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Synthesis and Activity of 6″-Deoxy-6″-thio-α-GalCer and Peptide Conjugates

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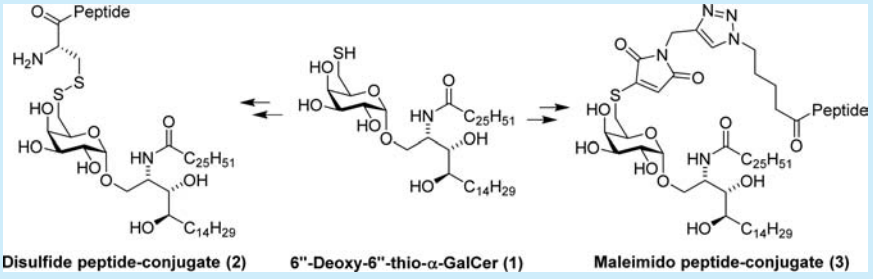
Supporting Information



ABSTRACT: A major challenge in the development of highly defined synthetic vaccines is the codelivery of vaccine components (i.e., antigen and adjuvant) to secondary lymphoid tissue to induce optimal immune responses. This problem can be addressed by synthesizing vaccines that comprise peptide antigens covalently attached to glycolipid adjuvants through biologically cleavable linkers. Toward this, a strategy utilizing previously unreported 6″-deoxy-6″-thio analogues of α-GalCer that can undergo chemoselective conjugation with peptide antigens is described. Administration of these conjugate vaccines leads to enhanced priming of antigen specific T cells. This simple vaccine design is broadly applicable to multiple disease indications such as cancer and infectious disease.

In the context of vaccine development, adjuvants are components that enhance antigen-specific immune responses. Of particular interest are chemically defined compounds that boost cellular immunity by known biological mechanisms.[1] Examples that have either reached market or are in late-stage development include monophosphoryl lipid,[2] cytosine phosphoguanosine,[3] and trehalose dimycolate.[4] These adjuvants all signal through pattern recognition receptors leading to the direct activation of antigen-presenting cells (APCs). This activation of APCs is necessary to drive adaptive immune responses including differentiation of antigen-specific $CD4^+$ T cells into T helper cells and $CD8^+$ T cells into cytotoxic T lymphocytes (CTLs). An alternative approach toward activating APCs is to specifically stimulate innate-like T cells, a class of semiactivated T cells with an invariant T cell receptor (TCR). The most studied innate-like T cells are the invariant natural killer T (NKT) cells, which are found in high numbers in the spleen, liver, and bone marrow in humans and animals.[5−7] Unlike conventional T cells that recognize peptide antigens presented by major histocompatibility complex (MHC) class I and II molecules, NKT cells respond to glycolipid antigens presented by the MHC class I-like molecule CD1d.[8,9] Activation of NKT cells with glycolipid antigens can drive potent stimulatory interactions with APCs such as providing ligands for CD40 signaling.[5,6] The most studied antigen of this class is α-galactosylceramide (α-GalCer, KRN7000), an optimized synthetic derivative of the agelasphins, isolated from the marine sponge *Agelas mauritianus*.[10] A number of subsequent structural modifications have produced an array of analogues with altered or improved immunological activities. These include a C-glycosidic α-GalCer analogue in which the anomeric oxygen was replaced with a hydrolytically stable methylene group,[11] substitution of the C-26 fatty acid acyl group (FA) with aryl containing FAs (e.g., 7DW8-5 and C-34),[12,13] and a number of derivatives resulting from modification of the galactose ring including modification of the primary hydroxyl group (6″-OH).[14] This latter group of compounds is of particular interest because the galactose 6″-OH can easily be accessed selectively for chemical modification. Crystallographic studies of the CD1d-α-GalCer-TCR complex show the galactose 6″-OH is solvent-exposed,[15] implying that chemical modifications at this position can be tolerated which have been supported by

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functional SAR studies.[14,16−21] Examples include the DMSO-soluble compound PBS-57, used for tetramer staining of NKT cells,[22] and a related derivative ABX196 that drives increased T cell responses compared to α-GalCer and induces adjuvant activity in humans.[23] Furthermore, solubilizing groups[17] or carbohydrate antigens[24] have been attached to the 6″-position. Both the 6″-amino and -azido compounds are particularly useful as they can be further modified in the presence of the hydroxyl groups associated with the active glycolipid. We considered 6″-deoxy-6″-thio-α-GalCer (**1**) would be equally versatile in conjugation strategies through its facile reaction with a wide range of substrates including alkenes, alkynes, enones, epoxides, haloacetamides, or cross-coupling reactions to form disulfides. We have reported that the conjugation of minimal peptide epitopes to an α-GalCer derivative where the C-26 FA is deliberately misorientated produces vaccines with enhanced T cell responses and efficacies in allergy[25] and cancer[26] models. We postulated that **1** would allow access to an analogous series of conjugates designed to favor intracellular release of the glycolipid and antigen and drive increased adaptive immune responses. This strategy, dependent on the stimulatory activity of **1**, exploits the endogenous reductant glutathione (GSH) found in circulation and within cellular compartments.[27] Typically, the extracellular concentration of GSH is relatively low (approximately 1−20 μM in plasma) compared to the intracellular concentration (approximately 0.5−10 mM). Such a gradient provides a level of stability for "thiol-reactive" conjugates outside the cell and promotes rapid degradation upon internalization. Various bioconjugation strategies incorporating disulfide bonds and thio-maleimide adducts have been designed to utilize this phenomenon. Herein, we report here the synthesis and biological activity of **1** and two strategically different peptide conjugates both reliant upon intracellular GSH to mediate the release of the glycolipid and peptide constituents (Figure 1).[28]

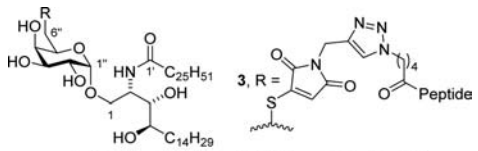
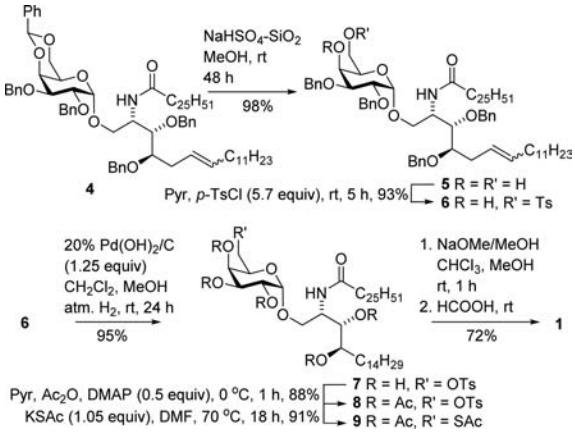


Figure 1. Structure of **1** and its peptide conjugates **2** and **3**.

The synthesis of **1** started from the well-characterized benzylidene acetal **4** which was prepared by the method of Plettenburg et al. (Scheme 1).[29] Attempts to reductively open the benzylidene acetal in **4** to the primary hydroxyl with Bu_2BOTf/BH_3·THF or $Sc(OTf)_3/BH_3$·THF resulted in the complete removal of the group or the recovery of starting material. A more successful choice may have been the use of $Cu(OTf)_2$ which has been used successfully on a similar substrate.[14] Instead, mild acidic hydrolysis of the benzylidene moiety, by stirring over $NaHSO_4$ adsorbed onto silica gel,[30] afforded diol **5**.[31] Selective tosylation at O-6″ over the less reactive axial secondary hydroxyl group at O-4″ was achieved in high yield. The position of the tosyl group was confirmed by 2D NMR experiments of a later synthetic intermediate (vide infra). Attempts to displace the tosyl group with potassium thioacetate or benzylate the free hydroxyl group in **6** under basic conditions resulted in 4,6-oxetane formation (SI, Scheme 1). In contrast, a *tert*-butyl thioether group could be readily installed. However, it

Scheme 1. Synthesis of 1



could then not be deprotected later in the synthesis.[32−35] Considering these findings, we decided to exchange the benzyl protection for acetyl groups. Hydrogenolysis of **6** over Pearlman's catalyst afforded compound **7**, which was subsequently peracetylated to give intermediate **8** suitable for nucleophilic substitution at C-6″ (Scheme 1). Reacting **8** with potassium thioacetate in DMF at elevated temperatures successfully installed a thioacetate moiety at C-6″ as evidenced by an HMBC correlation between the C-6″ methylene protons and the S-acetyl carbonyl carbon. Global deprotection of **9** using sodium methoxide in MeOH and quenching with formic acid afforded thiol **1**. In the absence of formic acid disulfide **10** (see the SI for the chemical structure) was isolated. The signals for the C-6″ methylene protons in the ^{1}H NMR spectra (3:1 $CDCl_3$/CD_3OD) provided good diagnostic method to differentiate the thiol from the disulfide form of these molecules. For **1**, the methylene resonances were observed upfield at 2.66 ppm (dd, J = 13.7, 6.5 Hz, 1H) and 2.79 ppm (dd, J = 13.7, 7.6 Hz, 1H) compared to those of **10** for which H-6″ resonated at 3.00 (d, J = 6.7 Hz, 2H). A more dramatic shift was observed in the ^{13}C NMR spectrum of each compound, in which the resonances for C-6″ were at 25.3 ppm for **1** and 40.2 ppm for **10**. Both compounds also displayed significantly different retention times when analyzed by HPLC with the more lipophilic disulfide eluting later on a C18 column using a gradient of 2-propanol with 90% methanol/water.

Before chemically synthesizing and testing vaccine constructs that incorporated **1**, we assessed whether the compound retained "α-GalCer-like" biological activity. First, the ability of compound **1** to bind to plate-bound CD1d and stimulate NKT cells from the hybridoma line DN32[36] was assessed. Marked activation of NKT cells was observed in this assay, with compound **1** inducing similar levels of IL-2 release to that seen when plate-bound CD1d was loaded with α-GalCer (58 pmol/well, 16 h at 37 °C). In contrast, disulfide **10** was not able to activate NKT cells (SI, Figure 1a). However, when injected i.v. (0.23 nmol) into mice, both thiol **1** and disulfide **10** (0.115 nmol) were able to induce activation of DCs, as indicated by upregulation of CD86 in spleens harvested 20 h post injection. This did not occur in CD1d-deficient mice, indicating that DC activation was dependent on activity of NKT cells (SI, Figure 1b). Consequently, the disulfide was able to become an effective NKT cell agonist *in vivo*, presumably through physiological reduction to the thiol. We next checked if the adjuvant activity for the sulfur derivatives was also retained. For this, we compared the

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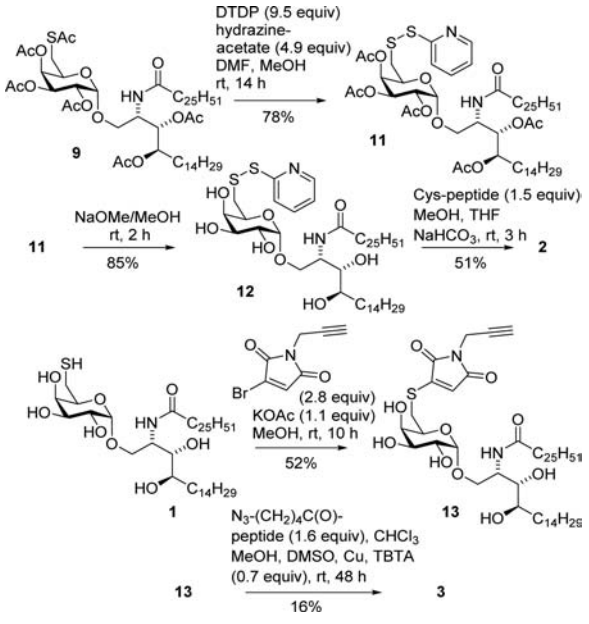
adjuvant activity of **10** alongside α-GalCer in cancer vaccines that use irradiated leukemia cells as a source of tumor antigens.[37] Loading irradiated C1498 leukemia cells with **10** (0.58 μM) or α-GalCer (1.16 μM) prior to intravenous injection resulted in induction of adaptive immune responses that completely protected mice from challenge with live C1498 cells; injection of irradiated cells alone provided no such protection (SI, Figure 2). Given the similar biological activity of **1** and **10** compared to α-GalCer, we next explored whether the thiol group could be exploited for conjugating peptide antigens in order to generate synthetic vaccines. Pyridyl disulfides have been utilized as suitable precursors to the formation of disulfide bridges by undergoing nonreversible sulfur exchange under benign conditions.[38] We reasoned that **1** modified in this manner would be a versatile intermediate that could be conjugated directly to cysteine containing peptides or proteins.

To exemplify this strategy, the pyridyl disulfide **12** was synthesized by treatment of **9** with hydrazine acetate to selectively cleave the *S*-acetyl group, followed by subsequent trapping of the thiol group with 2,2′-dithiodipyridine (DTDP) to afford **11** in good yield. Methanolysis of the remaining *O*-acetyl groups was achieved under Zemplèn conditions affording **12** in high yield (Scheme 2). A fusion peptide containing the

Scheme 2. Synthesis of Disulfide- and Maleimido-Linked Peptide Conjugates 2 and 3



ovalbumin MHC class I epitope ($OVA_{257–64}$; Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu), a proteasomal cleavage sequence to ensure the minimal MHC-binding epitope was released (Phe-Phe-Arg-Lys) and *N*-terminal cysteine for conjugation was selected as a model antigen. Reacting **12** with the modified peptide in THF/MeOH with the pH adjusted to 6.9 by the addition of sodium bicarbonate afforded the disulfide conjugate **2** after purification by semipreparative HPLC (MeOH−H_2O gradient modified with 0.05% TFA). Halomaleimides are useful reagents for bioconjugation in that they produce a hydrolytically stable chemical linkage that can undergo addition−elimination Michael-type reactions in conditions of high GSH concentration.[39] Incorporating a bioorthogonal reactive group such as an alkyne or azide through the maleimide nitrogen, achieved through reacting an appropriately functionalized amine with *N*-methoxycarbonyl-bromomaleimide, offers an alternative method for conjugation via a 1,3-dipolar cycloaddition (compound **13**).[28] To this end, the maleimide adduct **13** was prepared by reacting **1** with an equimolar amount of *N*-propargyl bromomaleimide[40] in the presence of potassium acetate (Scheme 2). Peptide conjugation of this compound was achieved by reacting **13** with 5-azidopentanoyl peptide in the presence of copper metal and TBTA ligand at ambient temperature. The conjugate precursors **12** and **13** both retained ability to stimulate NKT cells *in vivo*, as determined by examining activation of splenic DCs after i.v. administration (SI, Figure 3). The capacity of conjugates **2** and **3** to function as vaccines was tested *in vivo* (Figure 2). Single

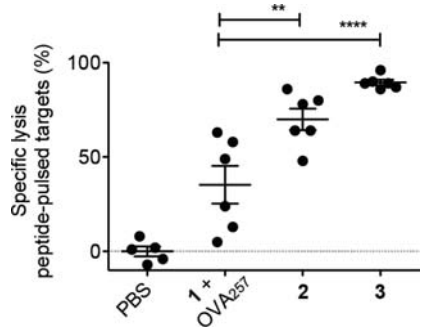


Figure 2. Conjugates **2** and **3** induce peptide-specific cytotoxic responses *in vivo*. Mice were administered (i.v.) vaccines **2** (1.30 μg), **3** (1.38 μg), unconjugated components (**1**, 0.444 μg + OVA_{257}, 0.500 μg) in PBS (200 μL), or PBS vehicle (200 μL). Cytolytic activity was assessed on day 8 by monitoring elimination of injected, day 7, populations of fluorescent syngeneic splenocytes that had been loaded with peptide. An additional population of fluorescent splenocytes without peptide served as internal negative control. Lysis is expressed as percent reduction in peptide loaded cells relative to unloaded control. Data from five to six mice per group are shown with a mean percentage of specific lysis ± SEM indicated. $^{**}P < 0.01$; $^{***}P < 0.001$ one way anova.

administration of either conjugate (day 0) induced potent peptide-specific cytotoxic T cell responses, assessed on day 8, that resulted in elimination of OVA_{257} loaded target cells (injected day 7). While some cytotoxic activity was also induced in animals injected with the unconjugated components, the levels observed were significantly lower. Conjugation therefore provides a mechanistic advantage that improves efficacy, presumably by optimizing delivery of antigen and adjuvant to the same APCs *in vivo*.

We have synthesized new analogues of the potent NKT cell agonist α-GalCer to probe the tolerability of incorporating bioorthogonal appendages for chemoselective conjugation by installing a thiol at position-6″ of the galactose unit. We show the base compound, thiol **1**, can be readily synthesized from intermediates used in the synthesis of α-GalCer and importantly retains NKT cell agonist and adjuvant properties. We also show **1** can be conveniently converted to the *S*-pyridyl disulfide **12** or maleimide derivative **13**, which also retain NKT cell agonist activity, for conjugation purposes. Accordingly, conjugation to a model MHC-binding peptide antigen was achieved via disulfide or triazole linkages respectively, providing two distinct vaccine constructs designed to be degraded intracellularly through GSH interactions. These conjugates induced potent T cell-mediated immune responses *in vivo* that were markedly improved over injection of the unconjugated components. Such conjugates may

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prove effective as vaccines for the prevention of infectious diseases or the treatment of cancer.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b02836.

Synthetic procedures, NMR spectra, HPLC traces, biological assay methods, and detailed figure captions (PDF)

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Notes

The authors declare the following competing financial interest(s): G.F.P. and I.F.H. are the CTO and CFO, respectively, of Avalia Immunotherapies. The company has licensed related technology for commercialization.

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