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Mannosylated saponins based on oleanolic and glycyrrhizic acids. Towards synthetic colloidal antigen delivery systems

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ABSTRACT

Immunostimulatory saponin based colloidal antigen delivery systems show promise as adjuvants for subunit vaccines. For this reason, allyl oleanolate was glycosylated at the 3-position using trichloroacetimidate donors to give monodesmodic saponins following deprotection. Bisdesmodic saponins were synthesized by double glycosylation at the 3- and 28-positions of oleanolic acid. When formulated together with cholesterol and phospholipids, ring-like, helical and rod-like nanostructures were formed depending on the saponin concentrations used. As an indication of adjuvant activity, the ability of these formulations, and the saponins by themselves, to induce dendritic cell maturation was measured, but no significant activity was observed.

1. Introduction

The use of saponins (triterpene and steroidal glycosides) as adjuvants in immunotherapy has potential for the prophylactic and therapeutic treatment of a range of human conditions ranging from cancer to tropical diseases.[1–3] Adjuvants decrease the quantities of antigens required for the generation of effective immune responses as well as biasing these responses down the CD4 or CD8 cellular pathways.[4] The most commonly used adjuvant, alum (aluminium phosphate or hydroxide) promotes a Th2 type immune response which results in antibody production, but no useful $CD8^+$ cytotoxic T-cell response, which is required for the treatment of intracellular pathogens and cancer.[5] Further concerns regarding the use of alum centre on vaccine allergy and also rare reports of a chronic inflammation syndrome known as macrophagic myofascitis.[5–7] There are a number of adjuvants under development such as oil in water emulsions (MF59),[8] TLR-4 receptor agonists (monophosphoryl lipid A),[9] and γ-inulin[10] which generate a Th1 response. Select saponin adjuvants, are also known to elicit long lasting T-cell immunity and show great promise for the therapeutic treatment of diseases such as melanoma, by immunological therapy.[11–14]

The major problems associated with the clinical application of saponins are their high haemolytic activity resulting in toxicity, and their limited availability as homogenous, chemically well-defined, entities. An example is Quil A, a complex mixture of saponins isolated from *Quillaja saponaria*, and one of its components, QS-21 (**1**) (Fig. 1),[15,16] which both possess high haemolytic and adjuvant activities. Fortuitously, these two properties are independent of each other, which means the development of a saponin adjuvant for use with humans may be possible.[17] For example, hydrolysis of the glycosidic ester in **1**, giving rise to QS-L1 (**2**) (Fig. 1), decreases the toxicity profile of the saponin while retaining most of the immune modulating properties.[18–20] With many saponins, their haemolytic activities can be modulated through incorporation into stable nanostructures by complexing with cholesterol and phosphatidylcholine (PC).[21–23]

Formulated mixtures of saponin, cholesterol and phosphatidylcholine (PC) can form an array of nanostructures such as helices, thread-like and ring-like micelles, pored sheets and lamellar structures.[24] The most described structure is the ISCOM™ (Immune Stimulating Complex) particle formed from Quil A or ISCOPREP™, cholesterol and PC. Two examples of how vaccines incorporating these particles have been used are as prophylactics against bovine syntectical virus in calves[25] and therapeutics against melanoma in human clinical trials.[2,16] Under electron microscopy the complexes appear as ~40 nm pored spheres thought to be aggregates of ring-like micelles or circular annelulae held together by hydrophobic and hydrogen bonding interactions, Figure 2.[24,26,27] The size and hydrophobicity of the particles allows multiple copies of

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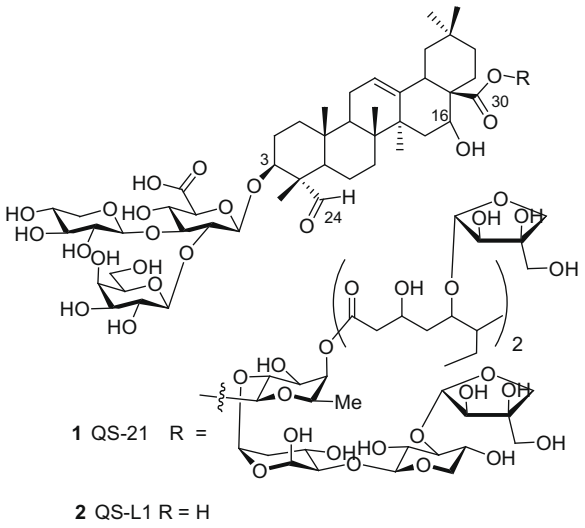


Figure 1. Adjuvant active Quillaja saponins.

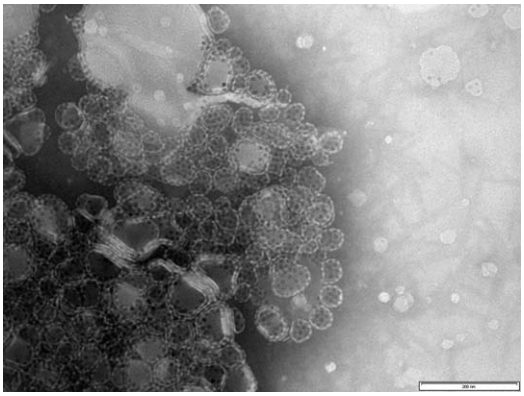


Figure 2. ISCOMs formed from Quil A/cholesterol/PC.

hydrophobic and amphipathic antigenic structures to associate with each individual particle. The close association of adjuvant and antigen means that when particles are endocytosed by antigen-presenting cells following administration of the vaccine formulation, the cell receives antigen and adjuvant at the same time thereby optimizing the immune response generated.[28]

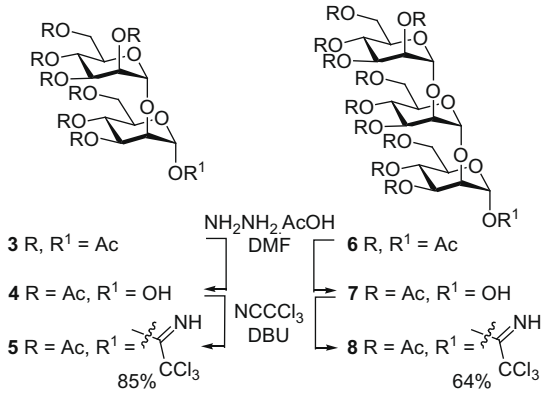
We have embarked upon a program to develop synthetic saponins that aggregate with cholesterol and lipids into colloidal particles that possess adjuvant properties without haemolytic activity. The finding that QS-L1 (**2**) is a potent adjuvant led us to target much simpler compounds than the highly complex **1**.[20] We have included mannopyranosides within the structures to target endocytotic mannose receptors located on antigen-presenting cells such as dendritic cells. These receptors are well utilised receptor targets for the delivery of antigens and furthermore, mannan, a mannose polymer is able to induce the functional maturation of dendritic cells.[29–32] Lectins (carbohydrate binding proteins) such as the mannose receptor have high affinity interactions with multivalent displays of mono- and oligosaccharides through the 'cluster glycoside effect'.[31] It was envisaged that ISCOMs incorporating *manno*-mono- and oligo-saccharides would display multiple copies of the saccharide on the particle surface, and would interact strongly with these receptors. We now wish to disclose the synthesis of some of the manno-saponins that have arisen from this project, and describe the colloidal structures formed from their association with cholesterol and phospholipid. The synthesis of a small set of manno-saponins has recently been reported by Cmoch et al. for use as potential anti-cancer agents.[33]

2. Results and discussion

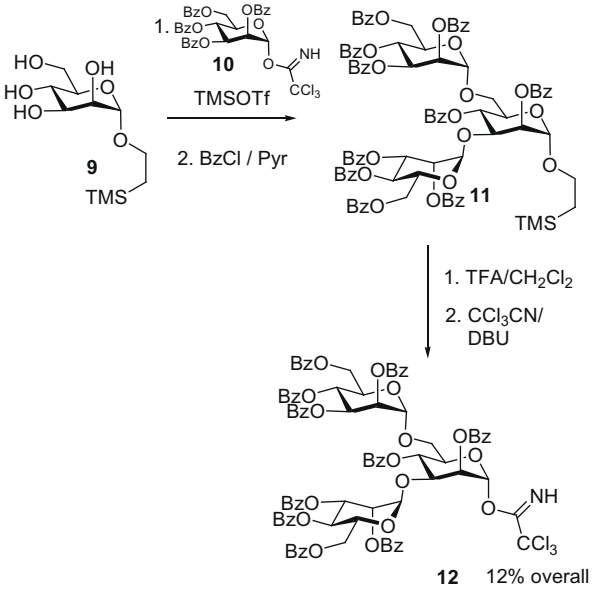
2.1. Synthesis of saponins

The α-(1→2)-mannobiose motif is recognized by the human mannose receptor.[30,34] The acetolysis of yeast mannan provides a simple route to (1→2)-substituted manno-oligosaccharides which can be conducted on a large scale, and this process was used to prepare gram scale quantities of (1→2)-mannobiose and α-(1→2)-mannotriose as their peracetates **3** and **6**.[35] These peracetates were deacylated at the anomeric sites using hydrazine acetate in DMF, then converted under standard conditions to the known trichloroacetimidates **5** and **8** in 85% and 64% yield, respectively, Scheme 1.

Mannose specific lectins such as Concanavalin A have been shown to tightly bind Man*p*-(1→3)-[Man*p*-(1→6)]-Man*p* trisaccharide containing motifs and the human mannose receptor has a preference for Man*p*-(1→6)-Man*p* structures.[30,36] In light of this, trisaccharide donor **12** was synthesized from 2-(trimethylsilyl)ethyl α-D-mannopyranoside (**9**) as indicated in Scheme 2.[37] Double glycosylation using the known mannose donor **10**,[38] and



Scheme 1.



Scheme 2.

subsequent benzoylation afforded the α,α-3,6-branched trisaccharide **11**. The configurations at the anomeric centres were assigned from the measured $^{1}J_{H\text{-}1,C\text{-}1}$ values of 171 and 173 Hz.[39] Trifluoroacetic acid mediated removal of the anomeric 2-(trimethylsilyl)ethyl group, followed by reaction with trichloroacetonitrile/DBU, gave α-configured trichloroacetimidate donor **12** in 12% overall yield in four steps. During glycosylation of the 3-hydroxy group of hindered triterpenes such as oleanolic acid, 2-acyl migration from donor to acceptor can be a significant problem. The introduction of the 2-benzoyl group into compound **12** was designed to minimize this side-reaction.[40]

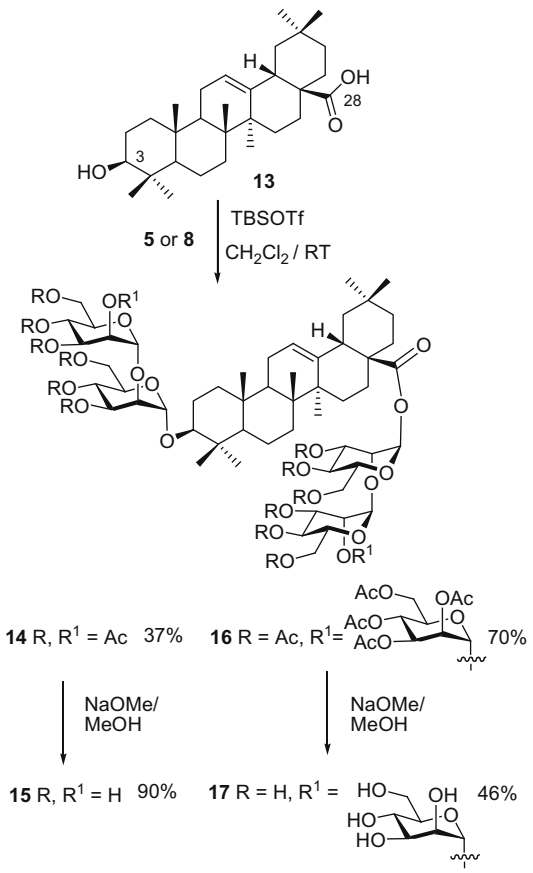
The choice of triterpene used in the synthesis of the saponins was governed by their availability for prospective scale-up and by their similarity to sapogenins found in other known adjuvants. The bioactive saponins found in Quil A are based upon the rare oleanolane-type triterpene quillaic acid which has the C-23 aldehyde functionality not often found in plant triterpenes. We substituted quillaic acid with commercially available oleanolic acid (**13**) and with glycyrrhizic acid (**27**) which has two glucuronic acids already installed as a disaccharide attached via the C-3 hydroxyl.

In one study on saponin adjuvants, Bomford et al. demonstrated that bisdesmodic saponins with branched sugars at positions 3- and 28- of the oleanane skeleton afforded ISCOM like structures.[41] Bisdesmodic saponins are most easily synthesised when the two oligosaccharides are identical, since standard glycosylations could be used at both the carboxylic acid and alcohol groups without the need for selective protection. We found the reaction of disaccharide donor **5** with acceptor **13** led to the doubly glycosylated product **14** in an acceptable yield of 37% (Scheme 3) when promoted by *tert*-butyldimethylsilyl triflate (TBSOTf). Analysis of the $^{1}J_{H\text{-}1,C\text{-}1}$ coupling constants indicated that both new anomeric centers were formed with α-selectivity with all four anomeric coupling constants being >170 Hz.[39] The selectivity of the reaction was attributed to the axial mannose at the 2-position of the donor, which effectively blocked acceptor approach from the β-face. Basic deprotection of the peracylated saponin **14** afforded the desired bisdesmoside **15** in a yield of 91% after silica-gel chromatography. There was no evidence of cleavage of the glycosidic ester under the reaction conditions. Applying the same sequence to the linear trisaccharide donor **8** gave hexa-alpha configured product **16** in a yield of 70% and subsequently **17** after methanolysis. Again the glycosidic bonds were shown to have the α-configuration.

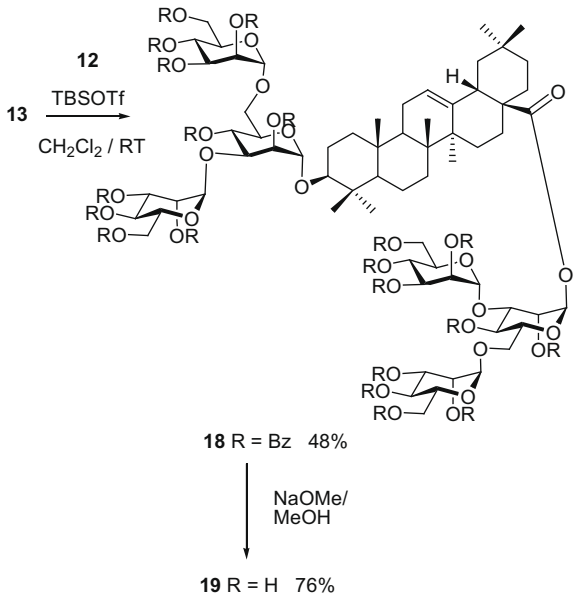
The reaction of oleanolic acid (**13**) with the branched trisaccharide donor **12** also afforded the expected hexa-glycosidic product **18** (Scheme 4). In this case, the selectivity of the glycosylation was governed by the participating 2-benzoyl group of the donor. Once the perester **18** had been deprotected, the expected molecular ion was observed by mass spectrometry. The homogeneity of the deprotected hexasaccharide **19** was established from ^{1}H and ^{13}C NMR data.

Synthesis of monodesmodic 3-glycosyl saponins using oleanolic acid is most easily performed when the C-28 acid group is protected as the allyl or benzyl ester which, respectively, enable palladium catalysed or hydrogenolytic removal of the base-stable ester groups.[42,43] Alkylation of the C-28 carboxyl group of oleanolic acid (**13**) with allyl bromide gave allyl oleanolate (**20**) (Scheme 5). Glycosylation of acceptor **20** using disaccharide donor **5** afforded ester glycoside **21** in 74% yield. The allyl ester group of **21** was removed using catalytic Pd(0)-mediated allylic displacement, and the intermediate **22** was saponified to give monodesmoside **23** in 79% yield following chromatography. Saponin purity was established using analytical RP-HPLC with a UV/VIS detector (220 nm), combined with NMR data. Likewise, the protected linear-trisaccharide substituted saponin **24** was synthesized from glycosyl donor **8** and allyl oleanolate (**20**) in a yield of 70%. Deprotection with catalytic Pd(0) afforded acid **25** which was deprotected to give the monodesmodic saponin **26**.

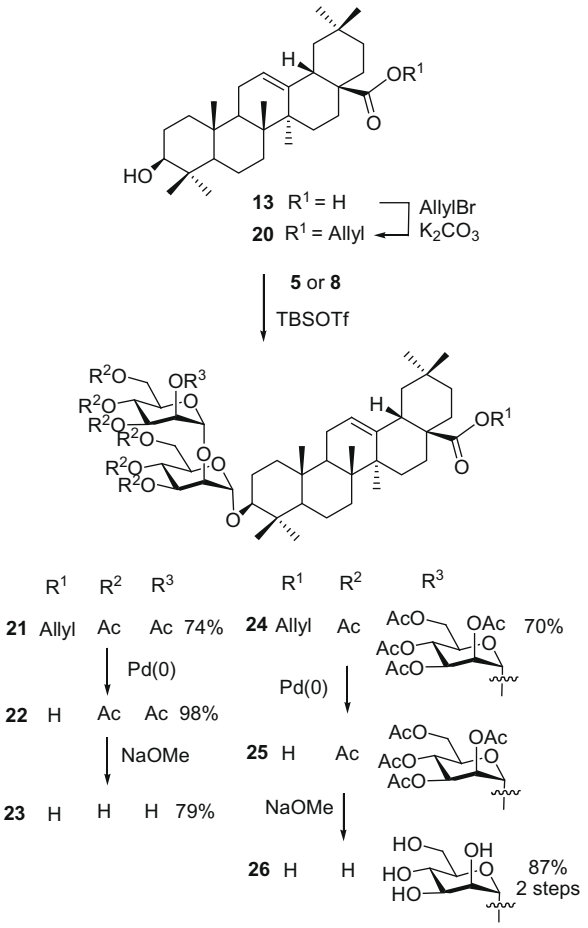
Glycyrrhizic acid is not immunogenic nor does it form ordered nanostructures with cholesterol and phospholipids. However, slight structural modifications can have dramatic effects on saponin properties, and synthetic immunomodulatory derivatives of glycyrrhizic acid have been described.[44] The effect of modifying



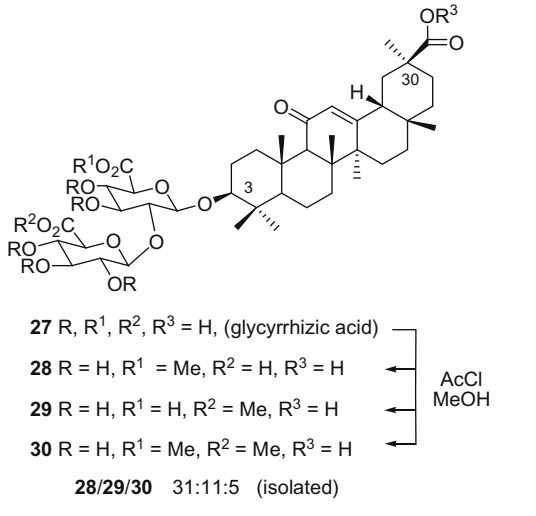
Scheme 3.



Scheme 4.



Scheme 5.



Scheme 6.

the charge on the uronic acid moieties by partial esterification was evaluated, as was the effect of incorporating mannose and glucose residues at the C-30 acid group.

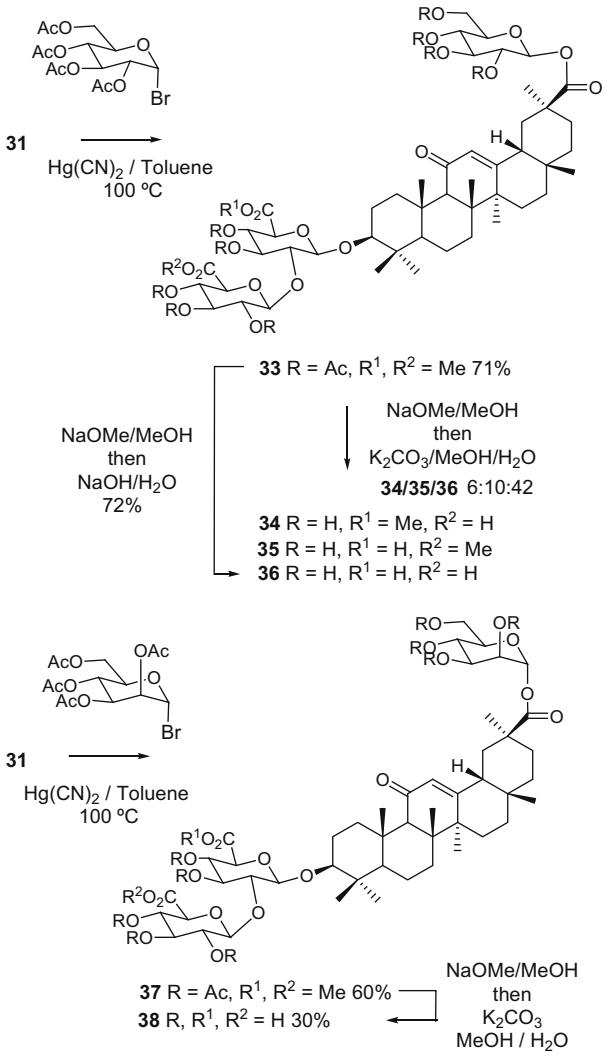
Under acidic conditions, the partial esterification of glycyrrhizic acid gave a mixture of 6′- and 6″-monomethyl esters (**28** and **29**, respectively, 20%) and a 6′,6″-dimethyl ester **30** (31%) (Scheme 6). These were separated by normal phase flash chromatography, and the monomethyl esters further purified by RP-HPLC. Assignment of the monomethyl esters was made using a combination of HSQC and HMBC spectra to establish coupling from C-6′ to C-5′ to C-1′ and thence to the C-3 of the triterpene of **28**. Similarly for **29** the C-3 resonance of the triterpene was correlated with the uronic acid carbonyl resonance. Extending the reaction time for the esterification provided an acceptable yield of 6′,6″-dimethyl ester **30** leaving the more hindered C-30 acid unreacted.[45] Peracetylation of this diester followed by an aqueous quench gave monoacid **31** ready for further glycosylation.

A low yielding synthesis of the C-30 methyl ester of glycyrrhizic acid **32** has been previously reported by alkylating the monopotassium salt with one equivalent of methyl iodide.[44] Due to the increased steric bulk about the C-30 centre, it was thought the ester at this position would have greater stability than the uronic esters of the disaccharide under basic conditions. Reaction of glycyrrhizic acid with excess methyl iodide and potassium carbonate in DMF gave the trimethyl ester which, following purification, was partially hydrolysed to give monomethyl ester **32** in good yield. The position of the methyl ester was assigned on the basis of HMBC NMR spectra, the ester carbonyl resonance showing long range interactions with several aliphatic triterpene protons.

The C-30 ester glucoside of glycyrrhizic acid, liquorice-saponin A3 (**36**), is naturally occurring in liquorice root.[45] In the present work, **36** and the corresponding mannosyl analogue **38** were synthesised by the procedure of Kitagawa et al.[45] as outlined in Scheme 7. Due to the failure of more standard conditions, $Hg(CN)_2$ was used to promote the reaction of free acid **31** with tetra-*O*-acetyl-α-D-glucopyranosyl bromide at 100 °C in toluene, affording the β-glucoside **33**. Methanolysis of this product, followed by partial saponification with potassium carbonate afforded a mixture of mono-methyl esters **34** and **35** in rather modest yields along with the diacid **36.** The separation of the two mono-methyl esters was achieved using RP-HPLC, and the assignment of the ester position was made on the basis of the HMBC spectra as above. Cleavage of the glycosidic ester bond at C-30 in these glycyrrhizic acid derivatives occurred under much milder conditions than for the C-28 oleanolyl esters, and reactions were therefore carefully monitored by TLC. Optimisation of the reaction conditions for the formation of diacid **36**, by the use of limiting amount of sodium hydroxide afforded a 72% yield following a resin neutralisation. Likewise the $Hg(CN)_2$ promoted glycosylation of **31** with tetra-*O*-acetyl-α-D-mannopyranosyl bromide afforded the α-glycoside **37** in moderate yield and subsequently the bisdesmoside **38** after monitored saponification.

2.2. TEM evidence of saponin interactions with cholesterol and phospholipids and formation of ordered nanostructures

The types and the yields of nanostructures formed from mixtures containing saponin, cholesterol and phospholipids are highly dependent on the concentration and ratios of the constituents and also the method of formulation. To probe structure/suprastructure relationships, the lipid film hydration method[46,47] was employed, whereby dried thin-films of cholesterol and phospholipids were hydrated with an aqueous solution of synthetic saponin. For some saponins, the method was modified whereby alcoholic solutions of the saponins were incorporated into the lipid film by solvent evaporation and subsequent rehydration in aqueous buffer. Previous



Scheme 7.

work by our group using Quil A had shown that with the hydration methods, the optimal formulation for ISCOM production was 60:20:20 phospholipid/cholesterol/saponin.[46] Therefore the phospholipids to cholesterol ratio was kept constant at 3:1, and varying concentrations of saponin were added. Samples were analysed by negative staining transmission electron microscopy at several time points during a 4 week time-course as these formulations may require an equilibration period. The types and numbers of nanostructures were noted. An in-depth description of the morphology of particles formed in this way from *Quillaja* saponins has been given by Rades and co- workers[24,47] and these definitions are used here to describe structures derived by the above method. The observations are summarized in Table 1.

The oleanolic acid-derived linear and branched bisdesmosides **15, 17** and **19**, all failed to interact with the lipid film. Only liposomal structures were observed which were morphologically identical to those present when the procedure was repeated in the absence of saponin**.** By contrast, formulations containing 10% of monodesmoside **23** yielded ring-like micelles associated with liposomal structures (Fig. 3a), which when left to equilibrate did not further aggregate into the characteristic ISCOM spheres. This is in contrast to Quil A derived formulations in which the ring-like micelles are not necessarily embedded in liposomal structures and can exist as discrete units.[24] At a concentration of 50% of disaccharide **23** occasional tubular structures were observed and by 70% these predominated along with helical micelles. The morphology of these helical micelles was significantly different to those seen with QS-21, Figure 3b. At a concentration of 20% of **26** initially no ring-like micelles were observed but these formed during equilibration (Fig. 3c). Increasing the concentration of saponin **26** to 50% gave rod-like or tubular structures of approximately 20 nm width, Figure 3d, and at 70% only tubular structures and thread-like micelles were present after equilibration. With the exception of ester **32,** which when formulated appeared semi-crystalline, the glycyrrhizic acid-derived structures (**28, 29, 30, 34, 35, 36** and **38**) appeared as lipidic particles at low concentration and thread-like micelles at high concentration, Figure 3e and f. Modifying the charge of the saponin by partial esterification of the uronic esters had no effect on the ability of these saponins to form ordered structures in solution.

We have also examined whether these saponins have the potential to induce the maturation of dendritic cells by measuring the expression of MHC class II and CD86, two markers expressed by dendritic cells once stimulated to mature. None of the saponins examined from this series had the ability to induce significant upregulation of CD86 or MHC class II relative to LPS or Quil A derived ISCOMs, known potent adjuvants. Incorporation of the saponins into complexes with cholesterol and PC gave a similar lack of activity (Table 1).

3. Conclusions

We have synthesized a number of neutral and negatively charged oleanolane-type saponins bearing mannose containing di- and trisaccharides, some of which form ordered nanostructures when formulated with cholesterol and phospholipids. It was found that the monodesmosides gave helical and ring-like micelles whereas the bisdesmosides did not interact significantly with the liposomal formulation. The presence of a hydrophilic group at the C-30 position of glycyrrhizic acid did not aid the formation of ordered nanostructures with this class of saponin. The saponins alone and when formulated did not induce significant dendritic cell maturation. Further studies on the immune stimulating potential of similar mannosylated saponins are underway and will be reported in due course.

4. Experimental

4.1. General

Thin layer chromatography (TLC) was performed on Silica Gel 60 F_{254} aluminium sheets (Merck) visualized under UV light and/or with a dip (reagent A: 10% w/v ammonium molybdate and 0.4% w/v ceric sulfate in an aqueous solution of 10% sulfuric acid; reagent B: 2% w/w *p*-anisaldehyde, 93% w/w ethanol, 5% w/w concd sulfuric acid) and subsequent heating. Flash column chromatography was carried out using Silica Gel 60 (230–400 mesh, Merck). All chromatography solvents were reagent grade. Anhydrous solvents were obtained from Aldrich or Acros. Specific optical rotations, given in 10^{-1} deg cm^2 g^{-1}, were measured at ambient temperature using a cell of path length 1.0 dm. Melting points (uncorrected) were measured on a Stuart® melting point apparatus SMP3. NMR spectra were recorded on a Bruker AC-300 instrument at 300 MHz (^{1}H) referenced to TMS δ 0.0 and 75 MHz (^{13}C) referenced to residual solvent peak, $CDCl_3$, δ 77.0 ppm; CD_3OD δ 49.0 ppm; pyridine-d_5 δ 124.0 ppm. High resolution mass spectra (HRMS) were recorded on a Waters Q-TOF Premier Mass Spectrometer with electro-spray ionisation (ESI). Disaccharide **3** and trisaccharide **6** were obtained from yeast mannan (Sigma) using a

Table 1
The saponins ability to form ordered nanostructures when combined with phospholipid and cholesterol and the activation of murine bone marrow derived dendritic cells (BMDC) by these formulations

Saponin	Structure[a]			Nanostructures observed after formulation using various concentrations of saponin[b]	Activation of murine BMDC[c]	
	C-3 glycoside	Triterpene	Ester glycoside		MHC class II	CD86
15	Man–Man	Olean	Man–Man	No interaction with the lipid film	ND[d]	ND[d]
17	Man–Man–Man	Olean	Man–Man–Man	No interaction with the lipid film	1	0.9
19	$(Man)_2$–Man	Olean	Man–$(Man)_2$	No interaction with the lipid film	1.3	2
23	Man–Man	Olean	H	10%: Ring-like micelles associated with liposomal structures 50%: Occasional tubular structures 70%: Mainly tubular structures along with helical micelles	1.6	1.2
26	Man–Man–Man	Olean	H	20%: Ring-like micelles form during equilibration 50%: Rod-like or tubular structures, 20 nm width 70%: Tubular structures and thread-like micelles after equilibration	1.1	0.8
28	GlcA-6MeGlcA	Glycyrr	H	Low concentration: lipidic particles High concentration: thread-like micelles	1.5	0.9
29	6MeGlcA-GlcA	Glycyrr	H	Low concentration: lipidic particles High concentration: thread-like micelles	1.4	0.9
30	6MeGlcA-6MeGlcA	Glycyrr	H	Low concentration: lipidic particles High concentration: thread-like micelles	1.1	0.9
32	GlcA-GlcA	Glycyrr	Me	Semi-crystalline	1	0.8
34	GlcA-6MeGlcA	Glycyrr	Glc	Low concentration: lipidic particles High concentration: thread-like micelles	1.3	1.3
35	6MeGlcA-GlcA	Glycyrr	Glc	Low concentration: lipidic particles High concentration: thread-like micelles	1.1	1
36	GlcA-GlcA	Glycyrr	Glc	Low concentration: lipidic particles High concentration: thread-like micelles	1.1	1
38	GlcA-GlcA	Glycyrr	Man	Low concentration: lipidic particles High concentration: thread-like micelles	1.1	1
LPS					2.7	4.8

[a] In abbreviated form. Refer to schemes for exact structure. Olean = oleanolic acid, Glycyrr = triterpenoid core of glycyrrhizic acid, Man = mannose, GlcA = glucuronic acid, Glc = glucose.
[b] Using the hydration method with the PC/cholesterol ratio set at 3:1. Refer to the text and experimental for more details.
[c] Formulations containing 10 μg of synthetic saponin or LPS were incubated with murine BMDC and the fold increase in mean fluorescence intensity of MHC class II and CD86 expression was recorded. Experiments were carried out 1–3 times.
[d] Not determined.

published procedure[34] and were converted via literature methods to trichloroacetimidates **5**[48,49] and **8.**[48,50]

4.2. 2,3,4,6-Tetra-*O*-benzoyl-α-D-mannopyranosyl-(1→3)-[2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzoyl-α-D-mannopyranosyl trichloroacetimidate (12)[51,52]

A suspension of acceptor **9**[37] (458 mg, 1.6 mmol), donor **10**[38] (2.55 g, 3.44 mmol) and flame-dried powdered 4 Å molecular sieves in acetonitrile (50 mL) under an Ar atmosphere was stirred at ambient temperature for 45 min. The flask was immersed in a dry-ice/acetone bath maintained at −40 °C and after 15 min TMSOTf (117 mg, 95 μL, 0.96 mmol) was added and the cooling bath was removed and the reaction left at ambient temperature for 45 min. The reaction was quenched by addition of triethylamine (0.5 mL, 37 mmol) and filtered through Celite. The mixture was concentrated under reduced pressure and purified by flash silica-gel column chromatography, eluting with a gradient of ethyl acetate/petroleum ether 3:7 to 1:1 giving impure trisaccharide (1.1 g) as a white foam. To a solution of this material in pyridine (5.0 mL), DMAP (30 mg, 0.25 mmol) and benzoic anhydride (860 mg, 3.8 mmol) were added and the stirred mixture was left at ambient temperature for 20 h then cooled to 0 °C and methanol (5 mL) added. After 5 min the reaction mixture was concentrated under reduced pressure and the residue taken up in CH_2Cl_2 (50 mL) and washed with 1 M HCl (3 × 25 mL) and saturated $NaHCO_3$ solution (25 mL). The organic layer was dried ($MgSO_4$) and concentrated and the residue purified by flash silica-gel column chromatography, eluting with a gradient of ethyl acetate/petroleum ether 1:4 to 1:3 to give **11** a white foam (511 mg, 19%). ^{1}H NMR ($CDCl_3$) δ 8.36–8.29 (m, 2H), 8.20–7.10 (m, 48H), 6.28–5.90 (m, 5H), 5.82–5.70 (m, 3H), 5.40–5.35 (m, 2H), 5.19–5.16 (m, 2H), 4.72–4.27 (m, 7H), 4.18 (dd, J = 5.4, 10.8 Hz, 1H), 4.07–3.94 (m, 1H), 3.82 (dd, J = 1.8, 10.8 Hz, 1H), 3.77–3.64 (m, 1H), 1.7–0.75 (m, 8H), 0.09 (s, 9H). ^{13}C NMR ($CDCl_3$) δ 166.4, 166.20, 166.16, 165.8, 165.7, 165.6, 165.32, 165.31, 165.2, 164.79, 164.77, 133.6, 133.5, 133.33, 133.32, 133.11, 133.08, 133.0, 130.3–128.2 (m), 99.7 (J_{CH} = 176 Hz), 97.9 (J_{CH} = 173 Hz), 97.2 (J_{CH} = 171 Hz), 76.7, 72.3, 70.4, 70.4, 70.3, 69.7, 69.7, 69.6, 69.5, 69.0, 68.8, 67.1, 66.8, 66.60, 66.55, 66.16, 66.11, 62.8, 62.6, 18.0, −1.17. MS (ESI): m/z 1662.8 (M+NH_4, 100%). To a solution of triethylsilyl glycoside **11** (483 mg, 0.29 mmol) in CH_2Cl_2 (5 mL) cooled in a ice/water bath was added TFA (5 mL) and the cooling bath removed. After 5 h the reaction mixture was concentrated under reduced pressure then to remove trace TFA, ethyl acetate/toluene (1:2, 15 mL) was added, removed under reduced pressure and the process repeated. To a solution of this crude hemiacetal and trichloroacetonitrile (634 mg, 440 μL, 4.4 mmol) in dry CH_2Cl_2 (5 mL) under argon at room temperature was added DBU (8 mg, 8 μL, 0.05 mmol). The solution developed a light brown colour and after 2 h was applied directly to silica-gel without concentration, eluting with ethyl acetate/petroleum ether 1:2 to give **12** as a white amorphous powder (304 mg, 62%). The ^{1}H NMR spectrum was in close agreement with the literature reports.[50,51] ^{13}C NMR ($CDCl_3$) δ 166.19, 166.16, 166.1, 165.7, 165.6, 165.3, 165.24, 165.22, 164.83, 164.76, 159.7, 134.0, 133.8, 133.51, 133.48, 133.4, 133.2, 133.1, 130.3–128.3 (m), 99.8,

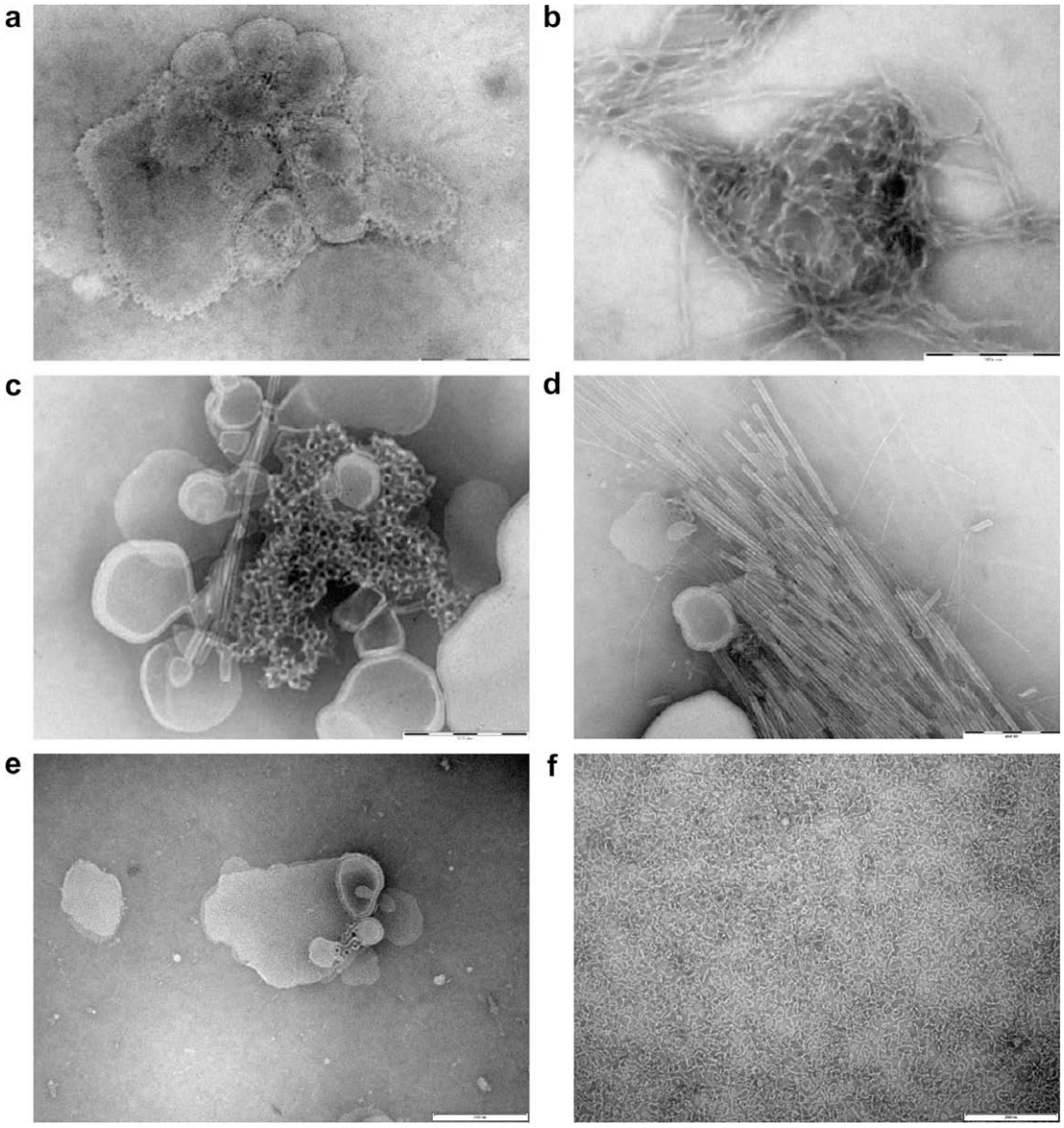


Figure 3. Transmission electron microscopy of (a) 10% formulation of **23** showing ring-like micelles associated with liposomes; (b) 70% formulation of **23** showing helical micelles; (c) 20% formulation of **26** showing ring-like micelles associated with liposomes; (d) 50% formulation of **26** showing rod-like structures; (e) lipidic particles obtained from saponin **38**; (f) worm-like micelles obtained from saponin **38**. Bar = 200 nm.

97.5, 94.5, 76.2, 72.4, 70.6, 70.4, 70.13, 70.1, 69.9, 69.4, 68.9, 67.9, 66.7, 66.5, 66.3, 62.6, 62.3.

4.3. 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl-(12)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl 3-*O*-[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(12)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl] oleanoate (14)

Oleanolic acid (**13)** (81 mg, 0.18 mmol), imidate **5** (310 mg, 0.39 mmol) and flame-dried powdered 4 Å molecular sieves (1.7 g) in CH_2Cl_2 (5 mL) were stirred under an argon atmosphere at room temperature for 45 min then TBSOTf (8 mg, 7 μL, 0.036 mmol) was added. After 45 min triethylamine (approx. 0.5 mL) was added and the mixture was filtered through Celite and concentrated. Purification of the residue by successive flash silica-gel chromatography, eluting with a gradient of ethyl acetate/petroleum ether 3:7 to 1:0 gave **14** as an amorphous colourless solid (113 mg, 37%). $[\alpha]_D^{20}$ +51.5 (*c* 1.1, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ 6.27 (d, J = 1.8 Hz, 1H, H-1′), 5.20–5.48 (m, 10H), 5.09 (d, J = 1.9 Hz, 1H, H-1‴), 4.90–4.97 (m, 2H H-1″, H-1⁗), 3.90–4.30 (m, 14H), 3.23 (dd, J = 3.6, 11.4, 1H), 2.85 (br dd, J = 3.2, 13.2 Hz, 1H), 2.15 (s, 6H), 2.13 (s, 3H), 2.12 (s, 3H), 2.09 (s, 3H), 2.08 (s, 9H), 2.06 (s, 3H), 2.04 (s, 9H), 2.01 (s, 3H), 2.00 (s, 3H), 1.95–0.70 (m, 44H). ^{13}C NMR ($CDCl_3$) δ 175.0, 170.9, 170.8, 170.8, 170.5, 170.4, 170.3, 170.0, 169.9, 169.7, 169.7, 169.6, 169.5 (×2), 169.3, 143.0, 123.2, 99.36 (J_{CH} = 176 Hz), 98.84 (J_{CH} = 173 Hz) (C-1″ and C-1⁗), 95.2 (C-1‴, J_{CH} = 171 Hz), 91.2 (C-1′, J_{CH} = 178 Hz), 84.2, 77.6, 76.0, 71.3, 70.3, 70.2, 69.8 (×2), 69.7, 69.3 (×2), 68.6, 68.5, 66.6, 66.4, 66.3, 65.7, 62.6, 62.5 (×2), 62.0, 55.7, 47.7, 47.4, 45.8, 41.8, 41.3, 39.5, 38.5 (×2), 38.2, 37.1, 33.9, 33.1, 32.8, 32.4 (×2), 30.8, 28.8, 27.6, 25.8, 23.53, 23.48, 23.33, 22.3, 20.9, 20.8 (×2), 20.8 (×2), 20.7, 20.7, 20.7, 20.7, 20.7, 20.7, 20.6, 18.4, 17.2, 16.6, 15.3. FAB-MS *m*/*z* 1736 (M^++Na, 1%), 619 (M^+−1074, 31%), 331 (M^+−1362, 50%), 169 (M^+−1524, 100%). HRMS (ESI) calcd for $[M+2(NH_4)]^{2+}$ $C_{82}H_{124}N_2O_3$: *m*/*z* 864.3936; found: 864.3956.

4.4. α-D-Mannopyranosyl-(12)-α-D-mannopyranosyl 3-*O*-[α-D-mannopyranosyl-(12)-α-D-mannopyranosyl] oleanolate (15)

To a solution of peracetate **14** (100 mg, 0.059 mmol) in methanol (10 mL) was added NaOMe (30% w/v solution in methanol, 0.2 mL). After stirring for 2 h at room temperature the solution was concentrated and the residue purified by flash silica-gel chromatography, eluting with acetonitrile/water/aq ammonia 6:1:1. The fractions containing product were concentrated to a glass then dissolved in water and the solution lyophilised to give **15** as an amorphous white solid (59 mg, 90%). $[\alpha]_D^{20}$ +82.9 (*c* 0.15, methanol). ^{1}H NMR (pyridine-d_5) δ 6.99 (br s, 1H), 6.12 (br s, 1H), 6.05 (br s, 1H), 5.80 (br s, 1H), 5.50 (br m, 1H), 4.95–4.30 (m, 24H), 3.38–3.48 (m, 1H, H-3), 3.10 (dd, *J* = 3.3, 13.3 Hz, 1H), 2.10–0.50 (m, 43H). ^{13}C NMR (pyridine-d_5) δ 175.0, 143.4, 122.4, 103.9 (J_{CH} = 170 Hz), 103.8 (J_{CH} = 170 Hz), (C-1″ and C-1‴″), 96.2 (J_{CH} = 168 Hz, C-1‴), 93.3 (J_{CH} = 175 Hz, C-1′), 82.2, 79.7, 77.7, 76.9, 75.4, 75.3, 75.1, 72.5, 72.4, 72.1, 72.0, 71.5, 71.4, 69.0, 68.9, 68.3, 68.0, 62.9, 62.8, 62.2, 62.0, 55.1, 47.3, 46.7, 45.5, 41.4, 41.2, 39.1, 38.0, 37.6, 36.4, 33.4, 32.5, 32.3, 32.0, 30.2, 28.4, 27.3, 25.4, 23.2, 23.0, 22.9, 21.8, 18.0, 16.8, 16.4, 14.8. HRMS (FAB) calcd for $[M+H]^+$ $C_{54}H_{89}O_{23}$: 1105.5795; found: 1105.5793.

4.5. 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl 3-*O*-[2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl] oleanolate (16)

Oleanolic acid **(13)** (62 mg, 0.14 mmol), imidate **8** (317 mg, 0.30 mmol) and flame-dried powdered 4 Å molecular sieves (1.8 g) in CH_2Cl_2 (30 mL) were stirred under an argon atmosphere at room temperature for 45 min then TBSOTf (6 mg, 5 μL, 0.027 mmol) was added. After 60 min. triethylamine was added (approx. 0.5 mL) and the reaction mixture was filtered through Celite and concentrated. The residue was purified by successive flash silica-gel chromatography, eluting with a gradient of methanol/$CHCl_3$ 1:400 to 1:200 to give **16** as an amorphous solid (222 mg, 70%). $[\alpha]_D^{20}$ +43.2 (*c* 0.95, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ 6.26 (d, *J* = 1.7 Hz, 1H), 5.15–5.48 (m, 15H), 5.10–5.13 (m, 2H), 5.04 (d, *J* = 2.8 Hz, 1H), 4.97 (d, *J* = 1.5 Hz, 1H), 4.95 (d, *J* = 1.6 Hz, 1H), 3.92–4.32 (m, 22H), 3.25 (dd, *J* = 3.7, 11.5 Hz, 1H), 2.84 (dd, *J* = 3.3, 13.0 Hz, 1H), 1.80–2.40 (m, 66H), 1.10–1.80 (m, 19H), 1.01 (s, 3H), 0.92 (s, 9H), 0.80 (s, 3H), 0.73 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 174.9, 170.9, 170.8, 170.8, 170.5, 170.4, 170.1, 170.0, 169.8, 169.7, 169.7, 169.5, 169.5, 169.5, 169.3, 169.3, 142.8, 123.1, 100.0 (J_{CH} = 173 Hz), 99.4 (J_{CH} = 172 Hz), 99.2 (J_{CH} = 172 Hz), 99.0 (J_{CH} = 176 Hz), 95.1 (J_{CH} = 169 Hz), 91.3 (J_{CH} = 178 Hz), 83.9, 77.4, 76.7, 75.5, 71.3, 70.4, 70.0, 69.9, 69.8, 69.7, 69.6 (×3), 69.5, 69.1 (×2), 68.5, 68.4, 67.3, 66.4 (×2), 66.3, 66.2, 66.1, 65.7, 65.6, 62.5 (×2), 62.2 (×2), 58.1, 55.7, 47.7, 47.3, 45.8, 41.8, 41.2, 39.4, 38.4, 38.1, 37.0, 33.9, 33.0 (×2), 32.9, 32.4, 30.7, 28.7, 27.6, 25.7, 23.5 (×2), 22.2, 20.88, 20.86, 20.78, 20.77, 20.74, 20.70, 20.67, 20.65, 20.63, 20.60, 18.4, 17.1, 16.5, 15.3. HRMS (ESI) calcd for $[M+2(NH_4)]^{2+}$ $C_{106}H_{156}O_{53}N_2$: *m/z* 1152.9798; found: 1152.9733.

4.6. α-D-Mannopyranosyl-(1→2)-α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl 3-O-[α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl-(1→2) α-D-mannopyranosyl] oleanolate (17)

Hexasaccharide **16** (198 mg 0.087 mmol) was dissolved and stirred in 7 N ammonia in methanol at ambient temperature. After 36 h the volatiles were removed and the residue purified by flash silica-gel chromatography, eluting with a gradient of acetonitrile/water/aq ammonia 6:1:1 to 3:1:1. This material was taken up in water (10 mL) and filtered (0.45 μm PTFE syringe filter) and freeze-dried to give **17** (58 mg, 46%). $[\alpha]_D^{20}$ +83.1 (*c* 0.18, methanol). ^{1}H NMR (pyridine-d_5) δ 6.28–5.85 (m, 20H), 6.88 (s, 1H), 6.25 (s, 1H), 6.19 (s, 1H), 6.02 (br s, 2H), 5.69 (s, 1H), 5.34 (br s, 1H), 5.15–4.80 (m, 36H), 3.37 (br dd, *J* = 3.9, 11.0 Hz, 1H), 3.06 (br dd, *J* = 3.5, 13.4 Hz, 1H), 2.05–0.45 (m, 22H), 1.13 (s, 3H), 1.07 (s, 3H), 0.91 (s, 3H), 0.88 (s, 6H), 0.75 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (pyridine-d_5) δ 174.9, 143.4, 122.4, 103.6 (J_{CH} = 171 Hz) (×2), 102.3 (J_{CH} = 172 Hz) (×2), 96.2 (J_{CH} = 167 Hz), 93.1 (J_{CH} = 175 Hz), 82.3, 79.8, 77.8, 77.6, 77.5, 76.8, 75.3, 75.2, 75.1, 75.1, 75.0, 72.5, 72.4, 72.3, 72.2, 72.0, 71.9, 71.5, 71.4, 69.1, 69.0, 68.9, 68.8, 68.6, 68.5, 67.9, 62.8, 62.7, 62.6, 62.2, 62.1, 55.1, 47.3, 46.7, 45.5, 41.4, 41.2, 39.1, 37.9, 37.6, 36.5, 33.4, 32.5, 32.4, 32.0, 30.2, 28.4, 27.4, 25.4, 23.2, 23.0, 22.9, 21.8, 17.8, 16.8, 16.3, 14.9. HRMS (FAB) calcd for $[M+H]^+$ $C_{66}H_{109}O_{53}$: 1429.6851; found: 1429.6868.

4.7. α-D-Mannopyranosyl-(1→3)-[α-D-mannopyranosyl-(1→6)]-α-D-mannopyranosyl 3-*O*-{α-D-mannopyranosyl-(1→3)-[α-D-mannopyranosyl-(1→6)]-α-D-mannopyranosyl} oleanolate (19)

Oleanolic acid (**13**), 35 mg, 0.077 mmol), imidate **12** (287 mg, 0.17 mmol) and flame-dried powdered 4 Å molecular sieves (1.6 g) in CH_2Cl_2 (20 mL) were stirred under an argon atmosphere at room temperature for 45 min and then TBSOTf (4 mg, 3 μL, 0.018 mmol) was added. Complete loss of donor was observed after 75 min at which time triethylamine was added (approx. 0.5 mL) and the reaction mixture was filtered through Celite and concentrated under reduced pressure. Purification by flash silica-gel chromatography, eluting with a gradient of ethyl acetate/petroleum ether 1:4 to 3:7 gave hexasaccharide **18** as a colourless foam (129 mg, 48%). $[\alpha]_D^{20}$ -39.4 (*c* 0.31, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ 8.4–7.50 (m, 100H, m), 6.47 (d, *J* = 1.6 Hz, 1-H, 1H), 6.24–5.92 (m, 5H), 5.86 (dd, *J* = 3.2, 10.1 Hz, 1H), 5.81–5.71 (m, 5H), 5.70–5.66 (m, 1H), 5.65–5.61 (m, 1H), 5.45–5.31 (m, 5H), 5.23 (br s, 1H), 5.14–5.10 (m, 2H), 4.67–4.24 (m, 16H), 4.23–4.07 (m, 3H), 3.90 (d, *J* = 9.7 Hz, 1H), 3.78 (d, *J* = 9.4 Hz, 1H), 3.23 (dd, *J* = 3.6, 11.1 Hz, 1H), 2.95 (dd, *J* = 3.2, 13.0 Hz, 1H), 2.00–0.40 (m, 22H), 1.11 (s, 3H), 1.08 (s, 3H), 0.94 (s, 3H), 0.85 (s, 3H), 0.78 (s, 3H), 0.61 (s, 3H), 0.58 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 175.0, 166.4, 166.2 (×2), 166.1, 166.0, 165.9, 165.8, 165.7, 165.6, 165.5, 165.29, 165.28 (×2), 165.21, 165.09, 165.07, 165.04, 165.01, 164.8, 164.7, 144.5, 133.9, 133.8, 133.7, 133.6, 133.5, 133.4, 133.3, 133.0, 130.3–128.2 (m), 122.3, 100.1 (J_{CH} = 178 Hz), 99.8 (J_{CH} = 172 Hz), 97.74 (J_{CH} = 174 Hz), 97.71 (J_{CH} = 174 Hz), 94.3 (J_{CH} = 172 Hz), 89.8 (J_{CH} = 179 Hz), 84.5 (C-3), 78.9 (CH), 78.0, 73.0, 72.5, 70.8, 70.7, 70.5, 70.4 (×2), 70.3, 70.2 (×2), 69.9 (×2), 69.7, 69.6, 68.9 (×2), 68.3, 67.5, 67.1, 67.0, 66.8, 66.5, 66.4, 66.1, 62.8, 62.6, 62.5, 62.3, 55.4, 47.6, 47.5, 45.9, 41.9, 41.6, 39.4, 38.4, 37.7, 36.7, 33.6, 33.1, 32.9, 32.5, 30.8, 29.0, 27.5, 25.7, 23.6, 23.4 (×2), 22.0, 18.3, 17.5, 16.6, 15.0. To a stirred solution of hexasaccharide **18** (129 mg, 0.037 mmol) in CH_2Cl_2 (5 mL) at ambient temperature was added NaOMe (1% solution in methanol, 10 mL). After 10 min a white precipitate formed and the suspension was stirred for 18 h. The reaction mixture was concentrated under reduced pressure to a volume of approximately 2 mL and methanol (10 mL) was added. The homogeneous solution was left at ambient temperature for 2 h then neutralized with Dowex® 50WX8–100 (H^+ form). The resin was filtered off and rinsed with methanol and the combined filtrates concentrated. The oil obtained was re-treated with NaOMe (1% solution in methanol, 15 mL) and left at ambient temperature for 18 h. The mixture was neutralised, filtered and concentrated in a manner similar to that described above. The target compound was purified using flash silica-gel chromatography, eluting with ethanol/ethyl acetate/water 1:2:0.5. The white foam thus obtained was taken up in water (10 mL), filtered (0.45 μm PTFE syringe filter) and freeze-dried to give **19** as a fluffy white solid (40 mg, 76%). $[\alpha]_D^{20}$ +104 (*c* 0.21, methanol). ^{1}H NMR (pyridine-d_5) δ

7.10–5.75 (m, 20H) 6.75 (d, J = 1.6 Hz, 1H), 6.09 (s, 2H), 5.65 (s, 2H), 5.46 (br s, 1H), 5.36 (s, 1H), 5.00–4.30 (m, 36H), 3.34 (dd, J = 4.0, 11.2 Hz, 1H), 3.16 (dd, J = 2.7, 13.0 Hz, 1H), 2.11–0.71 (m, 22H), 1.22 (s, 3H), 1.11 (s, 3H), 0.97 (s, 3H), 0.95 (s, 3H), 0.90 (s, 3H), 0.85 (s, 3H), 0.77 (s, 3H). ^{13}C NMR (pyridine-d_5) δ 175.3, 143.4, 122.5, 103.5 (J_{CH} = 172 Hz), 103.2 (J_{CH} = 168 Hz), 101.6 (J_{CH} = 169 Hz), 101.2 (J_{CH} = 169 Hz), 98.0 (J_{CH} = 167 Hz), 94.6 (J_{CH} = 173 Hz), 82.4, 80.5, 76.1, 74.9, 74.8, 74.6, 74.5, 73.9, 72.6, 72.54, 72.51, 72.5, 71.8, 71.7, 71.5 (×2), 71.4, 69.6, 69.0, 68.7, 68.6, 66.6, 66.5, 66.2, 65.7, 62.6, 62.5 (×3), 55.1, 47.3, 46.9, 45.5, 45.4, 41.5, 41.2, 39.2, 38.0, 37.6, 36.6, 33.5 (×2), 32.5, 32.2, 30.3, 28.4, 27.4, 25.4, 23.3, 23.0, 22.9, 21.9, 18.0, 17.0, 16.3, 14.9. HRMS (FAB) calcd for $[M+Na]^+$ $C_{66}H_{109}NaO_{33}$: m/z 1451.6671; found 1451.6634.

4.8. 3-*O*-[α-D-Mannopyranosyl-(1→2)-α-D-mannopyranosyl] oleanolic acid (23)

Allyl oleanolate (**20**) (145 mg, 0.291 mmol), imidate **5** (1.1 equiv, 250 mg, 0.32 mmol) and activated 4 Å molecular sieves were suspended in CH_2Cl_2 (10 mL) and stirred under an Ar atmosphere at room temperature for 45 min and then TBSOTf (0.1 equiv, 7 μL, 0.029 mmol) was added. After all donor had been consumed (TLC, 1 h) triethylamine (0.5 mL) was added and the reaction filtered through a pad of Celite. The volatiles were removed under reduced pressure and crude product purified by flash chromatography, eluting with a gradient of ethyl acetate/petroleum ether 3:7 to 6:4 to give **21** as a clear oil (240 mg, 74%). ^{1}H NMR ($CDCl_3$) δ 5.95–5.85 (m, 1H), 5.41 (dd, J = 3.3, 9.9 Hz, 1H), 5.35–5.1 8 (m, 7H), 5.09 (d, J = 2.0 Hz, 1H), 4.94 (d, J = 1.7 Hz, 1H), 4.54–4.51 (m, 2H), 4.27–3.97 (m, 5H), 3.86 (m, 1H), 3.26–3.21 (m, 1H), 2.92–2.86 (m, 1H), 2.15 (s, 3H), 2.12 (s, 3H), 2.08 (s, 6H), 2.04 (s, 3H), 2.00 (s, 3H), 1.99–1.82 (m, 2H), 1.72–0.96 (m, 24H), 1.13 (s, 3H), 1.01 (s, 3H), 0.93 (s, 3H), 0.92 (s, 3H), 0.90 (s, 3H), 0.82 (s, 3H), 0.73 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 177.7, 171.2, 170.8, 170.7, 170.2, 170.1, 169.8 (×2), 144.2, 132.9, 122.7, 118.1, 99.1, 95.4, 84.4, 77.9, 70.6, 70.1, 69.5 (×2), 68.8, 66.9, 66.7, 65.2, 62.9, 62.8, 56.0, 48.0, 47.1, 46.2, 42.0, 41.7, 39.8, 38.7, 38.5, 37.3, 34.3, 33.4, 33.1, 32.8, 31.1, 29.1, 28.0, 26.2, 24.0, 23.8, 23.4, 22.5, 21.4, 21.2, 21.0 (×5), 18.7, 17.4, 16.9, 15.7. To a solution of glycoside **21** (190 mg, 0.170 mmol) in CH_2Cl_2/methanol (1:1, 6 mL) under Ar was added $Pd(0)(PPh_3)_4$ (0.05 equiv, 10 mg, 0.008 mmol) and triphenylphosphine (0.2 equiv, 10 mg, 0.038 mmol). The reaction was stirred for 3 h at room temperature then an additional portion of $Pd(0)(PPh_3)_4$ (0.05 equiv, 10 mg, 0.008 mmol) and triphenylphosphine (0.2 equiv, 10 mg, 0.038 mmol) were added. After stirring for an addition 2 h, the solvent was removed under reduced pressure and crude product purified by flash chromatography, eluting with a gradient of ethyl acetate/petroleum ether 4:6 to 1:0 to afford **22** as a yellow oil (180 mg, 98%). ^{1}H NMR ($CDCl_3$) δ 5.41 (dd, J = 9.9, 3.3 Hz, 1H), 5.32–5.25 (m, 5H), 5.09 (d, J = 1.9 Hz, 1H), 4.94 (d, J = 1.6 Hz, 1H), 4.27–4.04 (m, 5H), 3.99–3.98 (m, 1H), 3.25–3.21 (m, 1H), 2.84–2.81 (m, 1H), 2.15 (s, 3H), 2.12 (s, 3H), 2.08 (s, 6H), 2.04 (s, 6H), 2.00 (s, 3H), 1.86–1.30 (m, 21H), 1.13 (s, 3H), 1.01 (s, 3H), 0.93 (s, 3H), 0.92 (s, 3H), 0.90 (s, 6H), 0.82 (s, 3H), 0.75 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 182.8, 170.9, 170.5, 170.3, 169.8, 169.7, 169.5 (×2), 143.8, 122.4, 98.8, 95.1, 84.1, 77.6, 70.3, 69.8, 69.2 (×2), 68.5, 66.5, 66.4, 62.6, 62.4, 55.6, 47.7, 46.5, 46.0, 41.7, 41.0, 39.4, 38.4, 38.1, 37.0, 33.8, 33.1, 32.7, 32.4, 30.7, 28.8, 27.7, 25.9, 23.6, 23.5, 23.0, 22.2, 20.7 (×7), 18.3, 17.1, 16.5, 15.3. To this oil (**22**) (175 mg, 0.163 mmol) dissolved in methanol (5 mL) was added sodium methoxide (30% w/v solution in methanol, 5 μL) and the mixture stirred at room temperature under Ar for 16 h. The reaction was neutralized with Dowex resin (H^+), filtered and solvent removed under reduced pressure. Purification by flash chromatography, eluting with $CHCl_3$/methanol/water 6:4:0.5 afforded title compound **23** as an amorphous white solid (100 mg, 79%). $[\alpha]_D^{24}$ +84.3 (c 0.48, methanol). ^{1}H NMR ($CDCl_3$) δ 5.23 (br s, 2H), 4.94 (br s, 2H), 4.52 (br s, 2H), 3.97–3.96 (m, 1H), 3.84–3.54 (m, 11H), 2.86–2.83 (m, 1H), 2.05–0.75 (m, 28H), 1.15 (s, 3H), 1.03 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.90 (s, 3H), 0.82 (s, 3H), 0.81 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 184.8, 148.2, 126.5, 107.1, 99.4, 86.8, 84.5, 78.1, 77.9, 75.4, 75.2, 74.8, 71.9, 71.8, 66.0 (×2), 59.8, 52.0, 50.6, 50.2, 45.8, 45.7, 43.5, 42.3, 42.2, 41.1, 37.8, 37.0, 36.8, 36.5, 34.5, 32.4, 31.8, 29.3, 27.5, 27.0, 26.9, 25.9, 22.3, 20.7, 20.0, 18.9; HRMS (ESI) calcd for $[M-H]^+$ $C_{42}H_{67}O_{13}$: 779.4582; found: 779.4595.

4.9. Allyl 3-*O*-[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl] oleanolate (24)

Oleanolic acid (**13**) (103 mg, 0.207 mmol), imidate **8** (201 mg, 0.188 mmol) and flame-dried powdered 4Å molecular sieves (1.8 g) in CH_2Cl_2 (20 mL) were stirred under an argon atmosphere at room temperature for 45 min then TBSOTf (6 mg, 5 μL, 0.027 mmol) was added. When no more donor was apparent (75 min, TLC), triethylamine (approx. 0.5 mL) was added and the reaction mixture was filtered through Celite and concentrated under reduced pressure. The residue was purified by flash silica-gel chromatography, eluting with ethyl acetate/petroleum ether 3:7 then 1:1 to give **24** as an amorphous solid (184 mg, 70%). An analytical sample of **24** was provided following further flash silica-gel chromatography, eluting with 1:99 methanol/$CHCl_3$. $[\alpha]_D^{20}$ +62 (c 0.62, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ 5.98–5.92 (m, 1H), 5.48–4.92 (m, 13H), 4.60–4.45 (m, 2H), 4.34–3.90 (m, 11H), 3.30–3.18 (m, 1H), 2.89 (dd, J = 3.8, 13.7 Hz, 1H), 2.18–0.66 (m, 22H), 2.15 (s, 3H), 2.13 (s, 3H), 2.11 (s, 3H), 2.08 (s, 3H), 2.07 (s, 6H), 2.06 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.26 (s, 3H), 1.14 (s, 3H), 1.01 (s, 3H), 0.93 (s, 3H), 0.92 (s, 3H), 0.90 (s, 3H), 0.80 (s, 3H). ^{13}C NMR ($CDCl_3$) δ 177.3, 170.8 (2x), 170.5, 170.1, 170.0, 169.8, 169.7, 169.5, 169.4, 169.3, 143.8, 132.6, 122.3, 117.7, 99.2 (J_{CH} = 172 Hz), 99.6 (J_{CH} = 173 Hz), 95.0 (J_{CH} = 170 Hz), 83.8, 76.7, 76.0, 69.93, 69.8, 69.8, 69.7, 69.4, 69.1, 68.5, 67.3, 66.2, 66.1, 64.8, 62.5, 62.4, 62.1, 55.7, 47.7, 46.8, 45.9, 41.7, 41.4, 39.5, 38.4, 38.1, 37.0, 33.9, 33.1, 32.8, 32.5, 30.7, 28.7, 27.7, 25.9, 23.6, 23.5, 23.1, 22.2, 20.8, 20.75, 20.74, 20.71, 20.70, 20.67 (×2), 20.65, 20.63 (×2), 18.4, 17.0, 16.5, 15.3. HRMS (ESI) calcd for $[M+Na]^+$ $C_{71}H_{102}O_{28}Na_1$: 1425.6455, found 1425.6466. Anal. Calcd for $C_{71}H_{102}O_{28}$: C, 60.76; H, 7.32. Found: C, 60.31; H, 6.83;

4.10. 3-*O*-[α-D-Mannopyranosyl-(1→2)-α-D-mannopyranosyl-(1→2)-α-D-mannopyranosyl] oleanolic acid (26)

A degassed solution of ammonium formate in dioxane (0.38 M, 2.0 mL, 0.75 mmol) was added to a round bottomed flask containing glycoside **24** (70 mg, 0.050 mmol), $Pd(OAc)_2$ (5.6 mg, 0.025 mmol) and PPh_3 (39 mg, 0.15 mmol). The mixture was stirred under an argon atmosphere at ambient temperature for 90 min then a further portion of PPh_3 (10 mg, 0.038 mmol) was added. After 4 h the mixture was concentrated in vacuo and partially purified by flash silica-gel chromatography to give impure acid **25** (76 mg). This material was dissolved in methanol (5 mL) and sodium methoxide (30% w/v solution in methanol, 100 μL) was added (pH ~10). After 4 h stirring at ambient temperature the mixture was neutralised with Dowex® 50WX8-100 resin, filtered, the resin washed with methanol and the crude reaction product was pre-adsorbed onto silica. Flash silica-gel chromatography, eluting with methanol/$CHCl_3$/water 6:4:0.25 gave **26** as a glass (41 mg, 87%). $[\alpha]_D^{20}$ +84 (c 0.21, methanol). ^{1}H NMR

(pyridine-d_5) δ 6.18 (s, 1H), 6.01 (s, 1H), 5.70 (s, 1H), 5.47 (br s, 1H), 4.92 (br s, 1H), 4.77 (br s, 1H), 4.73–4.45 (m, 12H), 4.44–4.24 (m, 4H), 3.40 (dd, J = 3.5, 11.2 Hz, 1H), 3.29 (dd, J = 3.2, 13.1 Hz, 1H), 2.26–1.70 (m, 9H), 1.59–0.57 (m, 13H), 1.24 (s, 3H), 1.10 (s, 3H), 1.02 (s, 3H), 0.97 (s, 3H), 0.96 (s, 3H), 0.75 (s, 3H), 0.72 (s, 3H). ^{13}C NMR (pyridine-d_5) δ 180.2, 144.9, 122.5, 104.1 (J_{CH} = 170 Hz), 102.8 (J_{CH} = 174 Hz), 96.7 (J_{CH} = 167 Hz), 82.8, 80.3, 78.4, 75.81, 75.78, 75.7, 73.0, 72.7, 72.5, 72.0, 69.6, 69.4, 69.3, 63.4, 63.2, 63.1, 55.8, 47.9, 46.7, 46.5, 42.1, 42.0, 39.7, 38.5, 38.2, 37.1, 34.3, 33.3, 33.2, 33.1, 31.0, 29.0, 28.3, 26.2, 23.79, 23.76, 23.72, 22.3, 18.5, 17.4, 16.9, 15.4. HRMS (ESI) calcd for $[M-H]^-$ $C_{48}H_{77}O_{18}$: 941.5110; found 941.5131.

4.11. Preparation of 28, 29 and 30 by partial esterification of glycyrrhizic acid

Acetyl chloride (0.86 mL, 0.95 g, 12.2 mmol) was added to stirred methanol (5 mL) cooled in an ice-water bath followed by glycyrrhizic acid (**27**) (1.0 g, mmol) in one portion giving a thick slurry. Further methanol (5 mL) was added and the reaction mixture became homogeneous after 5 min stirring. Analysis (TLC, $CHCl_3$/ethyl acetate/water, 6:4:1) indicated three main components of similar intensity attributed to unreacted starting material, monomethyl esters **28** and **29** (co-eluting) and dimethyl ester **30**. The reaction was quenched by the addition of NH_4OAc (1.1 g, 14 mmol) and after stirring for 5 min the reaction mixture was filtered through a porosity 3 sintered glass funnel. The mixture was adsorbed onto silica gel then loaded onto a column of silica gel and eluted initially with methanol/$CHCl_3$ 1:5 to give the dimethyl ester **30** (320 mg, 31%). Dimethyl ester **30** had properties consistent with those previously reported. Elution with $CHCl_3$/ethyl acetate/water 6:4:0.25 then gave a mixture of **28** and **29** (200 mg, 20%) as a mixture. Compounds **28** and **29** were separated by preparative RP-HPLC (methanol/ 0.1% TFA in water 1:1 to 1:0 gradient over 50 min) to give high R_f (reverse phase) **28** (115 mg 11%) followed by **29** (54 mg, 5%).

4.12. Glycyrrhizic acid 6′-methyl ester (28)

$[\alpha]_D^{20}$ +66 (c 0.3, methanol). ^{1}H NMR (pyridine-d_5) δ 5.96 (s, 1H), 5.42 (d, J = 7.4 Hz, 1H), 5.01 (d, J = 7.7 Hz, 1H), 4.68–4.56 (m, 2H), 4.54–4.14 (m, 6H), 3.71 (s, 3H), 3.33 (dd, J = 4.2, 11.5 Hz, 1H), 3.14–3.00 (m, 1H), 2.54 (br dd, J = 3.5, 13.0 Hz, 1H), 2.45 (s, 1H), 2.36–1.95 (m, 5H), 1.84–1.62 (m, 3H), 1.61–1.15 (m, 21H), 1.14–0.88 (m, 6H), 0.85–0.67 (m, 4H). ^{13}C NMR (pyridine-d_5) 199.5, 179.2, 172.1, 170.4, 169.6, 128.7, 106.9, 105.1, 89.3, 84.4, 78.4, 77.6, 76.8 (×2), 73.3, 72.7, 62.1, 55.5, 52.1, 48.8, 45.6, 44.1, 43.5, 41.7, 40.0, 39.5, 38.5, 37.2, 32.9, 32.2, 31.6, 28.8, 28.7, 28.1, 26.9, 26.69, 26.65 (×2), 23.6, 18.8, 17.6, 16.9, 16.8. HRMS (ESI) calcd for $[M-H]^-$ $C_{43}H_{63}O_{16}$: 835.4116; found: 835.4124. Anal. Calcd for $C_{43}H_{64}O_{16}\cdot 3H_2O$: C, 57.96; H, 7.92. Found: C, 57.67; H, 7.51.

4.13. Glycyrrhizic acid 6″-methyl ester (29)

$[\alpha]_D^{20}$ +60 (c 0.2, methanol). ^{1}H NMR (pyridine-d_5) δ 5.98 (s, 1H, H-12), 5.40 (br d, J = 5.4 Hz, 1H, H-1″), 5.03 (br d, J = 7.4 Hz, 1H, H-1′), 4.68–4.12 (m, 8H), 3.85 (s, 3H, CO_2CH_3), 3.35 (br dd, J = 3.1, 11.8 Hz, 1H, H-3), 3.06 (br d, J = 13.1 Hz, 1H), 2.55 (br d, J = 11.6 Hz, 1H, H-18), 2.49 (br s, 1H, H-7), 2.41–2.21 (m, 2H), 2.20–1.97 (m, 3H), 1.83–1.55 (m, 5H), 1.55–0.64 (m, 29H). ^{13}C NMR (pyridine-d_5) δ 199.6, 179.2, 172.4, 170.3, 169.6, 128.7, 107.0, 105.1, 89.3, 84.7, 77.8, 77.7, 77.5 (×2), 76.6, 73.0 (×2), 62.2, 55.5, 52.0, 48.8, 45.6, 44.1, 43.5, 41.7, 40.0, 39.6, 38.5, 37.3, 33.0, 32.2, 31.6, 28.8, 28.7, 27.9, 26.9, 26.8, 26.7, 23.6, 18.9, 17.7, 16.8, 16.5; HRMS (ESI) calcd for $[M-H]^-$ $C_{43}H_{63}O_{16}$ 835.4116, HRMS (ES); found 835.4136.

4.14. Glycyrrhizic acid 30-*O*-methyl ester (32)

To a stirred solution of glycyrrhizic acid (1.0 g, 1.21 mmol) dissolved in DMF (5 mL) was added methyl iodide (0.271 mL, 4.86 mmol) and potassium carbonate (400 mg, 2.89 mmol) and the mixture warmed to 70 °C for 3 h. The solution was cooled and then diluted with water (30 mL). The trimethyl ester was extracted with ethyl acetate (3 × 30 mL), the ethyl acetate extracts dried then concentrated under reduced pressure. Purification by flash chromatography, eluting with $CHCl_3$/methanol 9:1 to 5:1 afforded the trimethyl ester as a colourless gum. This gum (770 mg) was suspended in methanol/water/CH_2Cl_2 (30 mL, 1:0.5:0.5) and potassium carbonate (259 mg, 1.87 mmol) was added. After 24 h the mixture was quenched by the addition of acetic acid and then concentrated under reduced pressure. Purification of the residue by flash silica-gel chromatography, eluting with a gradient from 7:3 $CHCl_3$/methanol to 60:40:7 $CHCl_3$/methanol/water afforded a colourless amorphous solid (550 mg, 59%). mp 183 °C (darkens). Lit.[42] 182–185 °C. ^{1}H NMR (4:1 methanol-d_4/$CDCl_3$) δ 5.59 (s, 1H), 4.69 (d, J = 7.4 Hz, 1H), 4.49 (d, J = 7.2 Hz, 1H), 3.70 (s, 3H), 3.67–3.39 (m, 7H), 2.70 (m, 1H), 2.43 (br s, 1H), 2.20–1.00 (m, 21H), 1.41 (s, 3H), 1.16 (s, 3H), 1.14 (s, 3H), 1.13 (s, 3H), 1.08 (s, 3H), 0.85 (s, 3H), 0.82 (s, 3H). ^{13}C NMR (4:1 methanol-d_4/$CDCl_3$) δ 202.4, 178.4, 176.0, 175.3, 172.0, 128.9, 104.8, 104.3, 90.8, 80.9, 77.5, 77.2, 76.2, 76.0, 75.7, 73.3, 73.2, 62.9, 56.3, 52.3, 46.6, 45.1, 44.4, 42.1, 40.4, 40.2, 38.8, 37.8, 33.6, 32.8, 31.9, 29.1, 28.5, 28.2, 27.4, 27.2, 26.6, 23.8, 19.2, 18.2, 16.7.

4.15. Partial hydrolysis of glycoside 33

Sodium methoxide (30% w/v solution in methanol, 0.15 mL, 0.8 mmol) was added to a solution of **33** (prepared via **30** and **31** according to Ref. 43) (963 mg, 0.69 mmol) in methanol (15 mL) at ambient temperature. After 40 min the de-acetylated dimethyl ester intermediate was detected (TLC $CHCl_3$/ethyl acetate/water 6:4:1, R_f 0.50) with small amounts of lower and higher R_f material also present. The reaction mixture was neutralised by the addition of Dowex 50WX8-100 (H^+) resin then filtered and concentrated to give crude dimethyl ester as an oil (611 mg). To a solution of this oil in methanol (9 mL) and water (9 mL) was added potassium carbonate (41 mg, 0.30 mmol). After 15 min a further portion of potassium carbonate (41 mg, 0.30 mmol) was added (solution pH ~9) followed after 15 min by a further portion of potassium carbonate (41 mg, 0.30 mmol). After 45 min total reaction time, analysis (TLC) showed almost complete loss of the dimethyl ester and a spot corresponding to **34** and **35** (R_f = 0.21) was prominent. The reaction was quenched by the careful addition of HCl (1 M in water) until the pH tested neutral during which the reaction mixture changed from yellow to almost colourless. Silica (approx. 10 g) was added and the mixture concentrated and loaded onto a silica column. Elution with methanol/$CHCl_3$ 1:4 then $CHCl_3$/ethyl acetate/water 6:4:0.25 to 6:4:0.5 gave **34** and **35** (200 mg, 36%) as a mixture. Preparative HPLC (C18; gradient methanol/0.1%TFA in water 1:1 to 4:1 over 45 min) gave clean fractions of ester **34** (30 mg, 6%) and ester **35** (50 mg, 10%).

4.16. Glycyrrhizic acid 30-(β-D-glucopyranosyl) ester 6′-methyl ester (34)

$[\alpha]_D^{20}$ +72 (c 0.2, methanol). ^{1}H NMR (pyridine-d_5) δ 6.37 (d, J = 7.9 Hz, 1H, H-1‴), 5.95 (s, 1H, H-12), 5.42 (d, J = 7.1 Hz, 1H, H-1″), 5.01 (d, J = 7.8 Hz, 1H, H-1′), 4.68–4.12 (m, 13H), 4.09–3.98 (m, 1H), 3.71 (s, 3H, CO_2CH_3), 3.32 (br dd, J = 4.2, 11.1 Hz, 1H, H-3), 3.14–2.96 (m, 1H), 2.52 (br dd, J = 2.6, 12.4 Hz, 1H, H-18), 2.40 (s, 1H, H7), 2.34–1.81 (m, 6H), 1.80–0.60 (m, 33H). ^{13}C NMR (pyridine-d_5) δ 199.6, 175.8, 172.1, 170.4, 169.2, 128.8, 106.8, 105.1,

96.1 (C1‴, J = 164 Hz), 89.3, 84.3, 79.5, 78.8, 78.4 (×2), 77.6, 77.5, 76.8, 74.4, 73.3, 72.7, 71.3, 62.3, 62.1, 55.5, 52.1, 48.2, 45.5, 44.3, 43.4, 41.5, 40.0, 39.5, 37.9, 37.2, 32.9, 32.1, 31.3, 28.5, 28.1, 28.0, 26.8, 26.7 (×2), 23.4, 18.8, 17.6, 16.9, 16.7; HRMS (ESI) calcd for $[M-H]^-$ requires 997.4644, found 997.4636.

4.17. Glycyrrhizic acid 30-(β-D-glucopyranosyl) ester 6″-methyl ester (35)

$[\alpha]_D^{20}$ +75 (c 0.3, methanol). ^{1}H NMR (pyridine-d_5) δ 6.37 (d, J = 8.0 Hz, 1H, H-1‴), 5.96 (s, 1H, H-12), 5.40 (d, J = 5.4 Hz, 1H, H-1″), 5.02 (d, J = 7.6 Hz, 1H, H-1′), 4.69–4.11 (m, 13H), 4.08–3.97 (m, 1H), 3.85 (s, 3H, CO_2CH_3), 3.34 (br dd, J = 4.2, 11.9 Hz, 1H, H-3), 3.13–2.98 (m, 1H), 2.53 (br dd, J = 3.3, 12.9 Hz, 1H, H-18), 2.43 (1H, s, H-7), 2.38–2.17 (m, 2H), 2.17–1.83 (m, 4H), 1.82–1.52 (m, 4H), 1.52–0.98 (m, 24H), 0.98–0.85 (m, 1H), 0.84–0.61 (m, 4H); ^{13}C NMR (pyridine-d_5) δ 199.6, 175.8, 172.4, 170.3, 169.2, 128.8, 107.0, 105.0, 96.1 (C1‴, J_{CH} = 166 Hz), 89.3, 84.7, 79.5, 78.8, 77.8, 77.7, 77.5 (×2), 76.6, 74.4, 73.02, 72.99, 71.3, 62.3, 62.1, 55.5, 52.0, 48.2, 45.6, 44.3, 43.4, 41.5, 40.0, 39.5, 37.9, 37.3, 33.0, 32.1, 31.4, 28.5, 27.9 (×2), 26.8 (×3), 23.4, 18.8, 17.7, 16.8, 16.5; $[M-H]^-$ requires 997.4644, HRMS (ES) 997.4665.

4.18. Licorice saponin A3 (36)

Protected saponin **33** (100 mg, 72 μmol) was added to a solution of sodium methoxide (64.7 μmol) in methanol (1.4 mL) stirred under Ar. After 120 min, sodium hydroxide (4.1 mg, 72 μmol) in water (1.3 mL) was added. The reaction was neutralised via the addition of silica gel and solvent removed under reduced pressure. Purification by column chromatography, eluting with $CHCl_3$/methanol/water 6:4:0 to 6:4:1 afforded after freeze drying the desired product as a white fluffy solid (52 mg, 72%). ^{1}H NMR (D_2O) δ 5.64 (s, 1H), 5.52 (d, J = 7.8 Hz, 1H), 4.79–4.77 (m, 1H), 4.56 (br s, 1H), 3.90–3.86 (m, 1H), 3.74–3.67 (m, 1H), 3.53–3.45 (m, 7H), 3.34–3.23 (m, 2H), 2.54 (d, J = 12.5 Hz, 1H), 2.46 (s, 1H), 2.18–1.58 (m, 11H), 1.42–1.30 (m, 8H), 1.37 (s, 6H), 1.09 (s, 6H), 1.01 (s, 3H), 0.81 (s, 3H), 0.79 (s, 3H). ^{13}C NMR (D_2O) 204.5, 178.1, 174.1, 127.8, 103.7, 103.2, 94.6, 90.8, 79.9, 77.3, 76.6, 76.2, 75.9, 74.8, 72.2 (×2), 69.7, 62.2, 61.0, 55.3, 48.5, 46.1, 44.5, 43.8, 41.0, 39.7, 39.4, 37.6, 36.8, 32.9, 32.0, 30.8, 28.5, 27.7, 27.5, 26.7, 26.4, 25.9, 23.3, 18.8, 17.4, 16.6, 16.3. HRMS (ESI) calcd for $[M-2H]^{2-}$ $C_{48}H_{71}O_{21}$: 492.2210; found: 491.22.

4.19. Glycyrrhizic acid 30-*O*-(-D-mannopyranosyl) ester (38)

Peracetylated dimethyl ester **31** (200 mg, 188 μmol), tetra-*O*-benzoyl-α-D-mannopyranosyl bromide (323 mg, 490 μmol) and mercury (II) cyanide (95 mg, 377 μmol) were dried under vacuum in a flask containing 4 Å molecular sieves for 45 min. After this time toluene (4.2 mL) was added and the reaction heated to 100 °C for 4 h. The pale yellow reaction mixture was then cooled and filtered through Celite, the Celite washed with CH_2Cl_2 (20 mL) and combined filtrate and washings evaporated to dryness. Crude product was adsorbed onto silica and purified by column chromatography, eluting with ethyl acetate/petroleum ether 1:1 to afford protected saccharide **37** as a clear oil (185 mg, 60%). ^{1}H NMR ($CDCl_3$) 8.08–7.94 (m, 5H), 7.86–7.83 (m, 2H), 7.60–7.49 (m, 3H), 7.46–7.35 (m, 7H), 7.29–7.25 (m, 3H), 6.41 (d, J = 2.0 Hz, 1H), 6.16 (t, J = 10.0 Hz, 1H), 5.85 (dd, J = 3.2 and 10.2 Hz, 1H), 5.77 (s, 1H), 5.76–5.74 (m, 1H), 5.27–5.07 (m, 4H), 4.92 (t, J = 8.2 Hz, 1H), 4.75–4.67 (m, 1H), 4.52–4.45 (m, 2H), 4.00 (dd, J = 4.0 and 9.6 Hz, 2H), 3.85 (t, J = 7.7 Hz, 1H), 3.73 (s, 3H), 3.71 (s, 3H), 3.12 (t, J = 8.5 Hz, 1H), 2.81 (br d, J = 13.5 Hz, 1H), 2.33 (s, 1H), 2.31–2.30 (m, 1H), 2.16–2.05 (m, 2H), 2.11 (s, 3H), 2.03 (s, 3H), 2.00 (s, 6H), 1.99 (s, 3H), 1.89–1.81 (m, 3H), 1.7–1.61 (m, 5H), 1.40 (s, 3H), 1.35 (s, 3H), 1.30–1.18 (m, 4H), 1.15 (s, 3H), 1.12 (s, 3H), 1.04 (s, 3H), 0.98–0.80 (m, 5H), 0.84 (s, 3H), 0.79 (s, 3H), 0.76–0.67 (m, 1H). ^{13}C NMR ($CDCl_3$) 200.5, 180.7, 170.5, 170.2, 169.8, 169.6, 167.6, 166.8, 166.5.165.8, 133.8, 133.5, 133.4, 131.2, 130.2, 130.2, 130.1, 129.8, 129.5, 129.4, 129.2, 128.8, 128.6, 103.7, 101.1, 91.4, 77.2, 74.7, 73.2, 72.9, 72.6, 72.0, 71.6, 71.0, 70.5, 70.2, 69.7, 69.2, 69.0, 67.4, 64.7, 63.3, 62.2, 55.8, 53.1, 53.0, 45.8, 44.1, 43.6, 39.9, 39.5, 38.1, 37.2, 33.2, 32.2, 28.9, 28.8, 27.9, 26.9, 26.7, 26.1, 23.8, 21.2, 20.8 (×2), 19.1, 17.7, 16.7, 16.3. This oil (**37**) (0.225 g, 0.137 mmol) was dissolved in methanol/CH_2Cl_2 (1:1, 2.5 mL) and sodium methoxide (30% w/v solution in methanol, 25 μL) was added. After 2.5 h the reaction was quenched via the addition of Dowex 50Wx8 (H^+) resin, the reaction was filtered, the resin washed with methanol and the filtrate concentrated under reduced pressure. The resulting residue was suspended in potassium carbonate (10 mg) in methanol/H_2O (1:1, 4.9 mL) and the reaction was stirred at room temperature for 24 h. The reaction was quenched via the addition of silica gel, the solvent removed under reduced pressure and the crude product was dry loaded onto silica gel. Purification by column chromatography on silica gel, eluted with $CHCl_3$/methanol/H_2O 6:4:1 afforded the title compound **38** (40 mg, 30%) and separate fractions of partially deprotected **37**. ^{1}H NMR (D_2O) 6.03 (s, 1H), 5.58 (s, 1H), 4.80, (d, J = 7.5 Hz, 1H), 4.56 (br s, 1H), 3.98 (br s, 1H), 3.86 (dd, J = 3.1 and 9.6, 1H), 3.78–3.64 (m, 10H), 3.52–3.49 (m, 4H), 3.36–3.25 (m, 3H), 2.54 (d, J = 11.6 Hz, 1H), 2.45 (s, 1H), 2.12–1.18 (m, 15H), 1.36 (s, 3H), 1.25–1.18 (m), 1.19 (s, 3H), 1.08 (s, 6H), 1.01 (s, 3H), 0.81 (s, 3H), 0.79 (s, 3H). ^{13}C NMR (D_2O) 204.2, 177.1, 173.7, 127.8, 103.7, 102.9, 93.9, 90.7, 79.3, 76.7, 76.5, 76.0, 74.8, 72.3, 70.9, 69.7, 66.4, 62.2, 61.0, 55.2, 48.9, 46.1, 44.9, 43.8, 40.9, 39.8, 39.3, 37.9, 36.9, 32.9, 32.1, 31.0, 28.7, 28.0, 27.5, 26.8, 26.4, 25.9, 23.3, 18.9, 17.4, 16.6, 16.3. HRMS (ESI) calcd for $[M-2H]^{2-}$ $C_{48}H_{72}O_{21}$: 491.2210; found: 491.2198.

5. Formulation

Colloidal systems were prepared following the lipid film hydration method of Copland et al.[47] with some modifications. Briefly, 10 mg/mL stock solutions of cholesterol (Sigma) and phosphatidylcholine

(Sigma) were prepared in chloroform. Synthetic saponins were prepared as either 10 mg/mL solutions in 145 mM Tris buffer (pH 7.4) or ethanol dependent on solubility. Lipids were pipetted into sterile glass vials and made up to a total volume of 1 mL with chloroform. The solvent was evaporated at low temperature using a Speedvac (Savant Industries, NY). The obtained lipid films were hydrated with solutions of the synthetic saponins, made up to 1.5 mL with 145 mM Tris buffer, and stirred via magnetic fleas at room temperature for 24 h. The samples were subsequently freeze-dried (Feezone, Labconco, MO), at a condenser temperature of −82 °C and a pressure of less than 0.1 millibar. Freeze-dried samples were then resuspended in 1.5 mL of sterile Milli-Q water (Milli-Q water systems, Millipore Corp., MA) and stirred via magnetic fleas for a further 24 h to facilitate mixing. For Quil-A mimics solubilised in ethanol, the saponins were added with the lipids and the solvents evaporated to dryness. These systems were rehydrated in 145 mM Tris buffer (pH 7.4) alone, but were otherwise processed as above. The ratios of the lipids (PC:cholesterol) was kept constant at 3:1, which has previously been demonstrated to be optimal for the formation of ISCOM-like particles,[45] while the synthetic saponin was incorporated as 10%, 20%, 50% or 70% of the total formulation. The total lipid concentration in all systems was 6.67 mg/mL.

The particulate nature of the formulations was investigated by transmission electron microscopy (TEM). Briefly, samples were coated onto glow-discharged, carbon coated copper grids and

negatively stained with 2% phosphotungstic acid (pH 5.2). Samples were investigated using a Phillips CM100 electron microscope with an acceleration voltage of 100 kV and a magnification of 93,000×. Samples were investigated within 24 h of formulation and additionally following a 1 month equilibration time at 4 °C.

6. Generation and activation of bone marrow dendritic cells (BMDC)

Murine BMDC were generated by culturing bone marrow derived stem cells from C57Bl/6 J mice in complete Iscove's Modified Dulbecco's Medium (cIMDM; IMDM supplemented with 5% foetal bovine serum, 1% penicillin/streptomycin, 1% glutamax, and 0.01% 2-mercaptoethanol; all from Invitrogen, CA, USA) with 20 ng/ml recombinant granulocyte/macrophage colony stimulating factor (clone kindly supplied by Dr G. Buchan, University of Otago) for 6 days at 37 °C, 5% CO_2. Cells were seeded at 5×10^5 cells/mL in cIMDM for pulsing with colloidal formulations. Activation of BMDC was measured by flow cytometric analysis (FACScalibur, BD, USA). Propidium iodide was added to the cells to determine cell viability and the cells were stained with the BMDC marker CD11c. The expression of costimulatory activation markers CD86 and MHCII on propidium iodide^{-ve}, CD11c^{+ve} cells was investigated 48 h after the addition of titrations of Quil-A, Quil-A mimic or equivalent amounts of saponin in particulate formulations or 10 μg/mL lipopolysaccharide (LPS) as a positive control. Data was analysed using CellQuest Pro (BD, USA). The fold increase in the mean fluorescence intensity (MFI) of activation marker expression was determined by dividing the MFI of each sample by that of the negative control (media only, no formulation).

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