Conference paper

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Lipidated brartemicin adjuvant *p*-C18Brar is a promising α,α′-trehalose 6,6′-dilipid for use in ovine pneumonia vaccines

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Abstract: Ovine pneumonia is a disease in sheep that is associated with major animal welfare issues and economic losses and for which there is no effective vaccine. We tested the adjuvanticity of our most promising α,α′-trehalose 6,6′-glycolipids, lipidated brartemicin adjuvants *p*-C18Brar (**3**), *o*-C18Brar (**4**), and amide-TDB (**5**) in vaccines for ovine pneumonia containing *Mannheimia haemolytica* and *Mycoplasma ovipneumoniae* whole cell antigens. *p*-C18Brar (**3**) and *o*-C18Brar (**4**) led to strong antigen-specific IgG antibody titres that were better than those elicited by the prototypical α,α′-trehalose glycolipid trehalose dibehenate (TDB, **2**) and amide-TDB (**5**). T-cell responses, as determined by measuring IFN-γ and IL-17A production from antigen-stimulated whole blood cultures, revealed that *p*-C18Brar (**3**), but not TDB (**2**), *o*-C18Brar (**4**), or amide-TDB (**5**), led to statistically significant increases in these cytokines. We then optimised the synthesis of *p*-C18Brar (**3**) (3 steps, 72 % overall yield) and undertook further vaccination studies to determine the optimal dose of *p*-C18Brar (**3**) that would be used for future large scale ovine pneumonia field trials. At a dose of 3.75 mg per vaccine, the adjuvanticity of *p*-C18Brar (**3**), as measured by levels of anti-*M. haemolytica* IgG antibody and T-cell responses (IFN-γ and IL-17A) was better than that elicited by the commercially available adjuvant Quil-A, and had reduced reactogenicity. Taken together, the excellent immunological profile of *p*-C18Brar (**3**) and its ease and efficiency of synthesis makes it an attractive adjuvant for use in veterinary vaccines.

Keywords: Adjuvants; C18Brar; ICS-30; *Mannheimia haemolytica*; Mincle; *Mycoplasma ovipneumoniae*; ovine pneumonia; trehalose glycolipids.

Introduction

Ovine pneumonia is a disease in sheep that is associated with major animal welfare issues and causes considerable economic loss to livestock industries [1, 2]. There is no effective vaccine for the disease. Ovine pneumonia is multifactorial, with interactions between different bacterial and viral pathogens, as well as predisposing factors such as host defence, the environment, and stress, effecting the genesis of the disease [3, 4]. Two pathogens involved in the onset of ovine pneumonia are *Mannheimia haemolytica*, the main pathogen responsible for

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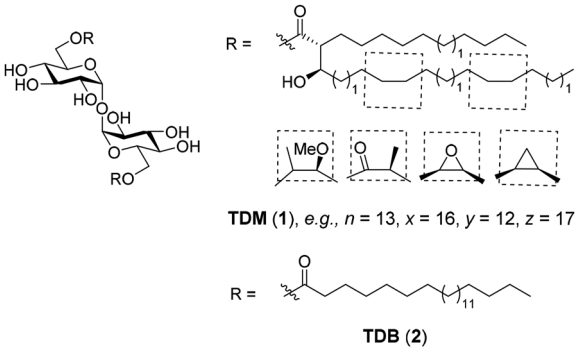


Fig. 1: α,α′-Trehalose diester Mincle agonists: trehalose dimycolate (TDM, **1**) and trehalose dibehenate (TDB, **2**).

causing lung damage, and *Mycoplasma ovipneumoniae*, which is considered a predisposing agent in sheep. Antibody responses against *M. haemolytica* and/or *M. ovipneumoniae* are considered essential for the development of effective vaccines against ovine pneumonia [4–6]. With the need for effective vaccines against ovine pneumonia, we became interested in the potential of α,α′-trehalose 6,6′-glycolipids as veterinary adjuvants. Many α,α′-trehalose 6,6′-glycolipids have been shown to signal through macrophage inducible C-type lectin (Mincle, Clec4e, or Clecsf9) [7, 8]. Mincle is a pattern recognition receptor (PRR) that is expressed on a variety of cells of the myeloid lineage and some B cells [7] and is highly conserved across mammalian species [9]. Prototypical Mincle ligands include the pathogen-associated molecular pattern (PAMP), trehalose dimycolate (TDM, **1**, Fig. 1), the major cell wall component of *Mycobacterium tuberculosis*, and the C22 derivative thereof, trehalose dibehenate (TDB, **2**) [10–13]. Upon Mincle binding, ligands such as TDM (**1**) and TDB (**2**), engage the Syk-CARD9 signalling pathway, ultimately leading to a Th1 or Th1/Th17-skewed immune response that can be harnessed to augment vaccine efficacy [7, 8, 14, 15].

Most studies have focused on the potential of Mincle ligands as adjuvants to improve human health. A notable adjuvant system involves the inclusion of TDB (**2**) as part of the cationic adjuvant formulation 01 (CAF01), a liposomal adjuvant system which also contains the cationic lipid, dimethyldioctadecylammonium bromide (DDA) [16]. CAF01 entered human trials based on promising pre-clinical results in several species [17, 18]. However, the adjuvanticity of Mincle ligands in the veterinary health space is more variable. For example, in cattle, CAF01 was found to only induce weak system immune responses [19], with vaccine efficacy being improved through the combination of multiple PAMPs [20]. In contrast, we determined that α,α′-trehalose 6,6′-glycolipids show promise as adjuvants for vaccines against ovine pneumonia when administered as single compounds in oil-in-water emulsions [21, 22]. Notably, the α,α′-trehalose 6,6′-glycolipid, *p*-C18-lipidated brartemicin (C18Brar, **3**, Fig. 2) [23], augmented antibody and T-cell responses to both *M. haemolytica* and *M. ovipneumoniae* antigens [21]. In this study, *p*-C18Brar (**3**) was compared to three commercially available adjuvants, Alum, Quil-A, and Alhydrogel + Quil-A, and found to exhibit adjuvanticity that was superior to that elicited by Alum and Alhydrogel + Quil-A, and adjuvanticity that was as good as, if not better than, that induced by Quil-A [21].

Despite the promise that α,α′-trehalose 6,6′-glycolipids have for use in vaccines against ovine pneumonia, studies to date have been limited and comparisons between the different adjuvants are difficult to make unless undertaken within the same vaccination trial. This is because the field animals used in our studies are naturally exposed to pathogens, such as *M. haemolytica* and *M. ovipneumoniae*. With this in mind, we sought to determine the optimal adjuvant for vaccines against ovine pneumonia by investigating the adjuvanticity of *p*-C18Brar (**3**) [21, 23],

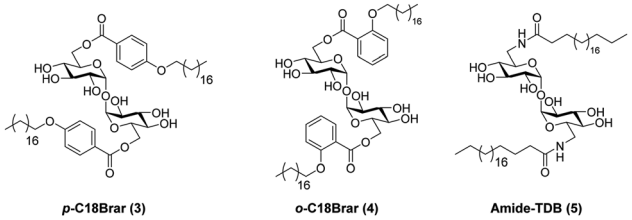


Fig. 2: Mincle agonists *p*-C18Brar (**3**), *o*-C18Brar (**4**), amide-TDB (**5**).

o-C18Brar (**4**), which was identified as a lead adjuvant from the *in vitro* screening of libraries of lipidated brartemicin derivatives [24, 25], amide-TDB (**5**) [22], and the prototypical Mincle ligand, TDB (**2**), within the same experimental set-up. The adjuvanticity of the Mincle ligands was determined by measuring antigen-specific IgG responses and T-cell responses, whereby the latter was assessed by determining IFN-γ and IL-17A production following the restimulation of whole blood cultures with antigen. The synthesis of the leading Mincle adjuvant was then optimized, and the ligand used in a further vaccination study to determine the optimal adjuvant dose and to assess how the efficacy of this adjuvant compared to Quil-A. The results of these studies are reported herein.

Materials and methods

Synthesis

General methods

Unless otherwise stated, all reactions were performed under an argon atmosphere. Methanol (Fisher Scientific), EtOAc (Pure Science), and Pet. Ether (40–60 °C, Pure Science) were distilled prior to use. Methyl 4-hydroxybenzoate (BDH) was recrystalised prior to use. TDB (**2**) [26], *o*-C18Brar (**4**) [24], amide-TDB (**5**) [22], and 2,2′,3,3′,4,4′-*hexa-O*-trimethylsilyl-α,α′-trehalose (**8**) [21, 26, 27] were synthesised as previously described. Dichloromethane (Fisher Scientific), 1-bromohexadecane (BDH), NaH (Aldrich), Pyridine (Fisher Scientific), DMF (Acros Organics, dried over molecular sieves), K_2CO_3 (Panreac), TBAI (Acros Organics), NaOH (Pure science), $MgSO_4$ (Fisher Scientific), Toluene (stored over Na wire, Carlo Erba), EDCI (Chem impex INT'L INC), DMAP (Aldrich), $NaHCO_3$ (Carlo Erba), Dowex-H^+ 50W X8 (Bio Rad), $CDCl_3$ (Sigma-Aldrich), d_5-pyridine (Cambridge Isotope Laboratories, Inc.), and AcOH (Scharlau), were used as received. All solvents were evaporated under reduced pressure. H_2O was purified using reverse osmosis (RiO Millipore). Reactions were monitored by TLC-analysis using Macherey-Nagel silica gel pre-coated plastic sheets (0.20 mm, fluorescent indicator UV-254) and visualised under UV absorption (254 nm), dipping in 10 % H_2SO_4 in EtOH followed by charring or dipping in $KMnO_4$ solution (2 % in H_2O). Column chromatography was performed using Pure Science silica gel (40–63 μm) or C18 column chromatography (Hypersep 500 mg/2.8 mL, thermoscientific). High resolution mass spectroscopic data were obtained on an Agilent 6530 Q-TOF mass spectrometer with a JetStreamTM electrospray ionisation source in positive or negative mode. Optical rotations were recorded on an Autopol II (Rudolph Research Analytical) at 589 nm (sodium D-line). Infrared spectra were obtained for the compounds (as a thin film) using a Bruker Platinum ATR and are reported in wave numbers (cm^{-1}). Nuclear magnetic resonance spectra were recorded at 20 °C in $CDCl_3$, or d_5-pyridine using a JEOL JNM-ECZ spectrometer operating at 500 or 600 MHz. Chemical shifts values given in ppm (δ) relative to residual solvent peaks. Peak assignments in NMR spectra were made using 2D-NMR experiments: COSY, HSQC, and HMBC. All synthesised adjuvants (**2**, **3**, **4** and **5**) were determined to be endotoxin free (≤0.1 EU/mL) prior to their use in the vaccination studies.

4-(Octadecyloxy)benzoic Acid (**7**). To a solution of methyl 4-hydroxybenzoate (**9**) (5.00 g, 32.9 mmol, 1 equiv.), 1-bromooctadecane (13.3 g, 39.4 mmol, 1.2 equiv.), K_2CO_3 (13.6 g, 98.6 mmol, 3 equiv.) and tetrabutylammonium iodide (1.28 g, 3.27 mmol, 0.1 equiv.) in dry DMF (120 mL) was heated to 80 °C and stirred overnight, at which point complete consumption of starting material was observed by TLC (petroleum ether/EtOAc, 24:1, v/v). The solution was cooled, and the product crashed out as a white solid. The solid was filtered, and then washed with water and ethyl acetate, and recrystalised from methanol to afford methyl 4-(octadecyloxy)benzoate as a white crystalline solid (11.8 g, 29.2 mmol, 89 %). R_f = 0.54 (petroleum ether/EtOAc, 24:1, v/v); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 (d, $J_{2,3}$ = 8.3 Hz, 2H, H-2), 6.90 (d, $J_{3,2}$ = 8.6 Hz, 2H, H-3), 4.00 (t, $J_{6,7}$ = 6.6 Hz, 2H, CH_2-6), 3.88 (s, 3H, CO_2Me), 1.79 (p, $J_{7,6} = J_{7,8}$ = 7.1 Hz, 2H, CH_2-7), 1.45 (p, $J_{8,7} = J_{8,9}$ = 7.3 Hz, 2H, CH_2-8), 1.42–1.26 (m, 28H, CH_2-9-CH_2-22), 0.88 (t, $J_{23,22}$ = 6.5 Hz, 3H, CH_3-23); ^{13}C NMR (125 MHz, $CDCl_3$) 166.9 (C-5), 163.0 (C-4), 131.6 (C-2), 122.3 (C-1), 114.0 (C-3), 68.2 (C-6), 51.8 (CO_2Me), 31.9, 29.7, 29.6, 29.5, 29.4, 26.0, 22.7 (C-8-C-22), 29.1 (C-7), 14.1 (C-23). IR (film): 2916, 2847, 1723, 1608, 1510, 1473, 1256, 850 cm^{-1}. HRMS (ESI) calcd for $[C_{26}H_{45}O_3]^+$ 405.3363; obsd, 405.3359. Characterisation data was consistent with that previously reported [23]. To a solution of methyl 4-(octadecyloxy)benzoate (3.7 g, 9.14 mmol) in MeOH (150 mL) was added NaOH (5 M, 30 mL), and the resulting solution refluxed for 48 h under atmospheric

air. Upon completion of the reaction [as gauged by TLC (petroleum ether/EtOAc, 5:1, v/v)], the excess MeOH was removed *in vacuo*. The resulting suspension was diluted with water, acidified with conc. HCl, extracted with hot toluene, dried with $MgSO_4$, filtered, and concentrated *in vacuo* to obtain 4-(octadecyloxy)benzoic acid (**7**) as a white solid (3.56 g, 9.11 mmol, 99 %). The resulting acid used without further purification. R_f = 0.40 (petroleum ether/EtOAc, 5:1, v/v); ^{1}H NMR (500 MHz, C_5D_5N) δ 8.49 (d, $J_{2,3}$ = 8.5 Hz, 2H, H-2), 7.17 (d, $J_{3,2}$ = 8.6 Hz, 2H, H-3), 4.00 (t, $J_{6,7}$ = 6.5 Hz, 2H, CH_2-6), 1.77 (p, $J_{7,6}$ = $J_{7,8}$ = 6.8 Hz, 2H, CH_2-7), 1.41–1.48 (m, 2H, CH_2-8), 1.32–1.26 (m, 28H, CH_2-9-CH_2-22), 0.88 (t, $J_{23,22}$ = 6.7 Hz, 3H, CH_3-23); ^{13}C NMR (125 MHz, C_5D_5N) δ 169.2 (C-5), 163.6 (C-4), 132.7 (C-2), 125.2 (C-1), 115.0 (C-3), 68.8 (C-6) 32.5, 30.42, 30.40, 30.37, 30.34, 30.30, 30.27, 30.1, 30.0 23.4 (C-9-C-22), 29.9 (C-7), 26.7 (C-8), 14.7 (C-23). IR (film): 2914, 2848, 1671, 1605, 1578, 1513, 1469, 1256, 1169, 846 cm^{-1}. HRMS (ESI) calcd for $[C_{25}H_{42}O_3 - H]^-$: 389.3061; obsd, 389.3062. Characterisation data was consistent with that previously reported [23].

2,2′,3,3′,4,4′-*Hexa*-*O*-trimethylsilyl-6,6′-di-(4-octadecyloxy-benzoyl)-α,α′-D-trehalose (10). 2,2′,3,3′,4,4′-*Hexa*-*O*-trimethlsilyl-α,α′-D-trehalose (**8**) [21, 26, 27] (1.00 g, 1.29 mmol) was co-evaporated with anhydrous toluene (2 × 20 mL), dissolved in anhydrous toluene (10 mL) and placed under an atmosphere of argon. EDCI (1.61 g, 8.39 mmol), DMAP (315 mg, 2.58 mmol) and carboxylic acid **10** (2.50 g, 6.4 mmol) were then added. The mixture was stirred at 70 °C for 48 h, with additional EDCI (0.49 g, 2.6 mmol) and carboxylic acid **4** (500 mg, 1.28 mmol) being added after 24 h. Upon reaction completion, the mixture was cooled and diluted with hot ethyl acetate (500 mL). The organic layer was washed with saturated NH_4Cl (200 mL), saturated $NaHCO_3$ (200 mL) and brine (200 mL), dried ($MgSO_4$), filtered and concentrated. The resultant yellow oil was purified by gradient flash silica gel chromatography (Petroleum ether to Petroleum ether:EtOAc, 95:5, *v/v*) to give the title compound as a white solid (1.75 g, 1.15 mmol, 89 %). R_f = 0.61 (Petroleum ether:EtOAc, 19:3, *v/v*); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 (d, $J_{2',3'}$ = 8.8 Hz, 4H, H-2′), 7.25 (d, $J_{2',3'}$ = 8.8 Hz, 4H, H-3′), 5.32 (d, $J_{1,2}$ = 3.1 Hz, 2H, H-1), 4.88 (dd, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6a}$ = 2.4 Hz, 2H, H-6a), 4.59 (dd, $J_{6a,6b}$ = 12.2 Hz, $J_{5,6b}$ = 3.4 Hz, 2H, H-6b), 4.46 (dt, $J_{4,5}$ = 9.4 Hz, $J_{5,6a}$ = $J_{5,6b}$ = 2.8 Hz), 4.35–4.29 (m, 6H, H-3 & H-5′), 3.99 (t, $J_{2,3}$ = $J_{3,4}$ = 9.0 Hz, 2H, H-4), 3.84 (dd, $J_{2,3}$ = 9.3 Hz, $J_{1,2}$ = 3.0 Hz, 2H, H-2), 2.13 (p, $J_{5',6'}$ = $J_{6',7'}$ = 7.1 Hz, 4H, H-6′), 1.82–1.76 (m, 4H, H-7′), 1.65–1.59 (m, 56H, H-8′ – H-21′), 1.22 (t, $J_{21',22'}$ = 6.7 Hz, 6H, H-22′), 0.52, 0.49, 0.46 (3 s, 54H, CH_3 TMS); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.4 (C=O), 163.2 (C-4′), 131.9 (C-2′), 122.3 (C-1′) 114.2 (C-3′), 94.8 (C-1), 73.8 (C-3), 72.9 (C-2), 72.1 (C-4), 71.0 (C-5), 68.4 (C-5′), 63.6 (C-6), 29.3 (C-6′), 26.2 (C-7′), 32.1, 29.85, 29.83, 29.81, 29.75, 29.72, 29.53, 29.52, 22.8 (C-8′ – C-21′) 14.3 (C-22′), 1.3, 1.1, 0.4 (TMS). Characterisation data was consistent with that previously reported [21].

6,6′-Di-(4-octadecyloxy-benzoyl)-α,α′-D-trehalose (C18Brar, 3). To 2,2′,3,3′,4,4′-*hexa*-*O*-trimethylsilyl-6,6′-di-(4-octadecyloxy-benzoyl)-α,α′-D-trehalose (0.973 g, 0.640 mmol) dissolved in CH_2Cl_2:Methanol (3:2, *v/v*, 25 mL) was added Dowex-H$^+$ (121 mg), under atmospheric air. The mixture was stirred slowly at room temperature for 3 h, at which time the reaction was deemed complete by TLC (EtOAc). The reaction mixture was then diluted with pyridine (20 mL) and filtered, and the Dowex resin washed with hot pyridine (3 × 10 mL). The filtrate was concentrated to give a white solid. This crude product was dissolved in boiling ethanol (200 mL) and then cooled. The resultant white precipitate was collected by filtration, washing with small portions of ice-cold ethanol, and dried in a desiccator, to give the title compound as a white solid (0.659 g, 0.606 mmol, 95 %). R_f = 0.31 (EtOAc) $[\alpha]_D^{20.0}$ = +67.8 (*c* = 0.1, pyridine); ^{1}H NMR (500 MHz, C_5D_5N) δ 8.29 (d, $J_{2',3'}$ = 8.6 Hz, 4H, H-2′), 6.96 (d, $J_{3',2'}$ = 8.9 Hz, 4H, H-3′), 5.98 (d, $J_{1,2}$ = 3.7 Hz, 2H, H-1), 5.27–5.31 (m, 2H, H-5), 5.21 (d, $J_{6a,5}$ = 11.4 Hz, 2H, H-6a), 5.06–5.09 (m, 2H, H-6b), 4.83 (t, $J_{3,2}$ = $J_{3,4}$ = 9.2 Hz, 2H, H-3), 4.40 (dd, $J_{2,3}$ = 9.6, $J_{2,1}$ = 3.6 Hz, 2H, H-2), 4.29 (t, $J_{4,3}$ = $J_{4,5}$ = 9.2 Hz, 2H, H-4), 3.91 (t, $J_{8,9}$ = 6.6 Hz, 4H, CH_2-8), 1.73 (p, $J_{9,10}$ = 6.8 Hz, 4H, CH_2-9), 1.39–1.45 (m, 4H, CH_2-10), 1.25–1.34 (m, 56H, CH_2-11-CH_2-24), 0.88 (t, $J_{25,24}$ = 6.8 Hz, 6H, CH_3-25); ^{13}C NMR (125 MHz, C_5D_5N) δ 166.3 (C-7), 163.1 (C-4′), 131.9 (C-2′), 124.2 (C-1′) 114.3 (C-3′), 95.7 (C-1), 74.8 (C-3), 73.2 (C-2), 71.9 (C-4), 71.5 (C-5), 68.2 (C-8), 64.7 (C-6), 31.9, 29.79, 29.76, 29.75, 29.70, 29.69, 29.65, 29.44, 29.39, 26.0, 22.7 (C-9-C-24), 14.6 (C-25). IR (film): 3428, 2917, 2850, 1710, 1686, 1607, 1511, 1469, 1254, 1168, 1100, 1077, 1052, 1036, 1021, 769 cm^{-1}. HRMS (ESI) calcd For $[C_{62}H_{102}O_{15} + Na]^+$: 1109.7111; obsd, 1109.7139. Characterisation data was consistent with that previously reported [23].

Animals

Romney cross male sheep, 6-months of age (*n* = 130), were sourced from a commercial farm and used for the comparative adjuvant study. These animals were selected from a larger group of lambs (*n* = 170) to eliminate

animals with high pre-existing serum antibody responses to *M. haemolytica* and *M. ovipnuemoniae* antigens. Animals with ELISA OD_{450} values < 0.3 at a 1:50 dilution of serum, were used for the vaccination study. All animals were grazed on pasture with water *ad libitum.* Animal ethics approval for the trial was obtained from the AgResearch Grassland's Animal Ethics Committee, Palmerston North, New Zealand.

Vaccine formulation and sampling of sheep

First vaccination study: The vaccines were prepared by combining bacterial antigens with adjuvants in a vaccine dose volume of 2 mL. *M. haemolytica* and *M. ovipneumoniae* antigens were obtained as previously described [21], with each vaccine dose containing *M. haemolytica* X387 (25 % v/v) and 0.6 mg of total protein from each of three isolates of *M. ovipneumoniae*. Trehalose glycolipids TDB **(2)**, *p*-C18Brar **(3)**, *o*-C18Brar **(4)** and amide-TDB **(5)** were dissolved in mineral oil by vortexing. Tween-80, followed by PBS, were added and the resulting emulsion mixed by vortexing and sonication for 30 min and stored at 4 °C prior to use. One day prior to vaccination, the preparation was diluted 1:1 with bacterial antigens in PBS by gentle mixing to form a homogeneous emulsion. For the first vaccination study, each vaccine dose contained 6.25 mg of either TDB **(2)**, *p*-C18Brar **(3)**, *o*-C18Brar **(4)**, or amide-TDB **(5)**. Sheep were allocated randomly to one of five groups, with each group containing 12 animals with the control group given antigens alone. Vaccines were administered by the intramuscular route in the anterior region of the neck. Animals were re-vaccinated with the same vaccine 4 weeks after the first vaccination.

Second vaccination study: For the second vaccination study, the vaccines were prepared by combining *M. haemolytica* bacterial antigens (50 % v/v) with adjuvants in a vaccine dose volume of 2.0 mL. Titrated amounts of *p*-C18Brar **(3)** (3.75 mg, 1.5 mg, and 0.5 mg per vaccine) were prepared, as noted for the first vaccination study. Sheep were allocated randomly to one of five groups, with each group containing 14 animals and one group given PBS alone as a control. Vaccines were administered *s.c.* in the anterior region of the neck at week 0. Animals were re-vaccinated with the same vaccine 4 weeks after the first vaccination.

Vaccination site monitoring

The vaccination sites for all animals were monitored daily for three days following the first and second vaccinations. Monitoring was also performed routinely on a weekly basis for up to 4 weeks after the second vaccination. There were no animal welfare concerns.

Measurement of *M. ovipneumoniae* and *M. haemolytica* antibodies

An ELISA assay was used to measure *M. ovipneumoniae* and *M. haemolytica* antigen-specific IgG responses, as previously described [21]. Blood samples were collected at week 0 (before vaccination) and 3, 4, 6, 9 and 12 weeks post-vaccination for the first vaccination study, and at week 0 (before vaccination) and weeks 4, 6, 9 and 13 weeks post-vaccination for the second vaccination study. Sera were prepared by centrifugation at 2000×*g* for 10 min at room temperature and stored at −20 °C until further analysis. For each animal, the antibody titre of each post-vaccination serum was calculated from the reciprocal of the highest dilution showing an OD_{450} value greater than the OD_{450} value of a 1:200 dilution of pre-vaccination serum.

Measurement of cellular immune responses

The ability of the adjuvants to induce cellular immune responses in the vaccinated animals was evaluated by measuring the release of IFN-γ and IL-17A from antigen-stimulated whole blood cultures, as previously described [21]. Blood samples were collected in heparinised blood collection tubes at weeks 0 and 6 for the determination of T-cell responses, with *M. haemolytica* and *M. ovipneumoniae* whole cell antigens being used at a final concentration of 14 and 10 μg/mL total cell protein, respectively. As a positive control, pokeweed mitogen (PWM) was

added to a well at a final concentration of 2.5 µg/mL, while PBS was added to another well as a negative control. Cytokine responses were measured by ELISA.

Statistical analysis

Analysis of antigen-specific antibody levels were based on mixed effects model using package 'nlme' in R version 4.1.1, as previously described [21]. Individual comparisons were made using a post-hoc multiple comparison test [28]. The *P* values from the test were adjusted by the "Benjamini-Hochberg" method to control for false discovery rate [29]. The level of significance was set at a *P* value of <0.05. For the IFN-γ and IL-17A responses a one-way ANOVA was applied to log-transformed values. *P* values <0.05 were considered statistically significant.

Results and discussion

Ovine pneumonia vaccination studies comparing different trehalose glycolipids

The ability of *p*-C18Brar (**3**), *o*-C18Brar (**4**), TDB (**2**), and amide-TDB (**5**) to augment the efficacy of vaccines for ovine pneumonia containing whole cell inactivated *M. ovipneumoniae* and *M. haemolytica* antigens was explored. In view of the poor adjuvanticity of the liposomal CAF01 adjuvant system when used for development of vaccines against bovine tuberculosis [19], we chose to deliver our adjuvants (6.25 mg per vaccine) in oil-in-water emulsions rather than in liposomes [21]. Sheep were injected subcutaneously with the ovine pneumonia vaccines twice at 4-week intervals (week 0 and week 4), and serum IgG antibody responses to *M. ovipneumoniae* and *M. haemolytica* were measured at weeks 3, 4, 6, 9 and 12.

When assessing *M. ovipneumoniae*-specific antibody titres, the sheep that were administered vaccines formulated with adjuvants had significantly higher IgG antigen-specific responses compared to antigen alone throughout the time course of the experiment ($P < 0.05$) (Fig. 3a). The adjuvants *p*-C18Brar (**3**), *o*-C18Brar (**4**), and amide-TDB (**5**) also led to higher *M. ovipneumoniae* IgG antibody titres compared to TDB (**2**), with this difference being significant at weeks 6, 9 and 12. There was no significant difference between the *M. ovipneumoniae* IgG antibody titres elicited by *p*-C18Brar (**3**), *o*-C18Brar (**4**), and amide-TDB (**5**) at any of the time-points measured.

In contrast, there was more variability in the *M. haemolytica*-specific IgG antibody titres upon vaccination of the sheep with the different adjuvants (Fig. 3b). Compared to antigen alone, *p*-C18Brar (**3**) and *o*-C18Brar (**4**) led to a significant increase in *M. haemolytica* IgG titres at all timepoints. In contrast, TDB (**2**) led to a significant increase in *M. haemolytica* IgG compared to antigen alone at weeks 6, 9 and 12, while amide-TDB (**5**) led to a significant

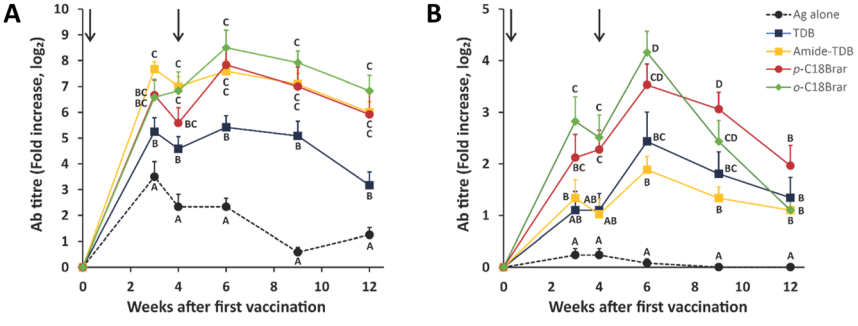


Fig. 3: IgG antibody responses to *M. haemolytica* and *M. ovipneumoniae* formulated in different adjuvants. Mean (+SE) serum IgG antibody responses to *M. ovipneumoniae* (a) and *M. haemolytica ovipneumonia* (b) in animals vaccinated with a mixture of *M. haemolytica* and *M. ovipneumoniae* whole cell antigens formulated with different adjuvants or given antigens (Ags) alone at week 0 and 4 (timing of vaccinations shown by arrows). Antibody responses were measured in serum samples collected at weeks 0, 3, 4, 6, 9, and 12 using ELISA. Different alphabetical letters indicate significant differences ($P < 0.05$) between the groups, while the same letter indicates no significant difference between groups.

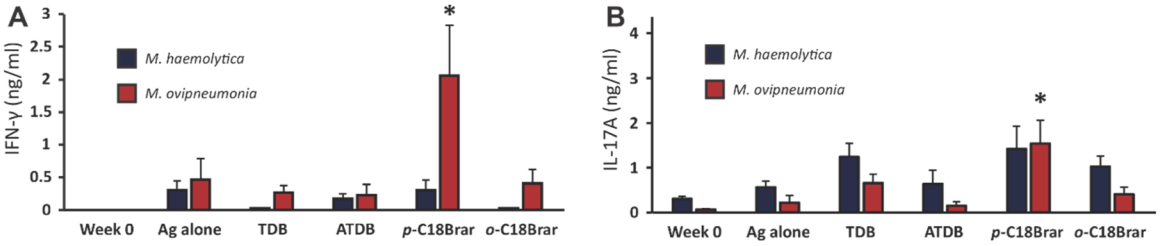


Fig. 4: Cytokine responses to *M. haemolytica* and *M. ovipneumoniae* whole cell antigens in a whole blood stimulation assay. Mean (+SE) IFN-γ (a) and IL-17A (b) responses to *M. haemolytica* and *M. ovipneumoniae* whole cell antigens at week 6 and pre-vaccination controls (Week 0). Blood samples from the vaccinated animals were stimulated for 40 h with *M. haemolytica* and *M. ovipneumoniae* whole cell antigens. Cytokine release was measured by ELISA. Significant differences are represented by * ($P < 0.05$) compared to antigens alone at week 6.

increase in IgG compared to antigen alone at weeks 3, 6, 9 and 12. Notwithstanding, there was no significance between the *M. haemolytica* IgG titres elicited by TDB (**2**) and amide-TDB (**5**). There was also no significant difference between the *M. haemolytica* IgG responses following the administration of *p*-C18Brar (**3**) or *o*-C18Brar (**4**). IgG titres for *p*-C18Brar (**3**) were significantly higher than those elicited by amide-TDB (**5**) at all time points except weeks 3 and 12, and were significantly higher than those elicited by TDB (**2**) at weeks 4, 6 and 9. IgG titres for *o*-C18Brar (**4**) were significantly higher than those elicited by amide-TDB (**5**) at all time points except that last (week 12) and were significantly higher than those elicited by TDB (**2**) at weeks 3, 4 and 6.

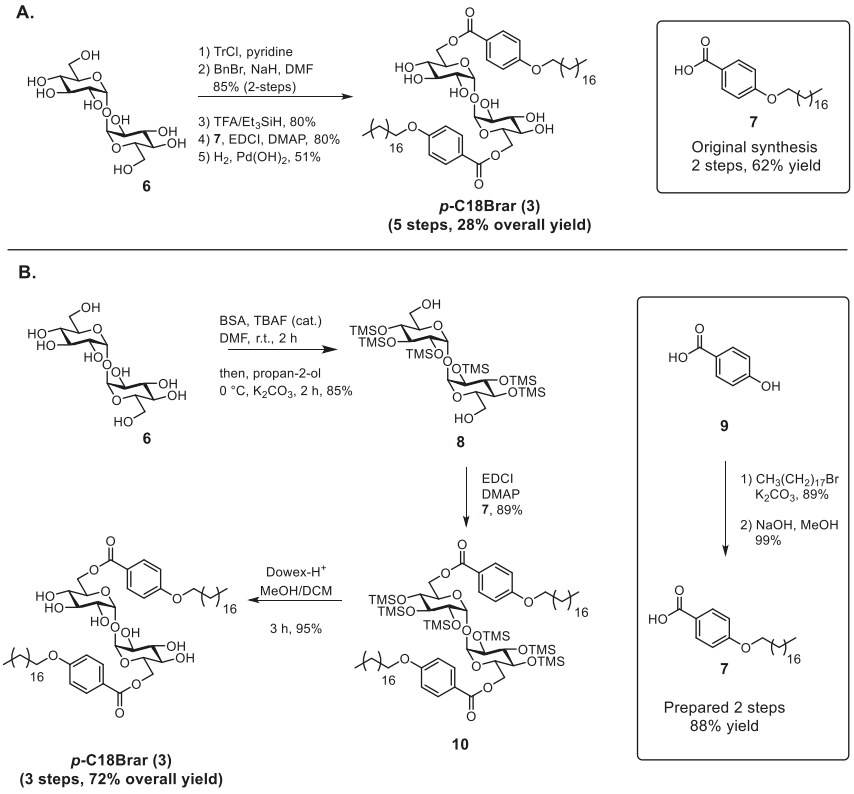
The ability of the adjuvants to induce cellular immune responses in the vaccinated animals was evaluated by measuring the release of IFN-γ and IL-17A cytokines from antigen-stimulated whole blood cultures taken at week 6. Only *p*-C18Brar (**3**) with antigen led to a statistically significant ($P < 0.01$) increase in IFN-γ and IL-17A compared to antigen alone following restimulation of the blood sample with *M. ovipneumoniae* antigens (Fig. 4a and b). None of the adjuvants led to an increase in IFN-γ or IL-17A when the blood samples were restimulated with *M. haemolytica* antigens (Fig. 4a and b).

Antibody responses against *M. ovipneumoniae* and/or *M. haemolytica* are essential for the development of effective vaccines against ovine pneumonia [4, 6]. Considering our data for the *M. ovipneumoniae* and *M. haemolytica* antigen-specific responses, it can be observed that the lipidated brartemicin derivatives *p*-C18Brar (**3**) and *o*-C18Brar (**4**) led to statistically significant IgG titres compared to antigen alone for both whole cell antigens. Of the other α,α′-trehalose 6,6′-glycolipids, TDB (**2**) resulted in adjuvanticity that was similar to that observed for *p*-C18Brar (**3**) and *o*-C18Brar (**4**) when using *M. haemolytica* antigens, while *M. ovipneumoniae* IgG responses were significantly lower ($P < 0.05$) for TDB (2) compared to the brartemicin derivatives. In contrast, amide-TDB (**5**) demonstrated good adjuvanticity when using *M. ovipneumoniae* whole cell antigens, but poor adjuvanticity when using *M. haemolytica* whole cell antigens.

In addition to strong IgG antibody responses, it is thought that a balanced humoural and cell-mediated (Th1 and Th17) immune response is best for the development of vaccines against pathogens such as *M. ovipneumoniae* and *M. haemolytica* [30–32]. Only *p*-C18Brar (**3**) led to statistically significant increases in IFN-γ and IL-17A upon the restimulation of blood samples with *M. ovipneumoniae* whole cell antigens. The lack of a strong cell-mediated response to *M. haemolytica* may be a consequence of the presence of PAMPs, such as lipopolysaccharide (LPS) [33], in the vaccine preparation, and/or the timing of the study, which may have seen sheep being naturally exposed to the pathogen while on the farm. Notwithstanding, considering the data as a whole, *p*-C18Brar (**3**) was deemed the most promising trehalose glycolipid for use in further ovine pneumonia vaccination studies. Given that whole cell antigen vaccines are often highly immunogenic and self-adjuvanting, the ability of *p*-C18Brar (**3**) to augment the humoural and cellular immune response to pathogens of relevance to ovine pneumonia was even more striking.

Improved synthesis of *p*-C18Brar (3)

In larger animals there are limited studies regarding the vaccine dose of Mincle-mediated adjuvants for optimal adjuvanticity. Moreover, cost of goods is an important limiting factor for veterinary applications, and while



Scheme 1: Synthesis of *p*-C18Brar (**3**) illustrating, a. Original route [23], and b. The optimised route.

changes to the ratio and quantity of antigen to adjuvant can be altered to improve immunogenicity, cost may be at an inhibitory factor. Insomuch, it is important that routes for the synthesis of vaccine adjuvants are optimised so that the cost-effectiveness of any potential on-farm application can be better assessed.

Previously we developed a route for the synthesis of *p*-C18Brar (**3**) commencing with α,α′-trehalose (**6**) that used a benzyl-protected trehalose intermediate [23] (Scheme 1a). While this route was fairly efficient, providing the target glycolipid in a 28 % yield over 5-steps, we thought that it could be further optimised. We also sought to improve the synthesis of methyl 4-(octadecyloxy)benzoate (**7**), which was used in the esterification reaction. To this end, we employed a new route for the synthesis of *p*-C18Brar (**3**) that commenced with the per-silylation of α,α′-trehalose (**6**) using *N,O*-bis(trimethylsilyl)acetamide (BSA) and catalytic (TBAF), followed by selective removal of the more labile primary TMS-group using K_2CO_3,to provide diol **8** in 85 % yield [26, 27] (Scheme 1b). Esterification of TMS-protective derivative with **7**, whereby the later was prepared from methyl 4-hydroxybenzoate (**9**) in 2-steps and an improved 88 % yield following the use of optimised reaction and workup conditions, then afforded the conjugated glycolipid product

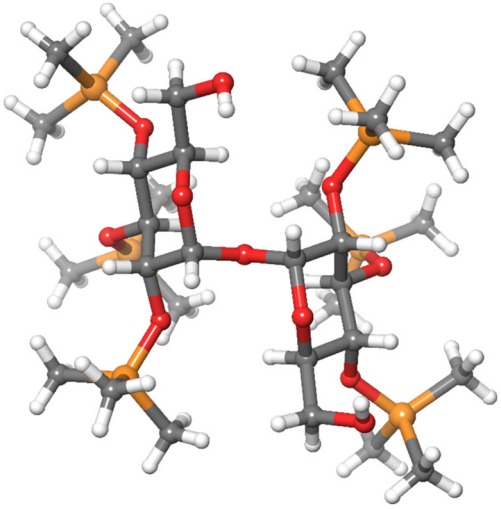


Fig. 5: X-ray single-crystal structure of 2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-α,α′-trehalose (**8**) [34].

10 in excellent yield. Finally, removal of the TMS groups under the mediation of Dowex-H^+ gave *p*-C18Brar (**3**) in a much improved 72 % yield over three linear steps. As part of the syntheses, we were also able to obtain 2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-α,α′-trehalose (**8**) as crystalline material, for which a single-crystal X-ray structure was obtained (Fig. 5).

Optimal dosage of *p*-C18Brar for ovine pneumonia vaccination studies

To determine the impact of adjuvant dose on the immune response, we then investigated the effect of different doses of *p*-C18Brar (**3**) using vaccines containing *M. haemolytica* antigens. Sheep were vaccinated with titrated doses of *p*-C18Brar (**3**) (3.75 mg, 1.5 mg, or 0.5 mg per vaccine). The efficacy of the adjuvants was compared to antigen alone, and to the immune response elicited when using Quil-A, which was the most promising commercially available adjuvant identified from our previous ovine pneumonia trials [21].

All adjuvants led to statistically significant increases *M. haemolytica* IgG titres ($P < 0.05$) compared to antigen alone, except for the lowest concentration of *p*-C18Brar (0.5 mg) at week 9 (Fig. 6a). There was no significant difference between the *M. haemolytica* IgG titres when using either 3.75 mg or 1.5 mg of *p*-C18Brar (**3**) at any of the time points measured ($P < 0.05$). However, the lowest concentration of *p*-C18Brar (0.5 mg) led to significantly lower

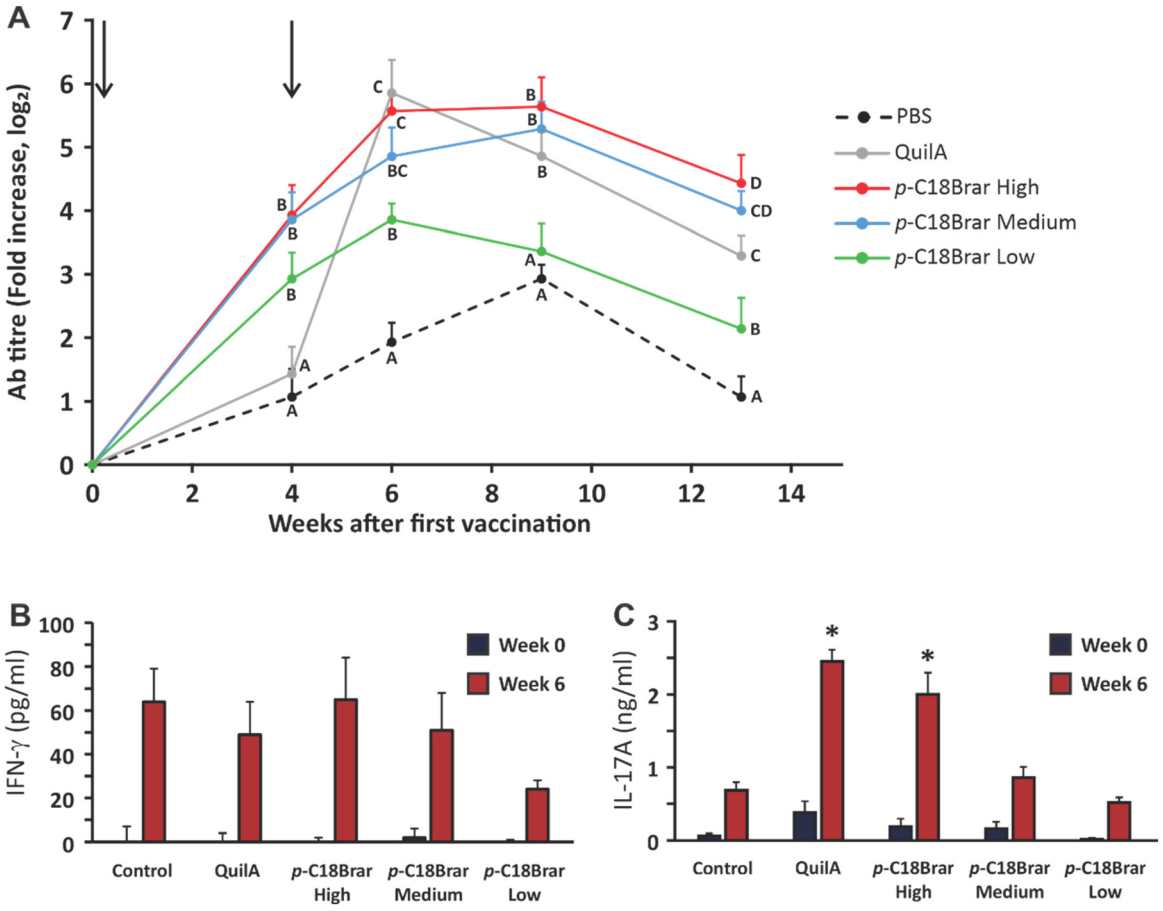


Fig. 6: Effect of the concentration of *p*-C18Brar (**3**) on *M. haemolytica* IgG titres and cytokine responses. a. Mean (±SE) serum IgG antibody responses to *M. haemolytica* in animals vaccinated with *M. haemolytica* whole cell antigens formulated with different concentrations of *p*-C18Brar (3.75 mg, 1.5 mg or 0.5 mg per vaccine), Quil-A or antigens (Ags) alone at weeks 4 and 6 (timing of vaccinations shown by arrows). Antibody responses were measured in serum samples collected at weeks 0, 4, 6, 9, and 13 using ELISA. Different alphabetical letters indicate significant differences ($P < 0.05$) between the groups, while the same letter indicates no significant difference between groups. b and c. Mean (±SE) IFN-γ (b) and IL-17A (c) responses to *M. haemolytica* whole cell antigens at week 6. Blood samples from the vaccinated animals were stimulated for 40 h with *M. haemolytica* whole cell antigens. Cytokine release was measured by ELISA. Significant differences are represented by * ($P < 0.05$) compared to Ag alone group at week 6.

levels of IgG compared to those elicited by 1.5 mg of *p*-C18Brar **(3)** at weeks 9 and 13, and significantly lower levels of IgG compared to those elicited by 3.75 mg of *p*-C18Brar **(3)** at weeks 6, 9, and 13. The IgG titres in response to Quil-A were significantly lower than those elicited by all concentrations of *p*-C18Brar **(3)** at week 4, and significantly lower than that elicited by 3.75 mg of *p*-C18Brar **(3)** at week 13.

With regard to T-cell responses, none of the adjuvant combinations led to significant increases in IFN-γ when blood samples were restimulated with *M. haemolytica* antigens (Fig. 6b). This was consistent with our findings from the first vaccination study where significant levels of IFN-γ were only observed in response to *M. ovipneumoniae* and *p*-C18Brar **(3)**. However, a significant increase in IL-17A compared to antigen alone was observed for both Quil-A and *p*-C18Brar (3.75 mg) following the restimulation of blood samples with *M. haemolytica* antigens (Fig. 6c). All concentrations of *p*-C18Brar **(3)** were also well tolerated and no animal welfare concerns were observed. Notably, at site reactogenicity for *p*-C18Brar (3.75 mg per vaccine) was lower than that observed following the administration of Quil-A (Table 1, Supporting information).

Taken together, our data demonstrates that the administration of *p*-C18Brar **(3)**, at a concentration of 3.75 mg per vaccine, appears the most promising in our ovine pneumonia vaccination studies. While *o*-C18Brar **(4)** also led to excellent *M. haemolytica* and *M. ovipneumoniae* IgG titres, *p*-C18Brar **(3)**, but not *o*-C18Brar **(4)**, led to a significant increase in IFN-γ and IL-17A following the restimulation of blood samples with *M. haemolytica,* and a significant increase in IFN-γ following the restimulation of blood samples with *M. ovipneumoniae.* This demonstrates how subtle changes to the position of the lipid (*e.g.*, *ortho*- vs. *para*-substitution) can alter immune responses.

In addition to vaccine efficacy, cost of goods plays an important role in vaccination research, particularly when developing vaccines for farm animals. With this in mind, we established a highly efficient synthesis of *p*-C18Brar **(3)** (3-steps, 72 % overall yield). Moreover, *p*-C18Brar **(3)** was synthesised as a single, homogenous compound, thus avoiding the need for complex purification procedures, such as those used for the extraction of Quil-A from the South-American tree, *Quillaja Saponaria* Molina. The immune response elicited by *p*-C18Brar **(3)** (3.75 mg per vaccine dose) was also better than that elicited by Quil-A; a finding supported by our earlier studies [21]. In the present study, *p*-C18Brar **(3)** was also found to have reduced at site reactogenicity compared to that elicited by Quil-A.

Conclusions

We demonstrated the efficacy α,α′-trehalose 6,6′-glycolipids as vaccine adjuvants when used in combination with antigens of relevance for ovine pneumonia. These studies were essential because formulations optimised for mice do not always lead to improved adjuvanticity in larger animals. Our lipidated brartemicin derivatives, *p*-C18Brar **(3)** and *o*-C18Brar **(4)** led to stronger *M. haemolytica*- and *M. ovipneumoniae*-specific IgG responses than those elicited by TDB **(2)**, a current leading Mincle-mediated adjuvant, and amide-TDB **(5)**, which had previously shown promise as a vaccine adjuvant for ovine pneumonia. When combined with antigen, *p*-C18Brar **(3)** also led to strong T-cell responses, as evidenced by statistically significant increases in IFN-γ and IL-17A following the restimulation of blood samples with *M. haemolytica,* and a significant increase in IFN-γ, following the restimulation of blood samples with *M. ovipneumoniae.* Given the optimal dose of *p*-C18Brar **(3)** required for vaccine efficacy (3.75 mg per vaccine dose) and its ease of synthesis (3 steps, 72 % overall yield), this bodes well for the potential uptake of this adjuvant for use in further vaccination trials for ovine pneumonia and potentially for other veterinary as well as human vaccine applications. Further pharmacokinetic studies will be undertaken in due course.

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Supplementary Material: This article contains supplementary material (https://doi.org/10.1515/pac-2023-0116).