH-C=O), 36.3 (CH₂-lipid), 32.1 (CH₂-lipid), 31.7 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 27.3 (CH₂-lipid), 22.8 (CH₂-lipid), 14.3 (CH₃-lipid). HRMS [M+H]⁺: 325.31015 found, 325.31011 calculated.

(3S)-5-methyl-3-tetradecyldihydrofuran-2 (3H)-one (side product 30).

In addition to the desired product also the cyclized side product **30** (41 mg, 0.13mmol, 16%) was isolated and characterized. Diastereoisomer a: ^1H NMR (850 MHz, CDCl_3) δ : 4.85 – 4.76 (m, 1H, CH-O), 2.81 – 2.70 (m, 1H, CH-C=O), 2.26 – 2.19 (m, 1H, CH_2 -ring), 2.15 (ddd, J = 12.8, 9.0, 5.0 Hz, 1H, CH_2 -ring), 2.12 – 2.03 (m, 1H, CH_2 -lipid), 2.01 – 1.93 (m, 1H, CH_2 -lipid), 1.90 – 1.66 (m, 1H, CH_2 -lipid), 1.65 – 1.58 (m, 1H, CH_2 -lipid), 1.58 – 1.53 (m, 1H, CH_2 -lipid), 1.51 (d, J = 6.4 Hz, 3H, CH_3 -ring), 1.50 – 1.46 (m, 2H, CH_2 -lipid), 1.46 – 1.27 (m, 21H, CH_2 -lipid), 1.02 (t, J = 7.1 Hz, 3H, CH_3 -lipid). ^{13}C -APT NMR (214 MHz, CDCl_3) δ : 179.3 (C=O), 75.3 (CH-O), 41.7 (CH-C=O), 35.2 (CH_2 -ring), 32.1 (CH_2 -lipid), 30.8 (CH_2 -lipid), 30.5 (CH_2 -lipid), 29.9 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.7 (CH_2 -lipid), 29.7 (CH_2 -lipid), 29.6 (CH_2 -lipid), 29.5 (CH_2 -lipid), 29.5 (CH_2 -lipid), 27.5 (CH_2 -lipid), 27.5 (CH_2 -lipid), 22.8 (CH_2 -lipid), 21.2 (CH_3 -ring), 14.3 (CH_3 -lipid). Diastereoisomer b: ^1H NMR (850 MHz, CDCl_3) δ : 4.67 – 4.57 (m, 1H, CH-O), 2.81 – 2.70 (m, 1H, CH-C=O), 2.61 (ddd, J = 12.4, 8.5, 5.5 Hz, 1H, CH_2 -ring), 1.90 – 1.66 (m, 2H, CH_2 -lipid), 1.65 – 1.58 (m, 2H, CH_2 -ring, CH_2 -lipid), 1.58 – 1.53 (m, 4H, CH_3 -ring, CH_2 -lipid), 1.50 – 1.46 (m, 2H, CH_2 -lipid), 1.46 – 1.27 (m, 22H, CH_2 -lipid), 1.02 (t, J = 7.1 Hz, 3H, CH_3 -lipid). ^{13}C -APT NMR (214 MHz, CDCl_3) δ : 179.6 (C=O), 75.1 (CH-O), 39.5 (CH-C=O), 37.2 (CH_2 -ring), 32.1 (CH_2 -lipid), 30.8 (CH_2 -lipid), 30.5 (CH_2 -lipid), 29.9 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.7 (CH_2 -lipid), 29.7 (CH_2 -lipid), 29.6 (CH_2 -lipid), 29.6 (CH_2 -lipid), 29.5 (CH_2 -lipid), 29.5 (CH_2 -lipid), 27.5 (CH_2 -lipid), 27.5 (CH_2 -lipid), 22.8 (CH_2 -lipid), 21.4 (CH_3 -ring), 14.3 (CH_3 -lipid). HRMS $[\text{M}+\text{H}]^+$: 325.30999 found, 325.31011 calculated.

(S)-2-(13-(acetylthio)tridec-2-en-1-yl)octadecanoic acid (15).

Compound **14** (630 mg, 1.94 mmol, 1 eq) was co-evaporated with toluene (2x) and dissolved in dry DCM (30 ml). Compound **31** (1.88 g, 7.76 mmol, 4 eq) was also co-evaporated with toluene (2x) before being dissolved in DCM (9 ml) and added to the carboxylic acid solution. After addition of Grubbs 1st generation catalyst (80mg, 0.1 mmol, 0.05 eq), the solution was heated to reflux for 2 days. Compound **15** was obtained after removal of volatiles followed by silicagel chromatography (Pentane/EtOAc 95:5→5:1; DCM for loading of crude) and size exclusion (LH-20, DCM/MeOH, 1/1, v/v) as a white solid (855 mg, 1, 46 mmol, 76%). ^1H NMR (400 MHz, CDCl_3) δ : 5.58 – 5.22 (m, 2H, CH=), 2.86 (t, J = 7.3 Hz, 2H, CH_2S), 2.52 – 2.09 (m, 6H, CH_3 -thioacetyl, CH_2 -allylic, CH-C=O), 1.98 (dt, J = 21.0, 7.0 Hz, 2H, CH_2 -allylic), 1.71 – 1.42 (m, 4H, CH_2 -lipid), 1.25 (d, J = 2.4 Hz, 42H, CH_2 -lipid), 0.92 – 0.82 (m, 3H, CH_3 -lipid). ^{13}C -APT NMR (101 MHz, CDCl_3) δ : 196.2 (S-C=O), 182.5 (C=O), 133.4 (CH=), 132.4 (CH=), 126.6 (CH=), 126.0 (CH=), 45.9 (CH-C=O), 35.2 (CH_2 -allylic), 32.7 (CH_2 -allylic), 32.1 (CH_2 -lipid), 31.7 (CH_2 -lipid), 31.6 (CH_2 -lipid), 30.7 (CH_3 -thioacetyl), 29.8 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.7 (CH_2 -lipid), 29.7 (CH_2 -lipid), 29.6 (CH_2 -lipid), 29.6 (CH_2 -lipid), 29.6 (CH_2 -lipid), 29.6 (CH_2 -lipid), 29.5 (CH_2 -lipid), 29.5 (CH_2 -lipid), 29.3 (CH_2 -lipid), 29.3 (CH_2 -lipid), 29.2 (CH_2 -S), 29.0 (CH_2 -lipid), 27.5 (CH_2 -lipid), 27.4 (CH_2 -lipid), 27.4 (CH_2 -lipid), 22.8 (CH_2 -lipid), 14.3 (CH_3 -lipid). HRMS $[\text{M}+\text{Na}]^+$: 561.4315 found, 561.4312 calculated.



S2 Figure - Synthetic scheme for the generation of glucose monomycolate analogues. a) TMSCl, HMDS, pyridine, y: quant. b) NH_4OAc , DCM/MeOH 1:1, y: 73%, c) lipid, EDCI, DMAP, y: 52%, d) 2-nitrobenzenesulfonyl hydrazide, Et_3N , DCM, e) amberlite H^+ , DCM/MeOH 1:1, f) TCEP.HCl, PBS, MeOH/ CH_3CN 1:1, y: 62% over 3 steps, g) lipid, EDCI, DMAP, y: 50%, h) 2-nitrobenzenesulfonyl hydrazide, Et_3N , DCM, i) amberlite H^+ , DCM/MeOH 1:1, l) TCEP.HCl, PBS, MeOH/ CH_3CN 1:1, y: 73% over three steps.

1, 2, 3, 4, 6-penta-trimethylsilyl- α -glucopyranoside (32).

α -D-glucose (1.8 g, 10 mmol, 1 eq) was dissolved in pyridine (100 ml). HMDS (18 ml, 86 mmol, 8.6 eq) and TMSCl (8.9 ml, 70 mmol, 7 eq) were added to this solution and heated up to 75 °C for 1 hour. The reaction mixture was allowed to cool to RT and ice-water (100 ml) was added to it. The mixture was then diluted with pentane and transferred to a separatory funnel. The water layer was extracted (3x) with pentane, then the combined organic layers were washed (3x) with water, dried over MgSO_4 , filtered and concentrated in vacuo. Compound **32** was obtained as a white solid (5.5 g, 10 mmol, 100%) without any further purification. NMR analysis confirmed purity of the product, whose ^1H NMR and ^{13}C NMR spectra were in agreement with published literature.⁵⁰

1, 2, 3, 4-tetra-trimethylsilyl-6-hydroxy- α -glucopyranoside (17).

Compound **32** (5.5 g, 10 mmol, 1 eq) was dissolved in a mixture of DCM/MeOH 1:1 (83 ml) and NH_4OAc (1.54 g, 20 mmol, 2 eq) was added. When TLC showed complete consumption of the starting material, the volatiles were removed by evaporation, the crude was dissolved in pentane and the mixture transferred to a separatory funnel. The organic layer was washed

(3x) with water, (1x) with brine, dried over MgSO_4 , filtered and concentrated in vacuo. Compound **17** was obtained after silicagel chromatography (Pentane/EtOAc 15:1; DCM for loading of crude) as a white solid (3.43 g, 7.31 mmol, 73%). ^1H NMR (400 MHz, CDCl_3) δ : 5.00 (d, J = 3.0 Hz, 1H, H-1), 3.87 – 3.59 (m, 4H, H-3, H-5, H-6), 3.45 (dd, J = 9.5, 8.6 Hz, 1H, H-4), 3.34 (dd, J = 9.1, 3.0 Hz, 1H, H-2), 1.76 (t, J = 6.1 Hz, 1H, -OH), 0.20 – 0.12 (m, 36H, CH_2 -TMS). ^{13}C -APT NMR (101 MHz, CDCl_3) δ : 94.0 (C-1), 74.1 (C-2), 73.6 (C-3), 72.0 (C-5), 71.8 (C-4), 61.9 (C-6), 1.2 (CH_3 -TMS), 0.9 (CH_3 -TMS), 0.4 (CH_3 -TMS), 0.2 (CH_3 -TMS). HRMS $[\text{M}+\text{Na}]^+$: 491.21040 found, 491.21072 calculated.

6-((R)-2-(13-(acetylthio)tridec-2-en-1-yl)octadecanoyl)-1, 2, 3, 4-tetra-trimethylsilyl- α -glucopyranoside (18).

Compound **17** (260 mg, 0.55 mmol, 1.26 eq) and compound **12** (260 mg, 0.44 mmol, 1 eq) were co-evaporated (3x) with toluene before being dissolved in dry toluene (8.8 ml). After addition of DMAP (54 mg, 0.44 mmol, 1 eq), the solution was cooled to 0 °C. EDCI (169 mg, 0.88 mmol, 2 eq) was added and the reaction mixture was heated up to 70 °C. After 4 hours the reaction was cooled to RT, diluted with water and EtOAc and transferred to a separatory funnel. The organic layer was washed (2x) with brine and the combined water layers were extracted (1x) with EtOAc. The combined organic layers were washed (1x) with brine, dried over MgSO_4 , filtered and concentrated in vacuo. Compound **18** was obtained after silicagel chromatography (Pentane/EtOAc 100:3→9:1; DCM for loading of crude) as a white solid (228 mg, 0.22 mmol, 50%). ^1H NMR (400 MHz, CDCl_3) δ : 5.47 – 5.27 (m, 2H, CH=), 4.98 (d, J = 3.0 Hz, 1H, H-1), 4.59 – 4.46 (m, 1H, H-6a), 3.97 – 3.85 (m, 2H, H-6b, H-4), 3.79 (pt, J = 8.8 Hz, 1H, H-3), 3.40 (td, J = 8.9, 2.6 Hz, 1H, H-5), 3.33 (dd, J = 9.1, 3.1 Hz, 1H, H-2), 2.86 (t, J = 7.4 Hz, 2H, CH_2 -S), 2.44 – 2.30 (m, 5H, CH_3 -thioacetyl, CH_2 -allylic, CH-C=O), 2.29 – 2.08 (m, 1H, CH_2 -allylic), 2.08 – 1.89 (m, 2H, CH_2 -allylic), 1.64 – 1.41 (m, 5H, CH_2 -lipid), 1.38 – 1.16 (m, 49H, CH_2 -lipid), 0.90 – 0.86 (m, 3H, CH_3 -lipid), 0.18 – 0.12 (m, 36H, CH_3 -TMS). ^{13}C -APT NMR (101 MHz, CDCl_3) δ : 196.2 (S-C=O), 175.9 (a C=O), 175.8 (b C=O), 133.1 (a CH=), 132.2 (b CH=), 126.9 (a CH=), 126.4 (b CH=), 94.0 (C-1), 74.2 (C-2), 74.2 (C-3), 73.9 (a C-5), 73.9 (b C-5), 72.8 (C-4), 70.3 (a C-6), 70.2 (b C-6), 63.5 (a CH-C=O), 63.4 (b CH-C=O), 46.0 (a CH_2 -allylic), 45.8 (b CH_2 -allylic), 35.3 (CH_2 -allylic), 32.8 (CH_2 -lipid), 32.1 (CH_2 -lipid), 31.9 (CH_2 -lipid), 31.9 (CH_2 -lipid), 30.8 (CH_3 -thioacetyl), 29.9 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.8 (CH_2 -lipid), 29.7 (CH_2 -lipid), 29.6 (CH_2 -lipid), 29.5 (CH_2 -lipid), 29.5 (CH_2 -lipid), 29.3 (CH_2 -lipid), 29.3 (CH_2 -lipid), 29.3 (CH_2 -lipid), 29.0 (CH_2 -lipid), 27.5 (CH_2 -lipid), 27.4 (CH_2 -lipid), 27.4 (CH_2 -lipid), 22.8 (CH_2 -lipid), 14.3 (CH_3 -lipid), 1.4 (CH_3 -TMS), 1.1 (CH_3 -TMS), 0.6 (CH_3 -TMS), 0.3 (CH_3 -TMS), 0.2 (CH_3 -TMS). HRMS $[\text{M}+\text{Na}]^+$: 1011.6424 found, 1011.6421 calculated.

6-((R)-2-(13-(mercapto)tridec-2-en-1-yl)octadecanoyl)- α -glucopyranoside (1).

Compound **18** (52 mg, 0.05 mmol, 1 eq) was co-evaporated (2x) with toluene and dissolved in dry DCM (0.25 ml). To this solution, Et_3N (0.25 ml) and 2-nitrobenzenesulfonylhydrazide (22 mg, 0.1 mmol, 2 eq) were added and the reaction was stirred overnight. The morning after TLC showed that the reaction was not complete and therefore an extra portion of 2-nitrobenzenesulfonylhydrazide (22 mg, 0.1 mmol, 2 eq) was added and the reaction was stirred for another day. After that the reaction mixture was diluted with EtOAc and brine and transferred to a separatory funnel. The organic layer was washed (2x) with brine, dried over MgSO_4 , filtered and concentrated in vacuo. This reaction intermediate was then dissolved in a mixture of DCM/MeOH 1:1 (9 ml) and a spatula of amberlite H^+ was added. The TMS-removal

reaction was complete after 30 minutes as verified by TLC. Amberlite H⁺ was removed from the solution by means of filtration and the volatiles were removed in vacuo. The crude was then dissolved in a mixture of MeOH/CH₃CN 1:1 (7 ml) and a solution of TCEP-HCl (143 mg, 0.5 mmol, 10 eq) in PBS (0.7 ml) was added. The reaction mixture was stirred for 15 minutes, then the volatiles were removed in vacuo. Compound **1** was obtained after silicagel chromatography (CHCl₃/MeOH 14:1; eluent used for loading of crude; N₂ flow was used for purification) as a white solid (24 mg, 0.036 mmol, 73%). **α-anomer**: ¹H NMR (600 MHz, MeOD/CDCl₃ 1:1) δ: 5.03 (d, J = 3.7 Hz, 1H, H-1), 4.40 – 4.36 (m, 1H, H-6a), 4.14 – 4.05 (m, 1H, H-6b), 3.88 (ddd, J = 10.1, 5.0, 2.2 Hz, 1H, H-5), 3.60 (t, J = 9.3 Hz, 1H, H-3), 3.32 – 3.27 (m, 1H, H-2), 3.27 – 3.19 (m, 1H, H-4), 2.46 – 2.38 (m, 2H, CH₂-S), 2.35 – 2.22 (m, 1H, CH-C=O), 1.68 – 1.45 (m, 4H, CH₂-lipid), 1.45 – 1.26 (m, 5H, CH₂-lipid), 1.26 – 1.13 (m, 43H, CH₂-lipid), 0.80 (t, J = 7.0 Hz, 3H, CH₃-lipid). **β-anomer**: ¹H NMR (600 MHz, MeOD/CDCl₃ 1:1) δ: 4.41 (d, J = 7.8 Hz, 1H, H-1), 4.37 – 4.33 (m, 1H, H-6a), 4.14 – 4.05 (m, 1H, H-6b), 3.40 (ddd, J = 9.8, 6.4, 2.0 Hz, 1H, H-5), 3.32 – 3.27 (m, 1H, H-3), 3.27 – 3.19 (m, 1H, H-4), 3.09 (dd, J = 9.3, 7.8 Hz, 1H, H-2), 2.46 – 2.38 (m, 2H, CH₂-S), 2.35 – 2.22 (m, 1H, CH-C=O), 1.68 – 1.45 (m, 4H, CH₂-lipid), 1.45 – 1.26 (m, 5H, CH₂-lipid), 1.26 – 1.13 (m, 43H, CH₂-lipid), 0.80 (t, J = 7.0 Hz, 3H, CH₃-lipid). **α-anomer**: ¹³C-APT NMR (151 MHz, MeOD/CDCl₃ 1:1) δ: 176.8 (C=O), 92.0 (C-1), 76.1 (C-2), 73.0 (C-3), 69.9 (C-4), 68.9 (C-5), 62.7 (C-6), 45.2 (CH-C=O), 33.5 (CH₂-lipid), 33.4 (CH₂-lipid), 31.7 (CH₂-lipid), 31.7 (CH₂-lipid), 31.6 (CH₂-lipid), 31.6 (CH₂-lipid), 31.5 (CH₂-lipid), 31.3 (CH₂-lipid), 29.1 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 28.9 (CH₂-lipid), 28.9 (CH₂-lipid), 28.9 (CH₂-lipid), 28.9 (CH₂-lipid), 28.8 (CH₂-lipid), 28.8 (CH₂-lipid), 28.8 (CH₂-lipid), 28.8 (CH₂-lipid), 28.7 (CH₂-lipid), 28.5 (CH₂-lipid), 27.7 (CH₂-lipid), 26.7 (CH₂-lipid), 26.7 (CH₂-lipid), 26.7 (CH₂-lipid), 26.7 (CH₂-lipid), 23.5 (CH₂-S), 22.0 (CH₂-lipid), 13.1 (CH₃-lipid). **β-anomer**: ¹³C-APT NMR (151 MHz, MeOD/CDCl₃ 1:1) δ: 176.8 (C=O), 96.2 (C-1), 74.2 (C-2), 73.6 (C-5), 71.9 (C-3), 69.9 (C-4), 63.2 (C-6), 45.1 (CH-C=O), 33.5 (CH₂-lipid), 33.4 (CH₂-lipid), 31.7 (CH₂-lipid), 31.7 (CH₂-lipid), 31.612 (CH₂-lipid), 31.6 (CH₂-lipid), 31.5 (CH₂-lipid), 31.3 (CH₂-lipid), 29.1 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 28.9 (CH₂-lipid), 28.9 (CH₂-lipid), 28.9 (CH₂-lipid), 28.8 (CH₂-lipid), 28.8 (CH₂-lipid), 28.8 (CH₂-lipid), 28.8 (CH₂-lipid), 28.7 (CH₂-lipid), 28.5 (CH₂-lipid), 27.7 (CH₂-lipid), 26.7 (CH₂-lipid), 26.7 (CH₂-lipid), 26.7 (CH₂-lipid), 26.7 (CH₂-lipid), 23.5 (CH₂-S), 22.0, 13.1 (CH₃-lipid). HRMS [M+Na]⁺: 683.4882 found, 683.4891 calculated.

6-((S)-2-(13-(acetylthio)tridec-2-en-1-yl)octadecanoyl)-1, 2, 3, 4-tetra-trimethylsilyl-α-glucopyranoside (19).

Compound **17** (105 mg, 0.23 mmol, 1 eq) and compound **15** (157 mg, 0.27 mmol, 1.2 eq) were co-evaporated (3x) with toluene before being dissolved in dry toluene (4.5 ml). After addition of DMAP (28 mg, 0.225 mmol, 1 eq), the solution was cooled to 0 °C. EDCI (86 mg, 0.45 mmol, 2 eq) was added and the reaction mixture was heated up to 70 °C. After 1 hour another portion of EDCI (43 mg, 0.23 mmol, 1 eq) was added to the reaction mixture and after 3 hours the reaction was cooled to RT, diluted with water and EtOAc and transferred to a separatory funnel. The organic layer was washed (2x) with brine and the combined organic layers were dried over MgSO₄, filtered and concentrated in vacuo. Compound **19** was obtained after silicagel chromatography (Pentane/EtOAc 100:3→9:1; DCM for loading of crude) as a white solid (122 mg, 0.12 mmol, 52%). ¹H NMR (400 MHz, CDCl₃) δ: 5.49 – 5.38 (m, 1H, CH=), 5.37 – 5.25 (m, 1H, CH=), 4.99 (d, J = 3.1 Hz, 1H, H-1), 4.55 – 4.40 (m, 1H, H-6a), 3.99 – 3.84 (m, 2H, H-6b, H-4), 3.79 (pt, J = 8.9 Hz, 1H, H-3), 3.44 – 3.36 (m, 1H, H-5), 3.36 – 3.30 (m, 1H, H-2), 2.86

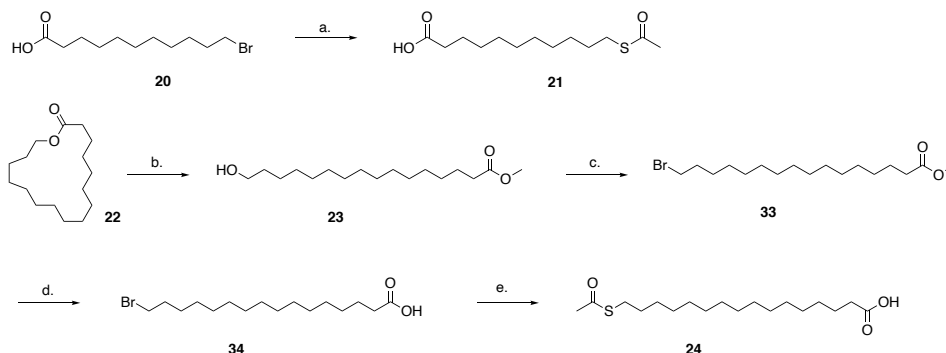
(t, J = 7.4 Hz, 2H, CH₂-S), 2.46 – 2.29 (m, 5H, CH₃-thioacetyl, CH₂-allylic, CH-C=O), 2.28 – 2.12 (m, 1H, CH₂-allylic), 2.07 – 1.90 (m, 2H, CH₂-allylic), 1.76 – 1.51 (m, 5H, CH₂-lipid), 1.49 – 1.14 (m, 49H, CH₂-lipid), 0.91 – 0.85 (m, 3H, CH₃-lipid), 0.18 – 0.13 (m, 36H, CH₃-TMS). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 196.2 (S-C=O), 175.9 (a C=O), 175.8 (b C=O), 133.1 (a CH=), 132.2 (b CH=), 126.8 (a CH=), 126.3 (b CH=), 94.0 (C-1), 74.2 (C-2), 74.0 (C-3), 73.9 (C-5), 72.8 (C-4), 70.3 (a C-6), 70.2 (b C-6), 63.5 (CH-C=O), 46.0 (a CH₂-allylic), 45.9 (b CH₂-allylic), 35.5 (CH₂-allylic), 32.8 (CH₂-lipid), 32.1 (CH₂-lipid), 31.6 (CH₂-lipid), 31.5 (CH₂-lipid), 30.8 (CH₃-thioacetyl), 30.1 (CH₂-lipid), 29.9 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.3 (CH₂-lipid), 29.3 (CH₂-lipid), 29.3 (CH₂-lipid), 29.0 (CH₂-lipid), 27.6 (CH₂-lipid), 27.5 (CH₂-lipid), 27.4 (CH₂-lipid), 22.8 (CH₂-lipid), 14.3 (CH₃-lipid), 1.4 (CH₃-TMS), 1.1 (CH₃-TMS), 0.6 (CH₃-TMS), 0.6 (CH₃-TMS), 0.3 (CH₃-TMS). HRMS [M+Na]⁺: 1011.64176 found, 1011.64212 calculated.

6-((S)-2-(13-(mercapto)tridec-2-en-1-yl)octadecanoyl)-α-glucopyranoside (2).

Compound **19** (100 mg, 0.1 mmol, 1 eq) was co-evaporated (2x) with toluene and dissolved in dry DCM (0.5 ml). To this solution, Et₃N (0.5 ml) and 2-nitrobenzenesulfonylhydrazide (84 mg, 0.39 mmol, 4 eq) were added and the reaction was stirred overnight. The morning after TLC showed that the reaction was not complete and therefore an extra portion of 2-nitrobenzenesulfonylhydrazide (22 mg, 0.1 mmol, 1 eq) was added and the reaction was stirred for 4 hours. After that the reaction mixture was diluted with EtOAc and brine and transferred to a separatory funnel. The organic layer was washed (2x) with brine, dried over MgSO₄, filtered and concentrated in vacuo. This reaction intermediate was then dissolved in a mixture of DCM/MeOH 1:1 (18 ml) and a spatula of amberlite H⁺ was added. The TMS-removal reaction was complete after 30 minutes as verified by TLC. Amberlite H⁺ was removed from the solution by means of filtration and the volatiles were removed in vacuo. A mixture of reduced and oxidised forms of the desired product was isolated after silicagel chromatography (CHCl₃/MeOH 14:1; eluent used for loading of crude; N₂ flow was used for purification) as a white solid (55 mg, 0.083 mmol, 85%). The product was then dissolved in a mixture of MeOH/CH₃CN 1:1 (12 ml) and a solution of TCEP·HCl (238 mg, 0.83 mmol, 10 eq) in PBS (1.2 ml) was added. The reaction was complete after 15 minutes and the volatiles were removed in vacuo. Compound **2** was obtained after silicagel chromatography (CHCl₃/MeOH 14:1; eluent used for loading of crude; N₂ flow was used for purification) as a white solid (41 mg, 0.062 mmol, 62% over 3 steps). α-anomer: ¹H NMR (400 MHz, MeOD/CDCl₃ 1:1) δ: 5.14 (d, J = 3.7 Hz, 1H, H-1), 4.50 – 4.45 (m, 1H, H-6a), 4.26 – 4.20 (m, 1H, H-6b), 3.98 (ddd, J = 10.0, 5.0, 2.2 Hz, 1H, H-5), 3.70 (pt, J = 9.3 Hz, 1H, H-3), 3.44 – 3.36 (m, 2H, H-2, H-4), 2.75 – 2.63 (m, 2H, CH₂-S), 2.43 – 2.34 (m, 1H, CH-C=O), 1.80 – 1.54 (m, 4H, CH₂-lipid), 1.54 – 1.36 (m, 4H, CH₂-lipid), 1.36 – 1.06 (m, 48H, CH₂-lipid), 0.89 (t, J = 6.7 Hz, 3H, CH₃-lipid). β-anomer: ¹H NMR (400 MHz, MeOD/CDCl₃ 1:1) δ: 4.51 (d, J = 7.8 Hz, 1H, H-1), 4.44 (d, J = 2.1 Hz, 1H, H-6a), 4.20 – 4.15 (m, 1H, H-6b), 3.57 – 3.47 (m, 1H, H-5), 3.44 – 3.36 (m, 2H, H-3, H-4), 3.24 – 3.15 (m, 1H, H-2), 2.75 – 2.63 (m, 2H, CH₂-S), 2.43 – 2.34 (m, 1H, CH-C=O), 1.80 – 1.54 (m, 4H, CH₂-lipid), 1.54 – 1.36 (m, 4H, CH₂-lipid), 1.36 – 1.06 (m, 48H, CH₂-lipid), 0.89 (t, J = 6.7 Hz, 3H, CH₃-lipid). α-anomer: ¹³C-APT NMR (101 MHz, MeOD/CDCl₃ 1:1) δ: 176.9 (C=O), 92.1 (C-1), 76.1 (C-2), 73.1 (C-3), 70.0 (C-4), 69.0 (C-5), 62.8 (C-6), 45.2 (CH-C=O), 33.5 (CH₂-lipid), 31.7 (CH₂-lipid), 31.4 (CH₂-lipid), 29.1 (CH₂-lipid), 29.1 (CH₂-lipid), 29.1 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 28.9 (CH₂-lipid), 28.8 (CH₂-lipid), 28.5 (CH₂-lipid), 27.8 (CH₂-lipid), 26.8 (CH₂-lipid), 23.7 (CH₂-S), 22.1 (CH₂-lipid), 13.2 (CH₃-lipid). β-anomer: ¹³C-APT NMR (101 MHz, MeOD/CDCl₃ 1:1) δ: 176.9 (C=O), 96.3 (C-1), 74.3 (C-2), 73.7 (C-5), 71.9 (C-3), 70.0 (C-4), 63.3

(C-6), 45.3 (CH-C=O), 33.5 (CH₂-lipid), 31.7 (CH₂-lipid), 31.4 (CH₂-lipid), 29.1 (CH₂-lipid), 29.1 (CH₂-lipid), 29.1 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 29.0 (CH₂-lipid), 28.914 (CH₂-lipid), 28.8 (CH₂-lipid), 28.5 (CH₂-lipid), 27.8 (CH₂-lipid), 26.8 (CH₂-lipid), 23.7 (CH₂-S), 22.1 (CH₂-lipid), 13.2 (CH₃-lipid). HRMS [M+Na]⁺: 683.48900 found, 683.48910 calculated.

Trehalose dimycolate analogues



S3 Figure - Synthetic scheme for the generation of lipid moieties of trehalose dimycolate analogues.

a) potassium thioacetate, DMF, y: 91%, b) NaOMe, MeOH, y: 85%, c) PPh₃, NaHCO₃, NBS, y: 88%, d) LiOH, THF, y: 91%, e) potassium thioacetate, DMF, y: 63%.

11-(acetylthio)undecanoic acid (21).

1-Bromoundecanoic acid (398 mg, 1.5 mmol, 1 eq) was dissolved in DMF (10ml) and cooled to 0 °C. Potassium ethanethioate (308 mg, 2.7 mmol, 1.8 eq) was added to the solution and the mixture was stirred at RT for 1 h and 30 minutes. At this point the reaction mixture was diluted with DCM and the organic layer was washed (3x) with water, dried over MgSO₄, filtered and concentrated in vacuo. Compound **21** was obtained as a brown solid (352 mg, 1.35 mmol, 91%) without any further purification. ¹H NMR (400 MHz, CDCl₃) δ: 2.86 (t, J = 7.3 Hz, 2H, CH₂-S), 2.42–2.24 (m, 5H, CH₂-C=O, CH₃-thioacetyl), 1.70–1.51 (m, 4H, CH₂-lipid), 1.36–1.21 (m, 12H, CH₂-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 196.3 (S-C=O), 179.7 (C=O), 34.1 (CH₂-C=O), 30.8 (CH₃-thioacetyl), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.4 (CH₂-lipid), 29.3 (CH₂-lipid), 29.3 (CH₂-lipid), 29.2 (CH₂-lipid), 29.1 (CH₂-S), 28.9 (CH₂-lipid), 28.9 (CH₂-lipid), 24.8 (CH₂-lipid). HRMS [M+Na]⁺: 283.1339 found, 283.1338 calculated.

methyl 16-hydroxyhexadecanoate (23).

Cyclohexadecanolide (3.61 g, 15 mmol, 1 eq) was co-evaporated with toluene and dissolved in dry MeOH (88 ml). After addition of NaOMe (4.05 g, 75 mmol, 5eq) the reaction mixture was heated up to reflux for 1 hour and 30 minutes. The reaction was quenched with a 1 M solution of HCl until pH 11, then brine and EtOAc were added and the mixture was transferred to a separatory funnel. The water layer was extracted (2x) with EtOAc. Compound **23** was obtained as a white solid (3.68 g, 12.8 mmol, 85%) without any further purification. ¹H NMR (400 MHz, CDCl₃) δ: 3.73–3.58 (m, 5H, CH₃-O, CH₂-OH), 2.30 (t, J = 7.6 Hz, 2H, CH₂-C=O), 1.65–1.52 (m,

4H, CH₂-lipid), 1.33 – 1.23 (m, 20H, CH₂-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 174.5 (C=O), 63.2 (CH₂-OH), 51.6 (CH₃-O), 34.3 (CH₂-C=O), 32.9 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.4 (CH₂-lipid), 29.3 (CH₂-lipid), 25.9 (CH₂-lipid), 25.1 (CH₂-lipid). HRMS [M+H]⁺: 287.2572 found, 287.2586 calculated.

methyl 16-bromohexadecanoate (33).

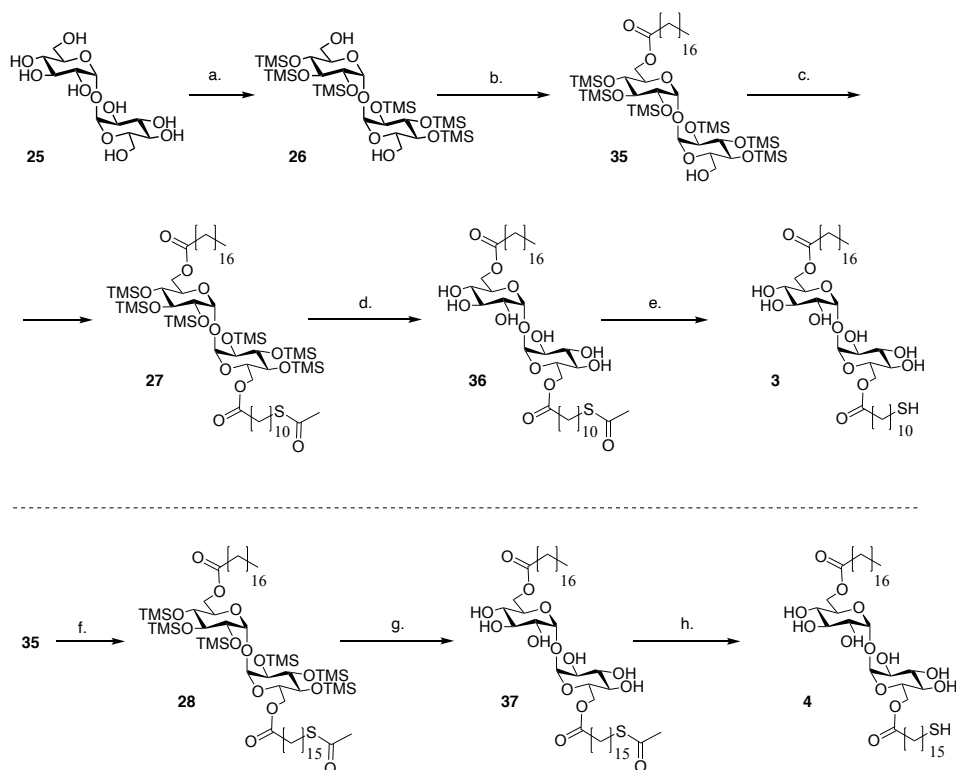
Compound **23** (3.44 g, 12 mmol, 1 eq) was co-evaporated with toluene and dissolved in DCM (60 ml). Triphenylphosphine (3.93 g, 15.6 mmol, 1.3 eq) and NaHCO₃ (90 mg, 1.08 mmol, 0.09 eq) were added and the solution was cooled to 0 °C. Subsequently, NBS (2.55 g, 21.6 mmol, 1.8 eq) was added in portions over 20 minutes and the reaction mixture was stirred for 2 hours at RT. Compound **33** was obtained after silicagel chromatography (Pentane/EtOAc 9:1→8:2; DCM loading of crude) as a white solid (3.69 g, 10.56 mmol, 88%). ¹H NMR (400 MHz, CDCl₃) δ: 3.67 (s, 3H, CH₃-O), 3.41 (t, J = 6.9 Hz, 2H, CH₂-Br), 2.30 (t, J = 7.5 Hz, 2H, CH₂-C=O), 1.90 – 1.81 (m, 2H, CH₂-lipid), 1.65 – 1.57 (m, 2H, CH₂-lipid), 1.46 – 1.38 (m, 2H, CH₂-lipid), 1.31 – 1.22 (m, 20H, CH₂-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 174.5 (C=O), 51.6 (CH₃-O), 34.2 (CH₂-Br), 34.2 (CH₂-C=O), 33.0 (CH₂-lipid), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.4 (CH₂-lipid), 29.3 (CH₂-lipid), 28.9 (CH₂-lipid), 28.3 (CH₂-lipid), 25.1 (CH₂-lipid). HRMS [M+Na]⁺: 371.1563 found, 371.1556 calculated.

16-bromohexadecanoic acid (34).

Compound **33** (3.49 g, 10 mmol, 1 eq) was dissolved in THF (100 ml). Subsequently a 1 M solution of LiOH (25 ml) was added and the reaction mixture was stirred overnight at RT. The reaction was quenched with a 1 M HCl solution until pH 2, after which this mixture was diluted with DCM and water and transferred to a separatory funnel. The water layer was extracted (3x) with DCM and the combined organic layers were dried over MgSO₄, filtered and concentrated in vacuo. Compound **34** was obtained as a brown solid (352 mg, 1.35 mmol, 91%) without any further purification. ¹H NMR (400 MHz, CDCl₃) δ: 3.41 (t, J = 6.9 Hz, 2H, CH₂-Br), 2.35 (t, J = 7.5 Hz, 2H, CH₂-C=O), 1.85 (dt, J = 14.5, 6.9 Hz, 2H, CH₂-lipid), 1.68 – 1.58 (m, 2H, CH₂-lipid), 1.46 – 1.39 (m, 2H, CH₂-lipid), 1.33 – 1.23 (m, 20H, CH₂-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 180.4 (C=O), 34.2 (CH₂-Br), 34.2 (CH₂-C=O), 33.0 (CH₂-lipid), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.4 (CH₂-lipid), 29.2 (CH₂-lipid), 28.9 (CH₂-lipid), 28.3 (CH₂-lipid), 24.8 (CH₂-lipid). HRMS [M+H]⁺: 319.1283 found, 319.1278 calculated.

16-(acetylthio)hexadecanoic acid (24).

Compound **34** (36 mg, 0.11 mmol, 1 eq) was dissolved in DMF (0.73 ml) and cooled to 0 °C. Potassium ethanethioate (22 mg, 0.19 mmol, 1.8 eq) was added to the solution and the mixture was stirred at RT for 4 hours. The reaction mixture was then diluted with DCM, the organic layer was washed (5x) with water, dried over MgSO₄, filtered and concentrated in vacuo. Compound **24** was obtained as a brown solid (22.8 mg, 0.07 mmol, 63%) without any further purification. ¹H NMR (400 MHz, CDCl₃) δ: 2.86 (t, J = 7.3 Hz, 2H, CH₂-S), 2.36 (t, J = 3.8 Hz, 2H, CH₂-C=O), 2.32 (s, 3H, CH₃-thioacetyl), 1.72 – 1.50 (m, 4H, CH₂-lipid), 1.35 – 1.23 (m, 20H, CH₂-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ: 196.4 (S-C=O), 179.4 (C=O), 34.1 (CH₂-C=O), 30.8 (CH₃-thioacetyl), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.4 (CH₂-lipid), 29.3 (CH₂-lipid), 29.3 (CH₂-S), 29.2 (CH₂-lipid), 29.0 (CH₂-lipid), 24.8 (CH₂-lipid). HRMS [M+H]⁺: 331.2310 found, 331.2307 calculated.



S4 Figure - Synthetic scheme for the generation of trehalose dimycolate analogues. a) i. N,O-bis(trimethylsilyl)acetamide, TBAF, DMF, ii. K₂CO₃, MeOH, y: 79%, b) stearic acid, EDCI, DMAP, toluene, y: 47%, c) 11-(acetylthio)undecanoic acid, EDCI, DMAP, toluene, y: 70%, d) amberlite H⁺, DCM/MeOH 1:1, y: quant., e) NH₂NH₂CH₂COOH, DCM/MeOH 1:1, y: quant., f) 11-(acetylthio)hexadecanoic acid, EDCI, DMAP, toluene, y: 85%, g) amberlite H⁺, DCM/MeOH 1:1, y: 87%, h) NH₂NH₂H₂O, DCM/MeOH 1:1, y: quant. (isolated as mixture of oxidized and reduced thiol).

2, 3, 4, 2', 3', 4'-hexaakis-O-(trimethylsilyl)- α , α' -trehalose (26).

D(+)-Trehalose dihydrate (684 mg, 2 mmol, 1 eq) was co-evaporated (2x) with toluene and dissolved in dry DMF (3 ml). Bis(trimethylsilyl)acetamide (3.56 g, 17 mmol, 8.7 eq) and a 1 M solution of TBAF (0.12 mmol, 0.06 eq) in THF were added and the reaction mixture was stirred for 2 hours at RT. The solution was cooled to 0 °C and quenched with i-propanol (1.2 ml). After diluting the mixture with MeOH (45 ml), K₂CO₃ (138 mg, 1 mmol, 0.5 eq) in MeOH (60 ml) was added and the reaction mixture was stirred for 2 hours at 0 °C. The solution was warmed up to room temperature, diluted with brine and EtOAc and transferred to a separatory funnel. The water layer was extracted (3x) with EtOAc. The combined organic layers were dried over MgSO₄, filtered and concentrated in vacuo. Compound **26** was obtained after silicagel chromatography (Pentane/EtOAc 4:1; DCM loading of crude) as a white solid (1.22 g, 1.58

mmol, 79%). NMR analysis confirmed purity of the product, whose ^1H NMR and ^{13}C NMR spectra were in agreement with published literature.²⁰

6-O-stearoyl-2, 3, 4, 2', 3', 4'-hexaakis-O-(trimethylsilyl)- α , α' -trehalose (35).

Compound **26** (533 mg, 0.69 mmol, 1.2 eq) and stearic acid (163 mg, 0.57 mmol, 1eq) were co-evaporated (2x) with toluene and dissolved in toluene (1.9 ml). After the solution was cooled to 0 °C, DMAP (7 mg, 0.057 mmol, 0.1 eq) and EDCI (132 mg, 0.69 mmol, 1.2 eq) were added and the reaction mixture was warmed first to room temperature over 1 hour and then to 70 °C. After 4 hours, the reaction mixture was diluted with EtOAc and transferred to a separatory funnel. The organic layer was washed (2x) with brine, dried over MgSO_4 , filtered and concentrated in vacuo. Compound **35** was obtained after silicagel chromatography (Pentane/EtOAc 20:1→9:1; DCM loading of crude) as a transparent oil (279 mg, 0.27 mmol, 47%). ^1H NMR (400 MHz, CDCl_3) δ : 4.95 – 4.88 (d (2x), J = 2.9, 2H, H-1, H-1'), 4.30 (dd, J = 11.8, 2.2 Hz, 1H, H-6a), 4.06 (dd, J = 11.8, 4.5 Hz, 1H, H-6b), 4.01 (ddd, J = 9.6, 4.4, 2.2 Hz, 1H, H-5'), 3.94 – 3.87 (dd (2x), J = 9.0, 4.2 Hz, 2H, H-3, H-3'), 3.87 – 3.81 (dd (2x), J = 9.5, 3.4 Hz, 1H, H-5'), 3.75 – 3.63 (m, 2H, H-6'), 3.52 – 3.39 (m, 4H, H-4, H-4', H-2, H-2'), 2.35 (td, J = 7.4, 3.1 Hz, 2H, $\text{CH}_2\text{-C=O}$), 1.84 – 1.51 (m, 4H, $\text{CH}_2\text{-lipid}$), 1.25 (m, 28H, $\text{CH}_2\text{-lipid}$), 0.90 – 0.85 (m, 3H, $\text{CH}_3\text{-lipid}$), 0.19 – 0.09 (m, 54H, $\text{CH}_3\text{-TMS}$). ^{13}C -APT NMR (101 MHz, CDCl_3) δ : 173.9 (C=O), 94.6 (C-1), 94.5 (C-1'), 73.6 (C-5), 73.4 (C-4), 73.1 (C-4'), 72.9 (C-2), 72.7 (C-2'), 72.0 (C-5'), 71.5 (C-3), 70.9 (C-3'), 63.4 (C-6), 61.8 (C-6'), 34.3 ($\text{CH}_2\text{-C=O}$), 32.1 ($\text{CH}_2\text{-lipid}$), 29.9 ($\text{CH}_2\text{-lipid}$), 29.8 ($\text{CH}_2\text{-lipid}$), 29.8 ($\text{CH}_2\text{-lipid}$), 29.6 ($\text{CH}_2\text{-lipid}$), 29.5 ($\text{CH}_2\text{-lipid}$), 29.5 ($\text{CH}_2\text{-lipid}$), 29.3 ($\text{CH}_2\text{-S}$), 25.0 ($\text{CH}_2\text{-lipid}$), 22.9 ($\text{CH}_2\text{-lipid}$), 14.3 ($\text{CH}_3\text{-lipid}$), 1.2 ($\text{CH}_3\text{-TMS}$), 1.2 ($\text{CH}_3\text{-TMS}$), 1.0 ($\text{CH}_3\text{-TMS}$), 1.0 ($\text{CH}_3\text{-TMS}$), 0.3 ($\text{CH}_3\text{-TMS}$), 0.3 ($\text{CH}_3\text{-TMS}$). HRMS $[\text{M}+\text{Na}]^+$: 1063.6024 found, 1063.6036 calculated.

6-O-stearoyl-6'-11-(acetylthio)undecanoyl-2, 3, 4, 2', 3', 4'-hexaakis-O-(trimethylsilyl)- α , α' -trehalose (27).

Compound **35** (97 mg, 0.1 mmol, 1 eq) was co-evaporated (2x) with toluene and dissolved in dry toluene (1.8 ml). Therefore, compound **21** (48.5 mg, 0.19 mmol, 2 eq) and DMAP (5.7 mg, 0.05 mmol, 0.5 eq) were added and the solution was cooled to 0 °C. EDCI (44.6 mg, 0.23 mmol, 2.5 eq) was finally added. The reaction mixture was warmed first to room temperature and then to 70 °C. After 2 days, the reaction mixture was diluted with EtOAc and transferred to a separatory funnel. The organic layer was washed (2x) with brine, dried over MgSO_4 , filtered and concentrated in vacuo. Compound **27** was obtained after silicagel chromatography (Pentane/EtOAc 18:1→9:1; DCM loading of crude) as a transparent oil (83 mg, 0.07 mmol, 70%). ^1H NMR (400 MHz, CDCl_3) δ : 4.92 (d, J = 3.1 Hz, 2H, H-1, H-1'), 4.27 (dd, J = 11.9, 2.2 Hz, 2H, H-6a, H-6a'), 4.06 (dd, J = 11.8, 4.4 Hz, 2H, H-6b, H-6b'), 4.00 (ddd, J = 9.6, 4.4, 2.2 Hz, 2H, H-5, H-5'), 3.90 (pt, J = 8.9 Hz, 2H, H-3, H-3'), 3.54– 3.39 (m, 4H, H-4, H-4', H-2, H-2'), 2.86 (t, J = 7.3 Hz, 2H, $\text{CH}_2\text{-S}$), 2.38 – 2.30 (m, 7H, $\text{CH}_2\text{-C=O}$, $\text{CH}_3\text{-thioacetyl}$), 1.65 – 1.53 (m, 6H, $\text{CH}_2\text{-lipid}$), 1.32 – 1.23 (m, 40H, $\text{CH}_2\text{-lipid}$), 0.88 (t, J = 6.7 Hz, 3H, $\text{CH}_3\text{-lipid}$), 0.18 – 0.09 (m, 54H, $\text{CH}_3\text{-TMS}$). ^{13}C -APT NMR (101 MHz, CDCl_3) δ : 207.9 (S-C=O), 173.9 (C=O), 94.5 (C-1, C-1'), 73.6 (C-3, C-3'), 72.8 (C-2, C-2'), 72.0 (C-4, C-4'), 70.8 (C-5, C-5'), 63.4 (C-6, C-6'), 34.3 ($\text{CH}_2\text{-C=O}$), 32.1 ($\text{CH}_2\text{-lipid}$), 30.8 ($\text{CH}_3\text{-thioacetyl}$), 29.8 ($\text{CH}_2\text{-lipid}$), 29.8 ($\text{CH}_2\text{-lipid}$), 29.6 ($\text{CH}_2\text{-lipid}$), 29.6 ($\text{CH}_2\text{-lipid}$), 29.5 ($\text{CH}_2\text{-lipid}$), 29.5 ($\text{CH}_2\text{-lipid}$), 29.4 ($\text{CH}_2\text{-S}$), 29.3 ($\text{CH}_2\text{-lipid}$), 29.2 ($\text{CH}_2\text{-lipid}$), 28.9 ($\text{CH}_2\text{-lipid}$), 24.9 ($\text{CH}_2\text{-lipid}$), 22.8 ($\text{CH}_2\text{-lipid}$), 14.3 ($\text{CH}_3\text{-lipid}$), 1.2 ($\text{CH}_3\text{-TMS}$), 1.0 ($\text{CH}_3\text{-TMS}$), 0.3 ($\text{CH}_3\text{-TMS}$). HRMS $[\text{M}+\text{Na}]^+$: 1305.7379 found, 1305.7376 calculated.

6-O-stearoyl-6'-(11-(acetylthio)undecanoyl)- α , α' -trehalose (36).

Compound **27** (932 mg, 0.72 mmol, 1 eq) was dissolved in a mixture of DCM/MeOH 1:1 (140 ml). Amberlite H⁺ was added until methanolysis of the TMS groups could be observed via TLC. The reaction was followed every 5 minutes, until after 30 minutes no starting material and only one lower running spot could be observed. The resin was removed via filtration and washed with DCM/MeOH 1:1. The filtered solution was concentrated and compound **36** was obtained as a white solid (620 mg, 0.72 mmol, quant.) without any further purification. ¹H NMR (400 MHz, CDCl₃) δ : 5.10 (d, *J* = 3.7 Hz, 2H, H-1, H-1'), 4.38–4.24 (m, 4H, H-6, H-6'), 4.08 (bs, 6H, OH), 3.99 (ddd, *J* = 10.2, 4.6, 2.5 Hz, 2H, H-5, H-5'), 3.88 (pt, *J* = 9.4 Hz, 2H, H-3, H-3'), 3.54 (dd, *J* = 9.8, 3.7 Hz, 2H, H-2, H-2'), 3.37 (dd, *J* = 10.2, 8.8 Hz, 2H, H-4, H-4'), 2.86 (t, *J* = 7.3 Hz, 2H, CH₂-S), 2.43–2.29 (m, 7H, CH₂-C=O, CH₃-thioacetyl), 1.65–1.53 (m, 6H, CH₂-lipid), 1.31–1.25 (m, 40H, CH₂-lipid), 0.90–0.86 (m, 3H, CH₃-lipid). ¹³C-APT NMR (101 MHz, CDCl₃) δ : 196.9 (S-C=O), 174.6 (C=O), 174.5 (C=O), 93.5 (C-1, C-1'), 72.9 (C-3, C-3'), 71.6 (C-2, C-2'), 70.2 (C-4, C-4'), 70.0 (C-5, C-5'), 63.1 (C-6, C-6'), 34.1 (CH₂-C=O), 31.9 (CH₂-lipid), 30.5 (CH₂-S), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.4 (CH₂-lipid), 29.4 (CH₂-lipid), 29.3 (CH₂-lipid), 29.3 (CH₂-lipid), 29.3 (CH₂-lipid), 29.2 (CH₂-lipid), 29.1 (CH₂-lipid), 29.1 (CH₂-lipid), 29.0 (CH₂-S), 28.7 (CH₂-lipid), 24.8 (CH₂-lipid), 24.8 (CH₂-lipid), 22.6 (CH₂-lipid), 14.0 (CH₃-lipid). HRMS [M+Na]⁺: 873.5020 found, 873.5005 calculated.

6-O-stearoyl-6'-(11-mercaptopundecanoyl)- α , α' -trehalose (3).

Compound **36** (51 mg, 0.06 mmol, 1 eq) was dissolved in a degassed solution of DCM/MeOH 1:1 (0.6 ml) and hydrazine monoacetate (14 mg, 0.15 mmol, 2.5eq) was added. After 5 hours an extra portion of hydrazine monoacetate (14 mg, 0.15 mmol, 2.5 eq) was added and the reaction mixture was stirred overnight. The morning after a white precipitate formed that was filtered and rinsed with a solution of degassed DCM/MeOH 9:1. The filtrate was dried in vacuo and compound **3** was obtained after silicagel chromatography (CHCl₃/MeOH 9:1→8:2; CHCl₃/MeOH 9:1 loading of crude; N₂ flow was used for purification) as a white solid (48 mg, 0.06 mmol, quant.). Reduced form: ¹H NMR (300 MHz, CDCl₃/MeOD 9:1) δ : 5.11 (d, *J* = 3.8 Hz, 2H, H-1, H-1'), 4.35–4.26 (m, 4H, H-6, H-6'), 4.00–3.92 (m, 2H, H-5, H-5'), 3.77 (pt, *J* = 9.3 Hz, 2H, H-3, H-3'), 3.53 (dd, *J* = 9.7, 3.7 Hz, 2H, H-2, H-2'), 3.40–3.32 (m, 7H, H-4, H-4', MeOH), 2.52 (t, *J* = 7.5 Hz, 2H, CH₂-SH), 2.39–2.31 (m, 4H, CH₂-C=O), 1.68–1.55 (m, 6H, CH₂-lipid), 1.43–1.19 (m, 40H, CH₂-lipid), 0.92–0.83 (m, 3H, CH₃-lipid). ¹³C-APT NMR (75 MHz, CDCl₃/MeOD 9:1) δ : 174.6 (C=O), 93.5 (C-1, C-1'), 72.9 (C-3, C-3'), 71.6 (C-2, C-2'), 70.2 (C-4, C-4'), 70.0 (C-5, C-5'), 63.1 (C-6, C-6'), 34.1 (CH₂-C=O), 31.9 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.4 (CH₂-lipid), 29.3 (CH₂-lipid), 29.2 (CH₂-lipid), 29.1 (CH₂-lipid), 29.0 (CH₂-lipid), 28.3 (CH₂-lipid), 24.8 (CH₂-lipid), 24.4 (CH₂-SH), 22.7 (CH₂-lipid), 14.0 (CH₃-lipid). HRMS [M+Na]⁺: 831.4907 found, 831.4899 calculated. Oxidized form: ¹H NMR (400 MHz, CDCl₃/MeOD 9:1) δ : 5.11 (d, *J* = 3.6 Hz, 2H, H-1, H-1'), 4.39–4.21 (m, 14H, H-6, H-6', OH), 4.05–3.94 (m, 2H, H-5, H-5'), 3.85 (pt, *J* = 9.4 Hz, 2H, H-3, H-3'), 3.55 (dd, *J* = 9.8, 3.7 Hz, 2H, H-2, H-2'), 3.43–3.31 (m, 3H, H-4, H-4', MeOH), 2.69 (t, *J* = 7.3 Hz, 2H, CH₂-S), 2.35 (t, *J* = 7.6 Hz, 4H, CH₂-C=O), 1.76–1.53 (m, 6H, CH₂-lipid), 1.43–1.24 (m, 40H, CH₂-lipid), 0.89 (t, *J* = 6.6 Hz, 3H, CH₃-lipid). ¹³C-APT NMR (101 MHz, CDCl₃/MeOD 9:1) δ : 174.5 (C=O), 93.5 (C-1, C-1'), 72.9 (C-3, C-3'), 71.5 (C-2, C-2'), 70.1 (C-4, C-4'), 69.9 (C-5, C-5'), 63.0 (C-6, C-6'), 39.0 (CH₂-S), 34.0 (CH₂-C=O), 31.8 (CH₂-lipid), 29.5 (CH₂-lipid), 29.4 (CH₂-lipid), 29.3 (CH₂-lipid), 29.3 (CH₂-lipid), 29.1 (CH₂-lipid), 29.1 (CH₂-lipid), 29.0 (CH₂-lipid), 28.9 (CH₂-lipid), 28.3 (CH₂-lipid), 24.7 (CH₂-lipid), 22.5 (CH₂-lipid), 13.8 (CH₃-lipid).

6-O-stearoyl-6'-(16-(acetylthio)hexadecanoyl)-2, 3, 4, 2', 3', 4'-hexakis-O-(trimethylsilyl)- α , α' -trehalose (28).

Compound **35** (211 mg, 0.2 mmol, 1 eq) was co-evaporated (2x) with toluene and dissolved in dry toluene (4 ml). Therefore, compound **24** (99 mg, 0.3 mmol, 1.5 eq) and DMAP (24 mg, 0.2 mmol, 1 eq) were added and the solution was cooled to 0 °C. EDCI (77 mg, 0.4 mmol, 2 eq) was finally added. The reaction mixture was warmed first to room temperature and then to 70 °C. The day after, the reaction mixture was diluted with EtOAc and transferred to a separatory funnel. The organic layer was washed (2x) with brine, dried over MgSO₄, filtered and concentrated in vacuo. Compound **28** was obtained after silicagel chromatography (Pentane/EtOAc 20:1→9:1; DCM loading of crude) as a transparent oil (232 mg, 0.17 mmol, 85%). ¹H NMR (400 MHz, CDCl₃) δ : 4.92 (d, J = 3.1 Hz, 2H, H-1, H-1'), 4.28 (dd, J = 11.8, 2.2 Hz, 2H, H-6a, H-6a'), 4.06 (dd, J = 11.8, 4.4 Hz, 2H, H-6b, H-6b'), 4.00 (ddd, J = 9.5, 4.4, 2.2 Hz, 2H, H-5, H-5'), 3.90 (pt, J = 9.0 Hz, 2H, H-3, H-3'), 3.53 – 3.39 (m, 4H, H-4, H-4', H-2, H-2'), 2.86 (t, J = 7.3 Hz, 2H, CH₂-SH), 2.38– 2.31 (m, 7H, CH₂-C=O, CH₃-thioacetyl), 1.66 – 1.53 (m, 6H, CH₂-lipid), 1.31– 1.22 (m, 50H, CH₂-lipid), 0.90 – 0.85 (m, 3H, CH₃-lipid), 0.17 – 0.12 (m, 54H, CH₃-TMS). ¹³C-APT NMR (101 MHz, CDCl₃) δ : 196.2 (S-C=O), 173.9 (C=O), 94.5 (C-1, C-1'), 73.6 (C-3, C-3'), 72.8 (C-2, C-2'), 72.0 (C-4, C-4'), 70.9 (C-5, C-5'), 63.4 (C-6, C-6'), 34.3 (CH₂-C=O), 32.1 (CH₂-lipid), 30.8 (CH₃-thioacetyl), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.7 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.5 (CH₂-lipid), 29.3 (CH₂-lipid), 29.3 (CH₂-lipid), 29.0 (CH₂-S), 24.9 (CH₂-lipid), 22.8 (CH₂-lipid), 14.3 (CH₃-lipid), 2.1 (CH₃-TMS), 1.2 (CH₃-TMS), 1.0 (CH₃-TMS), 0.4 (CH₃-TMS), 0.3 (CH₃-TMS). HRMS [M+Na]⁺: 1375.81640 found, 1375.81586 calculated.

6-O-stearoyl-6'-(16-(acetylthio)hexadecanoyl)- α , α' -trehalose (37).

Compound **28** (230 mg, 0.17 mmol, 1 eq) was dissolved in a mixture of DCM/MeOH 1:1 (36 ml). Amberlite H⁺ was added until methanolysis of the TMS groups could be observed via TLC. The reaction was followed every 5 minutes, until after 30 minutes no starting material and only one lower running spot could be observed. The resin was removed via filtration and washed with DCM/MeOH 1:1. The filtered solution was concentrated and compound **37** was obtained as a white solid (138mg, 0.15 mmol, 87%) without any further purification. ¹H NMR (400 MHz, CDCl₃/MeOD 3:1) δ : 5.11 (d, J = 3.7 Hz, 2H, H-1, H-2), 4.37 – 4.28 (m, 4H, H-6, H-6'), 3.98 (ddd, J = 10.2, 4.8, 2.5 Hz, 2H, H-5, H-5'), 3.83 (pt, J = 9.3 Hz, 2H, H-3, H-3'), 3.53 (dd, J = 9.8, 3.7 Hz, 2H, H-2, H-2'), 3.41– 3.32 (m, 3H, H-4, H-4', MeOH), 2.87 (t, J = 7.3 Hz, 2H, CH₂-S), 2.39 – 2.30 (m, 7H, CH₂-C=O, CH₃-thioacetyl), 1.67 – 1.54 (m, 6H, CH₂-lipid), 1.34 – 1.24 (m, 50H, CH₂-lipid), 0.88 (t, J = 6.7 Hz, 3H, CH₃-lipid). ¹³C-APT NMR (214 MHz, CDCl₃/MeOD 9:1) δ : 197.1 (S-C=O), 174.6 (C=O), 93.5 (C-1, C-1'), 77.3 (C-3, C-3'), 71.6 (C-2, C-2'), 70.2 (C-4, C-4'), 70.0 (C-5, C-5'), 63.1 (C-6, C-6'), 34.1 (CH₂-C=O), 34.1 (CH₂-C=O), 31.9 (CH₂-lipid), 30.5 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.5 (CH₂-lipid), 29.4 (CH₂-lipid), 29.4 (CH₂-lipid), 29.4 (CH₂-lipid), 29.4 (CH₂-lipid), 29.3 (CH₂-lipid), 29.2 (CH₂-lipid), 29.1 (CH₂-lipid), 29.1 (CH₂-lipid), 29.0 (CH₂-S), 28.7 (CH₂-lipid), 24.9 (CH₂-lipid), 24.8 (CH₂-lipid), 22.6 (CH₂-lipid), 14.0 (CH₃-lipid). HRMS [M+Na]⁺: 943.5804 found, 943.5787 calculated.

6-O-stearoyl-6'-(16-mercaptohexadecanoyl)- α , α' -trehalose (4).

Compound **37** (46 mg, 0.05 mmol, 1 eq) was dissolved in a degassed solution of DCM/MeOH 1:1 (0.5 ml) and hydrazine monohydrate (13 μ L, 0.25 mmol, 5 eq) was added. After 5 hours an

extra portion of hydrazine monohydrate (13 μ L, 0.25 mmol, 5 eq) was added and the reaction mixture was stirred overnight. The morning after the volatiles were removed in vacuo and compound **4** was obtained after silicagel chromatography (CHCl₃/MeOH 9:1 $\rightarrow$ 8:2; CHCl₃/MeOH 9:1 loading of crude; N₂ flow was used for purification) as a white solid (26 mg, 0.03 mmol, 50%). The remaining 50% was recovered as a mixture of free thiol and disulfide as confirmed by NMR analysis. ¹H NMR (300 MHz, CDCl₃/MeOD 1:1) δ : 5.10 (d, *J* = 3.7 Hz, 2H, H-1, H-1'), 4.43 – 4.20 (m, 4H, H-6, H-6'), 4.00 (ddd, *J* = 10.1, 5.0, 2.4 Hz, 2H, H-5, H-5'), 3.81 (pt, *J* = 9.3 Hz, 2H, H-3, H-3'), 3.53 (dd, *J* = 9.8, 3.8 Hz, 2H, H-2, H-2'), 3.46 – 3.29 (m, 3H, H-4, H-4', MeOH), 2.60 – 2.45 (m, 2H, CH₂-SH), 2.36 (t, *J* = 7.5 Hz, 4H, CH₂-C=O), 1.71 – 1.53 (m, 6H, CH₂-lipid), 1.41 – 1.21 (m, 50H, CH₂-lipid), 0.89 (t, *J* = 6.3 Hz, 3H, CH₃-lipid). ¹³C-APT NMR (75 MHz, CDCl₃/MeOD 3:1) δ : 174.4 (C=O), 93.3 (C-1, C1'), 73.1 (C-3, C-3'), 71.5 (C-2, C-2'), 70.2 (C-4, C-4'), 69.8 (C-5, C-5'), 63.0 (C-6, C-6'), 33.9 (CH₂-C=O), 31.6 (CH₂-lipid), 29.4 (CH₂-lipid), 29.4 (CH₂-lipid), 29.2 (CH₂-lipid), 29.0 (CH₂-lipid), 28.9 (CH₂-lipid), 28.8 (CH₂-lipid), 28.1 (CH₂-lipid), 24.6 (CH₂-SH), 24.1 (CH₂-lipid), 22.4 (CH₂-lipid), 13.6 (CH₃-lipid). HRMS [M+Na]⁺: 901.56788 found, 901.56813 calculated.

6, 6'-O-distearoyl-2, 3, 4, 2', 3', 4'-hexaakis-O-(trimethylsilyl)- α , α' -trehalose (38).

Compound **26** (311 mg, 0.4 mmol, 1 eq) and stearic acid (228 mg, 0.8 mmol, 2 eq) were co-evaporated (2x) with toluene and dissolved in toluene (1.3 ml). After the solution was cooled to 0 °C, DMAP (49 mg, 0.04 mmol, 0.1 eq) and EDCI (75 mg, 0.5 mmol, 1.2 eq) were added and the reaction mixture was warmed first to room temperature over 1 hour and then to 70 °C. After 4 hours, the reaction mixture was diluted with EtOAc and transferred to a separatory funnel. The organic layer was washed (2x) with brine, dried over MgSO₄, filtered and concentrated in vacuo. Compound **38** was obtained after silicagel chromatography (Pentane/EtOAc 20:1 $\rightarrow$ 9:1; DCM loading of crude) as a transparent oil (433 mg, 0.33 mmol, 82%). ¹H NMR (300 MHz, CDCl₃) δ : 4.99 (d, *J* = 3.1 Hz, 2H, H-1, H-1'), 4.35 (dd, *J* = 11.7, 2.0 Hz, 2H, H-6a, H-6b), 4.19 – 4.03 (m, 4H, H-6a', H-6b', H-5, H-5'), 3.98 (pt, *J* = 8.9 Hz, 2H, H-3, H-3'), 3.60 – 3.45 (m, 4H, H-2, H-2', H-4, H-4'), 2.41 (t, *J* = 7.3 Hz, 4H, CH₂-C=O), 1.69 (p, *J* = 7.4 Hz, 4H, CH₂-lipid), 1.43 – 1.25 (m, 56H, CH₂-lipid), 0.93 (t, *J* = 7.0 Hz, 6H, CH₃-lipid), 0.27 – 0.16 (m, 54H, CH₃-TMS). ¹³C-bbdec. NMR (101 MHz, CDCl₃) δ : 173.9 (C=O), 94.5 (C-1, C-1'), 73.6 (C-3, C-3'), 72.8 (C-2, C-2'), 72.0 (C-4, C-4'), 70.8 (C-5, C-5'), 63.4 (C-6, C-6'), 34.3 (CH₂-C=O), 32.1 (CH₂-lipid), 30.4 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.8 (CH₂-lipid), 29.6 (CH₂-lipid), 29.5 (CH₂-lipid), 29.5 (CH₂-lipid), 29.3 (CH₂-lipid), 24.9 (CH₂-lipid), 22.8 (CH₂-lipid), 14.3 (CH₃-lipid), 1.6 (CH₃-TMS), 1.2 (CH₃-TMS), 1.0 (CH₃-TMS), 0.7 (CH₃-TMS), 0.3 (CH₃-TMS), -0.1 (CH₃-TMS). HRMS [M+Na]⁺: 1329.6190 found, 1329.8645 calculated.

Trehalose-6, 6'-distearate (39).

Compound **38** (1.6 g, 1.23 mmol, 1 eq) was dissolved in a mixture of DCM/MeOH 1:1 (200 ml). Amberlite H⁺ was added until methanolysis of the TMS groups could be observed via TLC. After 2 hours, the resin was removed via filtration and washed with MeOH. The filtered solution was concentrated and compound **39** was obtained as a white solid (864 mg, 0.96 mmol, 78%) without any further purification. ¹H NMR (400 MHz, CDCl₃/MeOD 2:1) δ : 5.09 (d, *J* = 3.7 Hz, 2H, H-1, H-2), 4.32 (dd, *J* = 12.1, 2.4 Hz, 2H, H-6a, H-6b), 4.26 (dd, *J* = 12.0, 4.9 Hz, 2H, H-6a', H-6b'), 3.97 (ddd, *J* = 10.2, 4.9, 2.4 Hz, 2H, H-5, H-5'), 3.78 (pt, *J* = 9.3 Hz, 2H, H-3, H-3'), 3.52 (dd, *J* = 9.7, 3.7 Hz, 2H, H-2, H-2'), 3.39 – 3.30 (m, 3H, H-4, H-4', MeOH), 2.34 (t, *J* = 7.6 Hz, 4H, CH₂-C=O), 1.61 (p, *J* = 7.5 Hz, 4H, CH₂-lipid), 1.31 – 1.22 (m, 56H, CH₂-lipid), 0.87 (t, *J* = 6.8 Hz, 6H,

(CH₃-lipid). ¹³C-APT NMR (101 MHz, CDCl₃/MeOD 2:1) δ: 174.4 (C=O), 93.4 (C-1, C-1'), 73.03 (C-3, C-3'), 71.4 (C-2, C-2'), 70.1 (C-4, C-4'), 69.8 (C-5, C-5'), 63.0 (C-6, C-6'), 33.9 (CH₂-C=O), 31.7 (CH₂-lipid), 29.4 (CH₂-lipid), 29.4 (CH₂-lipid), 29.36 (CH₂-lipid), 29.2 (CH₂-lipid), 29.2 (CH₂-lipid), 29.1 (CH₂-lipid), 29.0 (CH₂-lipid), 28.9 (CH₂-lipid), 24.6 (CH₂-lipid), 22.4 (CH₂-lipid), 13.6 (CH₃-lipid). HRMS [M+H]⁺: 897.62662 found, 897.62736 calculated.

Peptide conjugates

Materials for the synthesis of peptides and conjugates

All reagents and solvents used in the solid phase peptide synthesis were purchased from Novabiochem (San Diego, CA, USA) and Biosolve (Valkenswaard, The Netherlands), with the exception of 3-maleimido-propionic acid (Bachem, Torrance, CA, USA), palmitoyl-Cys((RS)-2, 3-di(palmitoiloxy)-propyl)-OH (Bachem, Torrance, CA, USA) and tetradecyl isocyanate (Sigma-Aldrich, St.Louis, MO, USA). Tentagel resins were purchased from Rapp Polymere (Tuebingen, Germany). All chemicals were used as received. Analytical LC-MS was performed using an Agilent 1260 LC system (215 and 254 nm UV sensors included) coupled to Agilent 6120 single quadrupole mass analyzer. This LC-MS system was equipped with one of the following columns: Macherey-Nagel NUCLEODUR® C18 5 μm, 4.6 x 50 mm; Hichrom Vydac® 219TP Diphenyl 5 μm, 4.6 x 150 mm; Cosmosil 5C4-MS 120°A 5μm, 4.6 x 150 mm. Ternary solvent system: A - 100% water; B - 100% acetonitrile; C - 1% TFA in water. Gradients of B in A were employed in combination with 10% C. Purifications were performed on a Preparative HPLC system from Gilson equipped with one of the following columns: Macherey-Nagel NUCLEODUR® C18 5 μm, 10 x 250 mm; Hichrom Vydac® 219TP Diphenyl 5 μm, 10 x 250 mm; Cosmosil 5C4-MS 120°A 5μm, 10 x 250 mm. Binary solvent system: A - 1% TFA in water; B - 100% acetonitrile. Gradients of B in A were employed over 3 CV unless stated otherwise. MALDI-TOF spectra were recorded on an Ultraflextreme MALDI-TOF or a 15T MALDI-FT-ICR MS system.

General methods for the synthesis of peptides

The solid phase peptide synthesis was performed on a Liberty Blue™ Automated Microwave Peptide Synthesizer. TentaGel® S RAM resin and Fmoc based protocols were employed for the synthesis of the peptides, unless stated otherwise. The steps performed for each amino acid coupling were: a) DMF washes (2 times) and subsequent nitrogen purge of the solvent; b) Fmoc protecting group removal using 4 ml of a solution containing 20% piperidine in DMF (3 times 1.5 minutes) at 90 °C; c) DMF washes (3 times) and subsequent nitrogen purge of the solvent; d) Fmoc protected amino acid (5 eq) coupling in the presence of oxyma pure (1 M, 1 eq) and DIC (0.5 M, 1 eq), performed at 90 °C for 2.5 minutes; e) DMF washes (3 times) and subsequent nitrogen purge; f) washing of the resin with DMF (3 times), DCM (3 times), Et₂O (2 times) and subsequent nitrogen purge. After completion of all synthetic cycles, the resin was transferred to a polypropylene syringe equipped with a porous polypropylene disc at the bottom and treated for 1 hour and 30 minutes with a TFA/TIS/H₂O (38:1:1, v/v/v) cleavage cocktail (5 mL/100 μmol scale reaction). The reaction mixture containing the cleaved peptide was filtered into cold Et₂O/pentane (1:1, v/v) (50 ml/5 ml cleavage cocktail) and the resin was washed with 1 ml TFA (2 times) into the cold Et₂O/pentane solution. The solution was stored in a -20 °C freezer for 2 hours, then centrifuged (10 minutes, 4400 rpm, 3 x g); finally, the

supernatant was discarded and the precipitate was purified via RP-HPLC. Synthetic compounds were tested using LAL assay to exclude the possibility of LPS contamination.

Rv1733c p57-84 peptide: *Ile-Pro-Phe-Ala-Ala-Ala-Ala-Gly-Thr-Ala-Val-Gln-Asp-Ser-Arg-Ser-His-Val-Tyr-Ala-His-Gln-Ala-Gln-Thr-Arg-His-Pro-NH₂* (5)

The p57 peptide (compound **5**) was synthesized according to the general procedure for peptide synthesis described above. Pseudoproline dipeptides Fmoc-Asp(OtBu)-Ser (ψ Me, Mepro)-OH and Fmoc-Gly-Thr (ψ Me, Mepro)-OH were employed to enhance synthetic efficiency. Purification by RP-HPLC (linear gradient 20→30% B in 10 min) followed by lyophilization yielded compound **5** as a white powder (90.4 mg, 30.26 μ mol, 12.1% yield based on theoretical resin loading of 0.23 mmol/g). LC-MS analysis (C18 column, linear gradient 10→90% B, 11 min): Rt = 3.571 min, ESI-MS $[M+H]^+$ = 1494.3 found, 1493.8 calculated. MALDI-TOF $[M+H]^+$: 2986.4517 found, 2986.5143 calculated.

Rv1733c p57-84 maleimido-peptide: *Maleimido-Ile-Pro-Phe-Ala-Ala-Ala-Ala-Gly-Thr-Ala-Val-Gln-Asp-Ser-Arg-Ser-His-Val-Tyr-Ala-His-Gln-Ala-Gln-Thr-Arg-His-Pro-NH₂* (40)

Compound **40** was synthesised according to the general procedure for peptide synthesis described above. Pseudoproline dipeptides Fmoc-Asp(OtBu)-Ser (ψ Me, Mepro)-OH and Fmoc-Gly-Thr (ψ Me, Mepro)-OH were employed to enhance synthetic efficiency. Purification by RP-HPLC (linear gradient 20→35% B in 10 min) followed by lyophilisation yielded **40** as a white powder (20.99 mg, 6.68 μ mol, 6.7% yield based on theoretical resin loading of 0.23 mmol/g). LC-MS analysis (C18 column, linear gradient 10→50% B, 11 min): Rt = 6.395 min, ESI-MS $[M+H]^+$ = 1570.0 found, 1569.3 calculated.

3-(3-(trehalose-6-stearoyl-6'-undecanoylthio)-2, 5-dioxopyrrolidin-1-yl) propanoate-Ile-Pro-Phe-Ala-Ala-Ala-Ala-Gly-Thr-Ala-Val-Gln-Asp-Ser-Arg-Ser-His-Val-Tyr-Ala-His-Gln-Ala-Gln-Thr-Arg-His-Pro-NH₂ (6)

Trehalose glycolipid **3** (12 mg, 14.4 μ mol, 3 eq) was dissolved in degassed CHCl₃ (0.9 ml) and added to a mixture of Rv1733c p57-84 maleimido-peptide **40** (15 mg, 4.7 μ mol, 1 eq) in degassed DMF (1.2 ml) and H₂O (0.3 ml). The reaction was stirred overnight under Argon atmosphere. Compound **6** was obtained after purification by RP-HPLC (C18 column, linear gradient 25→75% B, 12 min) as a white powder (14.8 mg, 3.7 μ mol, 79%). LC-MS analysis (C18 column, linear gradient 10→90% B, 11 min): Rt = 7.906 min, ESI-MS $[M+H]^+$ = 1974.5 found, 1973.5 calculated. MALDI-TOF $[M+H]^+$: 3946.121 found, 3946.038 calculated.

3-(3-(trehalose-6-stearoyl-6'-palmitoylthio)-2, 5-dioxopyrrolidin-1-yl) propanoate-Ile-Pro-Phe-Ala-Ala-Ala-Ala-Gly-Thr-Ala-Val-Gln-Asp-Ser-Arg-Ser-His-Val-Tyr-Ala-His-Gln-Ala-Gln-Thr-Arg-His-Pro-NH₂ (7)

Trehalose glycolipid **4** (0.9 mg, 1.02 μ mol, 1 eq) was dissolved in degassed CHCl₃ (0.19 ml) and added to a mixture of Rv1733c p57-84 maleimido-peptide **40** (8 mg, 2.5 μ mol, 2.5 eq) in degassed DMF (0.25 ml) and H₂O (0.06 ml). The reaction was stirred overnight under Argon atmosphere. A precipitate formed, which was separated after centrifuging from the mother liquor, and redissolved in a 3:3:1 mixture of t-BuOH/CH₃CN/H₂O (500 μ l) with addition of TFA (50 μ l) to improve solubility. Compound **7** was obtained after purification by RP-HPLC (C18 column, linear gradient 30→100% B, 15 min) as a white powder (0.9 mg, 3.7 μ mol, 22%). LC-MS analysis (C18 column, linear gradient 10→90% B, 11 min): Rt = 9.081 min, ESI-MS

[M+H]³⁺= 1339.9 found, 1339.4 calculated. MALDI-TOF [M+H]⁺: 4016.0244 found, 4016.1202 calculated.

Immunological methods

Culturing and stimulation of HEK-Blue mMincle cell line

The HEK-Blue mMincle cell line was purchased from InvivoGen (San Diego, United States) and cultured according to manufacturer's instructions in Dulbecco's Modified Eagle Medium DMEM (Gibco, PAA, Linz, Austria) + 4.5 g/l glucose, 10% (v/v) heat-inactivated fetal calf serum (FCS) (HyClone, GE Healthcare Life Sciences, Eindhoven, the Netherlands), 50 U/ml penicillin, 50 mg/ml streptomycin, 100 mg/ml Normocin and 2 mM L-glutamine (Life Technologies-Invitrogen, Bleiswijk, the Netherlands). All compounds used for stimulation were dissolved in iso-propanol and transferred to 96 well plates (Microton high binding, Greiner Bio-One International). The solvent was evaporated completely at 50 °C. HEK-293 cells were suspended in Quantibblue (InvivoGen, San Diego, United States) medium and approximately 50.000 cells/well were transferred to 96 well plate. Reference peptide and UPam were used as negative controls. After overnight stimulation with novel adjuvants and conjugates, sample absorbance at 635 nm was measured using a Spectramax i3x (Molecular Devices, CA, USA) spectrometer.

ELISA using murine Mincle-Fc receptor

Recombinant Murine CLEC4E Fc Chimera Protein was purchased from RD systems (Minneapolis, MN, USA) and used at concentrations of 0.83 µg/ml. Control compound PAA-Lewis X was purchased from Lectinity (MW approx. 20 KDa, Carbohydrate content around 20% mol). Laminarin (from *Laminaria digitata*) and Mannan (from *Saccharomyces cerevisiae*) were purchased from Sigma-Aldrich (St.Louis, MO, USA). All synthetic compounds were dissolved in iso-propanol and were transferred to 96 well plates (Nunc MaxiSorp, Biolegend, London, UK) at three different concentrations (5 - 1 - 0.2 nmol/well) for a total volume of 50 µL/well. The solvent was evaporated at 50 °C. Control compounds Laminarin (3 µg/ml), PAA-Lewis X (1 µg/ml) and Mannan (1 µg/ml) were diluted in PBS and used to coat remaining wells for a total volume of 50 µL/well. After coating for 2 hours at room temperature, all the wells were washed twice with TMS (20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.0; 150 mM NaCl; 1 mM CaCl₂; 2 mM MgCl₂) (150µL) and blocked with 100 µL TMS with 1% of BSA (Fraction V, Merk Millipore, Burlington, MA, USA) for 30 minutes at room temperature. The plates were then incubated for 1 hour at room temperature with 50 µL soluble receptor in TMS with 1% of BSA. The wells were washed two times with TMS (150µL) and incubated at room temperature with 50 µL of Goat-anti human HRP (0.8 µg/mL, Jackson Immuno Research, Germany) in TMS with 1% of BSA for 30 minutes. After two washes with TMS (150µL), 50 µL of substrate solution (3, 3', 5, 5'-Tetramethylbenzidine, TMB, in citric/acetate buffer, pH=4, and H₂O₂) were added and after 2 minutes at room temperature the reaction was stopped with 50 µL of H₂SO₄ (0.8M). Sample absorbance at 450 nm was measured using a Spectramax i3x (Molecular Devices, CA, USA) spectrometer.

Culturing and stimulation of murine D1 DC cell line

The D1 cell line⁵¹ was obtained from the department of Immunohematology and Blood Transfusion of the Leiden University Medical Centre. The cells were cultured in IMDM medium (Lonza, Belgium) containing 10% heat inactivated FCS (HyClone, GE Healthcare Life Sciences, Eindhoven, the Netherlands), 2 mM GlutaMAX™ (Gibco, PAA, Linz, Austria), 50 μ M β -mercaptoethanol (Sigma, St.Louis, MO, USA) and 30% supernatant from R1 cells (mouse fibroblast NIH/3T3 cells transfected with mouse GM-CSF gene), which was collected from confluent cultures and filtered. Cells were harvested using PBS containing 2 mM EDTA, counted and transferred to 96 well plates (round bottom, Corning Costar TC-Treated Microplates, Corning, NY) at approximately 50.000 cells/well. Immediately after plating, the cells were stimulated at a concentration of 50 μ M of trehalose 6-6'-distearate and synthetic compounds. Synthetic compounds were dissolved in DMSO (Sigma, St.Louis, MO, USA) at a concentration of 5 nmol/ μ L, further diluted and premixed in culture medium. ODN1826 (1 μ g/ml; 5'-TCCATGACGTTCCCTGACGTT-3'; InvivoGen, San Diego, CA) was used as positive control for stimulated cells. Supernatants were harvested 20 hours after the addition of stimuli for subsequent analysis of cytokines and cells were stained as described below.

Flow cytometric analysis of D1 DC cell line

After 20 h stimulation, murine D1 cells were incubated for 30 minutes at 4 °C with the following dye-labelled antibodies: PE anti-mouse CD40 clone 3/23 (Biolegend, London, UK) and FITC anti-mouse CD86 clone B7-2 (eBioscience, San Diego, CA). Samples containing the stained cells were characterized on a BD FACSLytic™ flow cytometer and analysed using FlowJo v10 software (Treestar Inc).

Generation and stimulation of immature human moDCs and macrophages

Buffy coats of healthy human Blood Bank donors were purchased from Sanquin, Amsterdam, The Netherlands. CD14⁺ monocytes were isolated from whole blood using Ficoll-Paque density gradient followed by purification on autoMACS® Pro Separator instrument using CD14 MicroBeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). The monocytes were differentiated to monocyte-derived dendritic cells (moDCs) at a concentration of 10⁶ cells/mL in RPMI 1640 (Gibco, PAA, Linz, Austria) medium containing 10% FCS (Hyclone, GE Healthcare Life Sciences, Eindhoven, the Netherlands), 2 mM GlutaMAX™ (Gibco, PAA, Linz, Austria), 100 U/ml penicillin, and 100 μ g/ml streptomycin and 10 ng/mL GM-CSF (Life Technologies-Invitrogen, Bleiswijk, the Netherlands) and 10 ng/mL IL-4 (Peprotech, Rocky Hill, NJ). Differentiation into macrophages was done in the presence of a final concentration of 5 ng/ml GM-CSF for type 1 macrophages and 50 ng/ml M-CSF (R&D Systems, Abingdon, UK) for type 2 macrophages. On day 3 of culturing, all cultures were replenished with fresh culture media with the appropriate concentrations of GM-CSF and IL-4 for the moDC's and GM-CSF/M-CSF for the different subtypes of macrophages. Cells were incubated for a total of 5 days at 37 °C and in a 5% CO₂ atmosphere incubator. Immature dendritic cells were then harvested, counted and transferred to 96 well plates (round bottom, Corning Costar TC-Treated Microplates, Corning, NY) at approximately 50.000 cells/well; macrophages type 1 and type 2 were harvested, counted and transferred to 96 well plates (flat bottom, Corning Costar TC-Treated Microplates, Corning, NY) at approximately 30.000 cells/well. The next day, cells were stimulated at fixed concentrations (20 – 1 -0.05 μ M) of trehalose 6-6'-distearate and synthetic compounds. Synthetic compounds were dissolved in DMSO (Sigma, St.Louis, MO, USA) at a

concentration of 5 nmol/ μ L, further diluted and premixed in RPMI 1640 medium containing 10% FCS, 2 mM GlutaMAX™, 100 U/ml penicillin, and 100 μ g/ml streptomycin. LPS (InvivoGen, San Diego, United States), at a concentration of 100 ng/ml, was used as positive control for stimulated cells. Supernatants were harvested 20 hours after the addition of stimuli for subsequent analysis of cytokines and cells were stained as described below.

Flow cytometric analysis of human moDCs and macrophages

Cells were incubated for 10 minutes at room temperature with 5% human serum (Sigma, Merck, Darmstadt, Germany) in PBS to prevent nonspecific binding of the antibodies. Subsequently, cells were stained for 10 minutes at 4 °C using LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (Thermo Fisher scientific, Merelbeke, Belgium), followed by surface staining (30 minutes at 4 °C) with CD40 - APC (clone 5C3), CD80 - APC-R700 (clone L307.4), CD83 - PE (clone HB15e), CD86 - BB700 (clone 2331 (FUN-1)), HLA-DR-DP-DQ - BV510 (clone Tu39); all antibodies were purchased from BD Biosciences. All samples were characterized on a BD FACSLytic™ flow cytometer and analysed using FlowJo v10 software (Treestar Inc).

ELISA human cytokines

Human IL-12/IL-23 (p40) and human IL-10 ELISA kits were purchased from Biolegend (ELISA MAX™ Standard Set; London, UK). All supernatants were tested in duplicates according to manufacturer's instructions. Microton high binding 96 well plates (Greiner Bio-One International) were used for the assays. Sample absorbance was measured using a Spectramax i3x (Molecular Devices, CA, USA) spectrometer.

Luminex human Th1/Th17

Cytokines and chemokines in culture supernatants were measured with the Milliplex magnetic bead kit (Merck, USA) on 96 well multiscreen filter plates (Millipore, USA) using the Bio-Plex-100-suspension-array-system (Bio-Rad Laboratories, Veenendaal, The Netherlands) and analyzed using the Bio-Plex Manager software 6.1 (Bio-Rad Laboratories, Veenendaal, The Netherlands). Cytokines/chemokines included: IL-6, GM-CSF, TNF- α , IFN- γ . After pretreating the filter with assay solution supernatant samples (25 μ L) were added to the plates, together with 25 μ L assay buffer and 25 μ L beads, and the plates were incubated for overnight at 4 °C. After two washing steps with 200 μ L wash buffer (Millipore, USA), 25 μ L detection Ab mixture was added per well, and plates were incubated at room temperature in the dark for 1 hour on a plate shaker at 300 rpm. Per well 25 μ L streptavidin-PE solution was added and incubated for 30 min at room temperature in the dark. After two washes, 150 μ L Sheath Fluid was added to each well, and the plates were placed in the Bio-Plex System. From each well, a minimum of 50 analyte-specific beads was analyzed for fluorescence. A curve fit was applied to each standard curve according to the manufacturer's manual. Sample concentrations were interpolated from these standard curves. Analyte concentrations outside the upper or lower limits of quantification were assigned the values of the limits of quantification of the cytokine or chemokine.

Mice

HLA-DRB1*0301/DRA transgenic (tg), murine class II-deficient (HLA-DR3/Ab⁰) mice were bred under specific-pathogen-free conditions at the Leiden University Medical Centre (LUMC) animal facility. During breeding, PBMCs of each mouse were typed for expression and

segregation of the transgene by flow cytometry for HLA-DR PE-labelled mouse IgG2a anti-HLA-DR (clone G46-6; BD Biosciences, Franklin Lakes, NJ, USA) and murine CD4 FITC-labelled rat IgG2a, κ anti-mouse CD4 (clone H129.19; BD Biosciences). Littermates lacking HLA-DR expression were excluded from these experiments.

Immunizations

Mice (3 to 6 animals per group; 6 weeks old) were injected subcutaneously (s.c.) in the right flank with conjugate, or mixtures of p57-peptide and trehalose adjuvant, in 200 μ l phosphate-buffered saline (PBS) at 2 weeks interval. Two weeks after the last immunization, splenocytes were harvested.

In vitro cultures of splenocytes

Splenocytes were isolated from individual animals by homogenizing spleens through a 70 μ m cell strainer (Falcon; Fisher Scientific, Loughborough, UK), and were resuspended in Iscove's modified Dulbecco's medium (Invitrogen, Thermo Fisher Scientific, Bleiswijk, the Netherlands) + Glutamax (Gibco, PAA, Linz, Austria), supplemented with 100 U/100 μ g/ml penicillin-streptomycin solution (Invitrogen) and 8% FCS (Hyclone, GE Healthcare Life Sciences, Eindhoven, the Netherlands) at 3×10^6 cells/ml in a 96-well round-bottom microtiter plates (Costar; Corning Incorporated, Corning, NY). Cells were incubated in quadruplicate with 100 μ l of medium, peptide (final concentration 10 μ g/ml), or relevant recombinant *Mtb* protein (10 μ g/ml). The mitogen concanavalin A (ConA; 2 μ g/ml; Sigma, St.Louis, MO, USA) was used as a positive control for cell viability. After 6 days, supernatants were taken from each well and quadruplicates were pooled and frozen at -20°C.

Intracellular cytokine staining

Splenocytes (6×10^6 /well) were cultured *in vitro* with medium, peptide (5 μ g/ml) or protein (5 μ g/ml) in a 24 well plate. After 4 h, BrefeldinA (2.5 μ g/ml, Sigma, St.Louis, MO, USA) was added and incubated overnight. The following day cells were stained with the following surface markers CD8 α -Horizon V500 (clone 53-6.7), CD62L APC (clone MEL-14) (BD Biosciences, San Diego, CA), CD44-BV605 (clone IM7), HLA-DR-AF700 (clone L243), PD-1-PE-Cy7 (clone RMP1-30) (Biolegend, London, UK), KLRG1-V450 (clone 2F1) (ThermoFisher Scientific, Waltham, MA, USA). After fixation cells were permeabilized with Cytofix/Cytoperm reagents (BD Bioscience, San Diego, CA) according to manufacturer's instructions and stained intracellularly with IFN- γ -AlexaFluor488 (clone XMG1.2) (Biolegend, London, UK), TNF- α -PE (clone MP6-XT22), IL-2-APC Cy7 (clone JES6-5H4) (BD Biosciences, San Diego, CA) and IL-17A-PerCP-Cy5.5 (clone eBio17B7) (ThermoFisher Scientific, Waltham, MA, USA).

Antibody detection

Antibodies against the Rv1733c p57 peptide, Rv1733c protein and *Mtb* sonicate in serum from immunized mice were determined by ELISA: High binding 96 well plates (Microton, Greiner Bio-One International, Alphen a/d Rijn, The Netherlands) were coated overnight at 4 °C with Bovine Serum Albumine (0.4%, BSA, Roche, Woerden, The Netherlands) to determine the non-specific background, peptide (5 μ g/ml), protein (5 μ g/ml) or *Mtb* sonicate (5 μ g/ml) for the specific antibody responses; washed three times with PBS/0.05% Tween-20 and blocked with 1% BSA/1% Tween-20 for 2h at 37 °C. After washing three times, serum dilutions in 1% BSA were incubated at 37 °C for 2h, followed by three times washing and incubated for 2h at 37 °C

with horse radish peroxidase (HRP)-labelled Ig antibodies (total Ig, IgG1, IgG2a, IgG2b, IgG2c, IgG3, IgM and IgA (all Dako, Glostrup, Denmark)) in PBS/1% BSA. After incubation, plates were washed five times and TMB substrate (Sigma, St.Louis, MO, USA) was added for 10 minutes at RT, H₂SO₄ (1M) was added to stop the colorimetric reaction and OD450 was determined.

IFN- γ ELISA

Splenocytes (6 x 10⁶/well) were cultured *in vitro* with medium, peptide (5 μ g/ml) or protein (5 μ g/ml) in a 24 well plate. IFN- γ ELISA (BD Bioscience, San Diego, CA) was performed on supernatants according to manufacturer's instructions. Absorbance (OD450) was determined and Microplate Manager software version 5.2.1 (Biorad Laboratories, The Netherlands) was used to convert the values into concentrations using a standard curve.

Cytokine-chemokine analysis

Cytokines and chemokines in supernatants from splenocytes were measured using a Milliplex magnetic bead kit (Merck, USA) on 96 well multiscreen filter plates (Millipore, USA) using the Bio-Plex-100-suspension-array-system (Bio-Rad Laboratories, Veenendaal, The Netherlands) and analyzed using the Bio-Plex Manager software 6.1 (Bio-Rad Laboratories, Veenendaal, The Netherlands). Cytokines/chemokines included: IL-2, IL-6, IL-15, IFN- γ , IL-1 β , IL-4, IL-5, IL-10, IL-13, IL-17A, IL-17F, IL-21, IL-22, CD40L, TNF- α , TNF- β , MIP-3 α , GM-CSF. After pretreating the filter with assay solution supernatant samples (25 μ l) were added to the plates, together with 25 μ l assay buffer and 25 μ l beads, and the plates were incubated for overnight at 4 °C. After two washing steps with 200 μ l wash buffer (Millipore, USA), 25 μ l detection Ab mixture was added per well, and plates were incubated at room temperature in the dark for 1 hour on a plate shaker at 300 rpm. Streptavidin-PE solution (25 μ l/well) was added and incubated for 30 min at room temperature in the dark. After two washes, 150 μ l Sheath Fluid was added to each well, and the plates were placed in the Bio-Plex System. From each well, a minimum of 50 analyte-specific beads was analyzed for fluorescence. A curve fit was applied to each standard curve according to the manufacturer's manual. Sample concentrations were interpolated from these standard curves. Analyte concentrations outside the upper or lower limits of quantification were assigned the values of the limits of quantification of the cytokine or chemokine.

BCG immunization

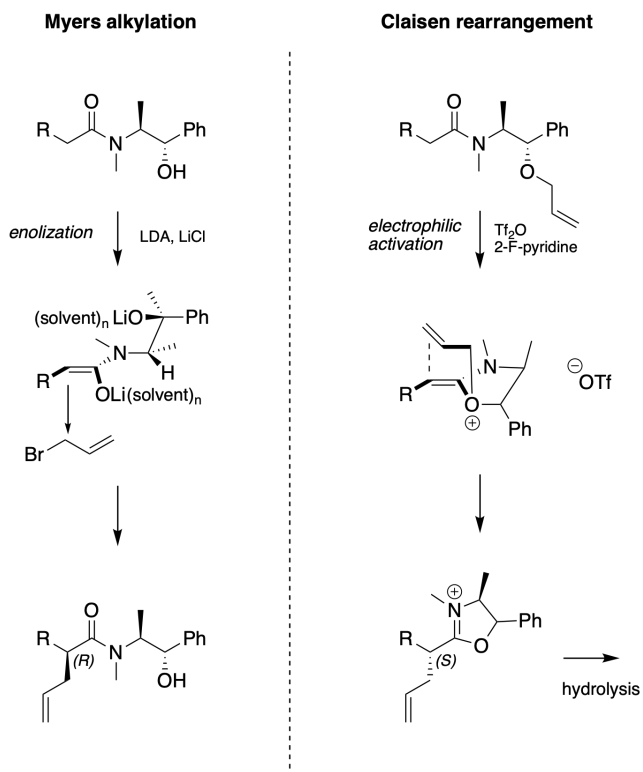
Mice were injected s.c. in the right flank with 10⁶ CFU BCG1331 (*M. bovis* bacillus Calmette Guérin; Statens Serum Institut, Copenhagen, Denmark) from glycerol stocks, 12 weeks before *Mtb* challenge.

Intranasal infection of mice with live *Mtb*

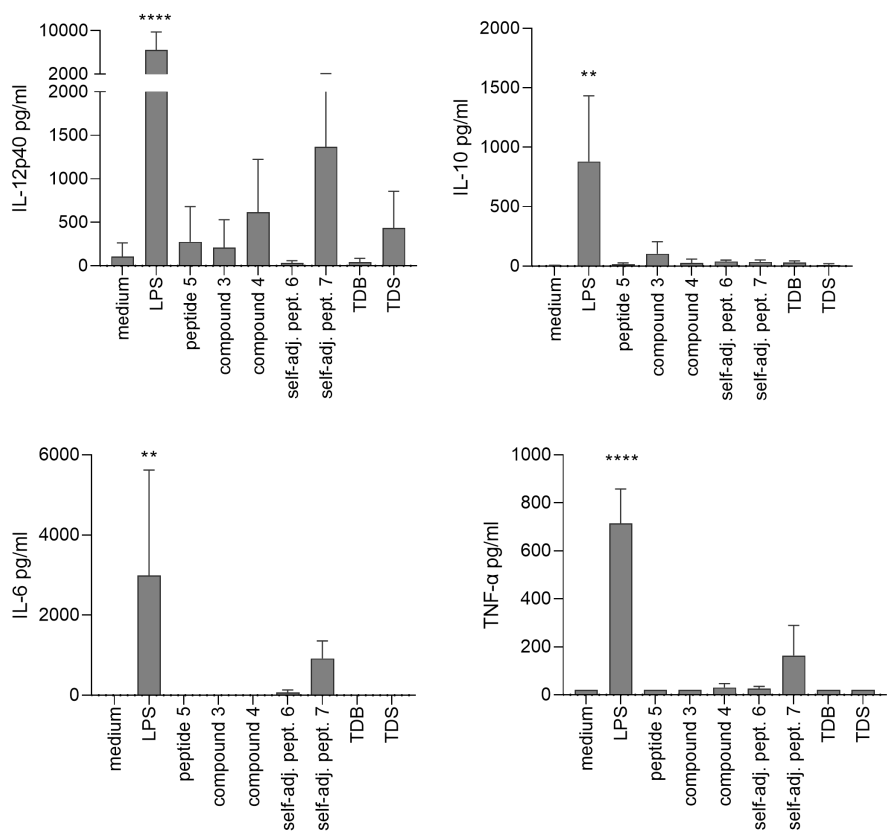
Mice were anesthetized with isoflurane (2-chloro-2-(difluoromethoxy)-1, 1, 1-trifluoroethane; Pharmachemie BV, The Netherlands) and intranasally (i.n.) infected with 10⁵ CFU live *Mtb* strain H37Rv from glycerol stocks, 2 weeks after the third antigen immunization or 12 weeks after BCG immunization. Mice were daily monitored for ethical requirements, and weighed once a week. They were sacrificed 6 weeks after *Mtb* infection, lungs and splenocytes were aseptically removed. Organs were homogenized using 70 μ M cell strainers (Fisher Scientific, Loughborough, UK) and the numbers of *Mtb* bacteria were determined by plating serial dilutions of the homogenates on 7H11 agar plates (BD Bioscience, San Diego, CA),

supplemented with BBL Middlebrook OADC enrichment and PANTA (all BD Bioscience, San Diego, CA). Colonies were counted after 3 weeks of incubation at 37 °C.

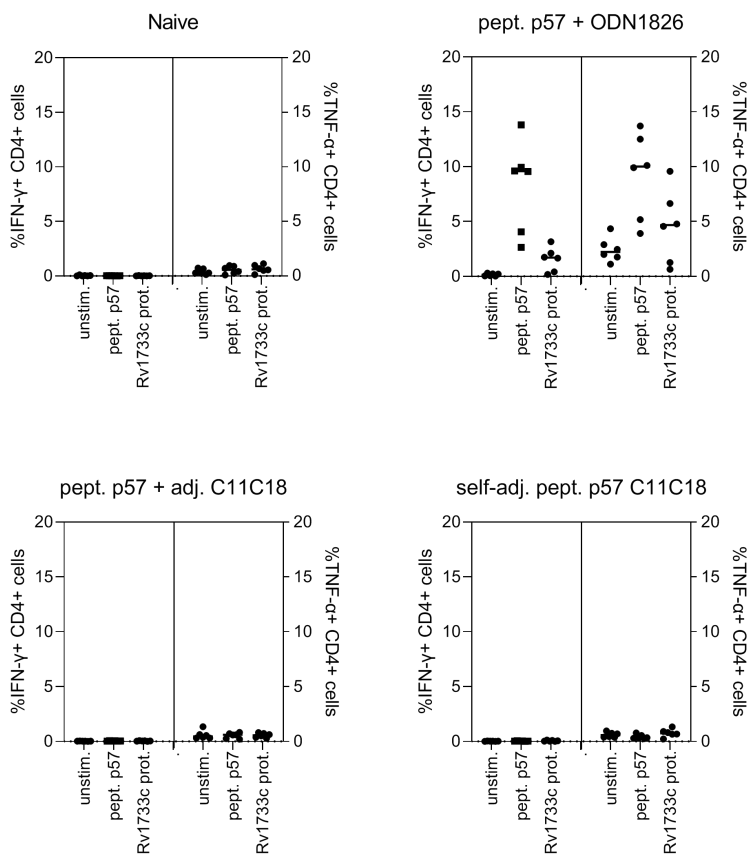
Supporting figures



S5 Figure - Schematic comparison of the reaction mechanisms for Myers alkylation and Peng's strategy employing Claisen rearrangement.⁵²



S6 Figure - Cytokine production profile of human moDCs stimulated for 20 hours using TDM analogues 3 and 4, and self-adjuvanting peptides 6 and 7, as measured by ELISA (IL-12p40 and IL-10) or Luminex (IL-6 and TNF-α). All synthetic compounds were used at a final concentration of 20 μ M in medium. LPS (100 ng/ml) is used as a positive control. Bars indicate the mean value + SD of duplicates from three donors as calculated using GraphPad Prism.



S7 Figure - IFN-γ released in cell supernatant after in vitro stimulation of splenocytes of immunized mice with either purified peptide p57 (5 µg/ml) or recombinant protein (5 µg/ml), as measured by ELISA assay. Mice (n=6) were injected three times with either PBS as negative control, peptide p57 (40 nmol) in admixture with ODN1826 (8 nmol) as positive control, peptide p57 (40 nmol) in admixture with adjuvant C11C18 (40 nmol) or self-adjuvanted peptide p57 C11C18 (40 nmol). Splenocytes were obtained two weeks after the last immunisation.

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