Development of Ribityllumazine Analogs as Mucosal-associated Invariant T Cell Activators

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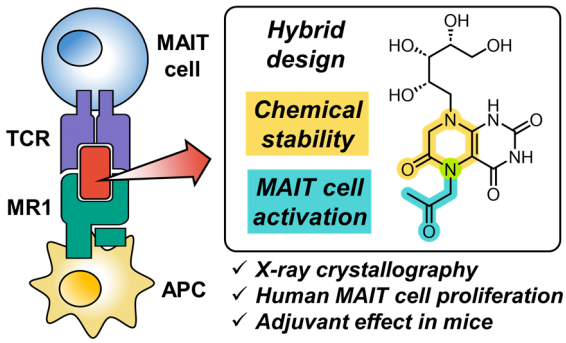


ABSTRACT:

Mucosal-associated invariant T (MAIT) cells are a subset of innate-like T cells abundant in human tissues that play a significant role in defense against bacterial and viral infections and in tissue repair. MAIT cells are activated by recognizing microbial-derived small-molecule ligands presented by the MHC class I related-1 protein. Although several MAIT cell modulators have been identified in the last decade, potent and chemically stable ligands remain limited. Herein, we carried out a structure-activity relationship study of ribityllumazine derivatives and found chemically stable MAIT cell activators with a pteridine core and a 2-

oxopropyl group as the Lys-reactive group. The activators showed high potency toward a co-cultivation assay using model cell lines of antigen-presenting cells and MAIT cells (EC_{50} = 20 nM). The X-ray crystallographic analysis revealed the binding mode of the activator to MR1 and T cell receptor, indicating that it forms a covalent bond with MR1 via Shiff base formation. Furthermore, we found that one activator stimulated proliferation of human MAIT cells in human peripheral blood mononuclear cells and showed an adjuvant effect in mice. Our developed activator is one of the most potent among chemically stable MAIT cell activators, contributing to accelerating therapeutic applications of MAIT cells.

INTRODUCTION

Mucosal-associated invariant T (MAIT) cells are a representative class of innate-like T cells abundant in human gut mucosal tissues, skin (~2% of $CD3^+$ cells), liver (~45% of α/β T cells) and peripheral blood (~10% of α/β T cells).[1–5] MAIT cells are activated by recognizing a complex of MHC class I related-1 protein (MR1) and ligand presented on antigen-presenting cells (APC) in a T cell receptor (TCR)-dependent manner (Figure 1). MAIT cells play a crucial role in the initial response to infection[6–8] by promoting dendritic cell maturation[9] and the production of inflammatory cytokines (IFN-γ, TNF) and cytotoxic effector molecules (perforin, granzyme).[4,10] Furthermore, MAIT cells have been implicated in various pathological conditions such as cancer[11] and autoimmune diseases.[12,13] Therefore, in drug discovery, the modulation of MAIT cell functions has recently attracted attention as a potential therapeutic approach for treating these disorders.[1,14–16]

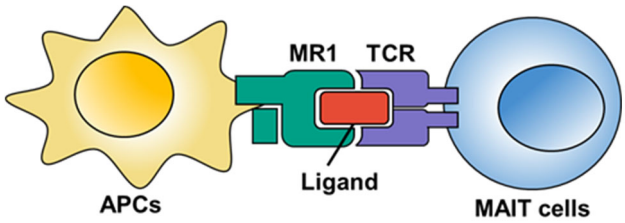


Figure 1. The APC–MAIT cell axis. MAIT cells recognize the MR1-ligand complex with their TCR.

Innate-like T cells, such as MAIT cells and natural killer T (NKT) cells, have a highly conserved and restricted TCR that detects specific antigens, including non-peptide molecules.[17,18] Regarding MAIT cells, although it has been shown that their development and accumulation are highly dependent on the presence of MR1 and microbiota,[19] the MAIT cell antigen bound to MR1 had not been identified for a long time. In the last decade, however, a class of compounds derived from microbial vitamin biosynthesis has been shown to bind to MR1 and function as MAIT cell activators.[20,21] Among them, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU, Figure 2a) is the most potent and well-known activator of MAIT cells (EC_{50}: 1–8 pM).[21,22] 5-OP-RU is formed transiently from 5-amino-6-D-ribitylaminouracil (5-A-RU), an intermediate in the biosynthesis of vitamin B_2 (riboflavin) produced by various

bacteria and fungi, which then reacts with methylglyoxal derived from human and microbial metabolism through non-enzymatic condensation to give 5-OP-RU.

Notably, 5-OP-RU forms a covalent bond (Schiff base) via its α-iminocarbonyl moiety with the ε-amino group of MR1 Lys43 (Figure 2a).[21] In general, covalent drugs show increased affinity toward target proteins because of their slow dissociation dynamics, enhancing their apparent potency.[23] Furthermore, forming this covalent bond promotes the localization of MR1 from the endoplasmic reticulum to the plasma membrane, where it can interact with MAIT cells.[24] This unique binding mode through Schiff base formation contributes to the remarkable bioactivity of 5-OP-RU. However, 5-OP-RU is chemically unstable under physiological conditions ($t_{1/2}$: *ca.* 90 min at 37 °C) because its α-iminocarbonyl moiety is liable to hydrolysis and intramolecular cyclization.[22] Thus, 5-OP-RU is generally prepared in situ by pre-mixing 5-A-RU and methylglyoxal for biological use, and the difficulty in isolating and storing 5-OP-RU hampers its facile use in MAIT cell research.

To address this instability issue, Mak et al. replaced the two nitrogen atoms of 5-OP-RU, the triggers of decomposition, with carbon atoms (JYM72, Figure 2b).[22] JYM72 showed improved stability under physiological conditions and activity toward MAIT cell stimulation in human peripheral blood mononuclear cells (hPBMC) and mice lungs.[22,25] Lange et al. developed the prodrug of 5-A-RU (Figure 2b),[26] which prevents oxidative decomposition of the electron-rich diaminouracil ring of 5-A-RU. This prodrug undergoes enzymatic cleavage upon cell entry to form 5-OP-RU via an intracellular reaction of the released 5-A-RU with methylglyoxal.

Despite these pioneering studies, there remains a limited array of practical MAIT cell activators for drug discovery applications, and there is also a lack of information on rational ligand design. These issues are in striking contrast to CD1d-restricted NKT cells, another representative class of innate-like T cells with similar transcriptional and functional characteristics to MAIT cells. NKT cells are activated by non-peptide antigens such as glycolipids,[17,18,27,28] and structure-activity relationship studies on NKT cell activators have revealed that their structural variations influence downstream responses from NKT cells.[29–31] Consequently, researchers have concentrated on optimizing the structures of activators capable of eliciting desired effects from NKT cells, resulting in the identification of numerous effective activators.[32,33] These advancements have enabled NKT cell modulator applications for therapeutic agent development and vaccine adjuvants.[31,34,35] This leading situation in NKT cell activator development underscores the significance of exploring ligand design strategies for activating MAIT cells, which are more abundant than NKT cells in humans,[17,28] and creating a diverse range of easy-to-handle MAIT cell activators that can be used as potent tools in therapeutic approaches.

In this study, we designed and synthesized chemically stable MAIT cell activator candidates and found that particular ribityllumazine derivatives exhibited high chemical stability and potency toward MAIT cells. Further evaluation revealed that one derivative stimulated MAIT cells in hPBMCs and functioned as a vaccine adjuvant in a mouse model.

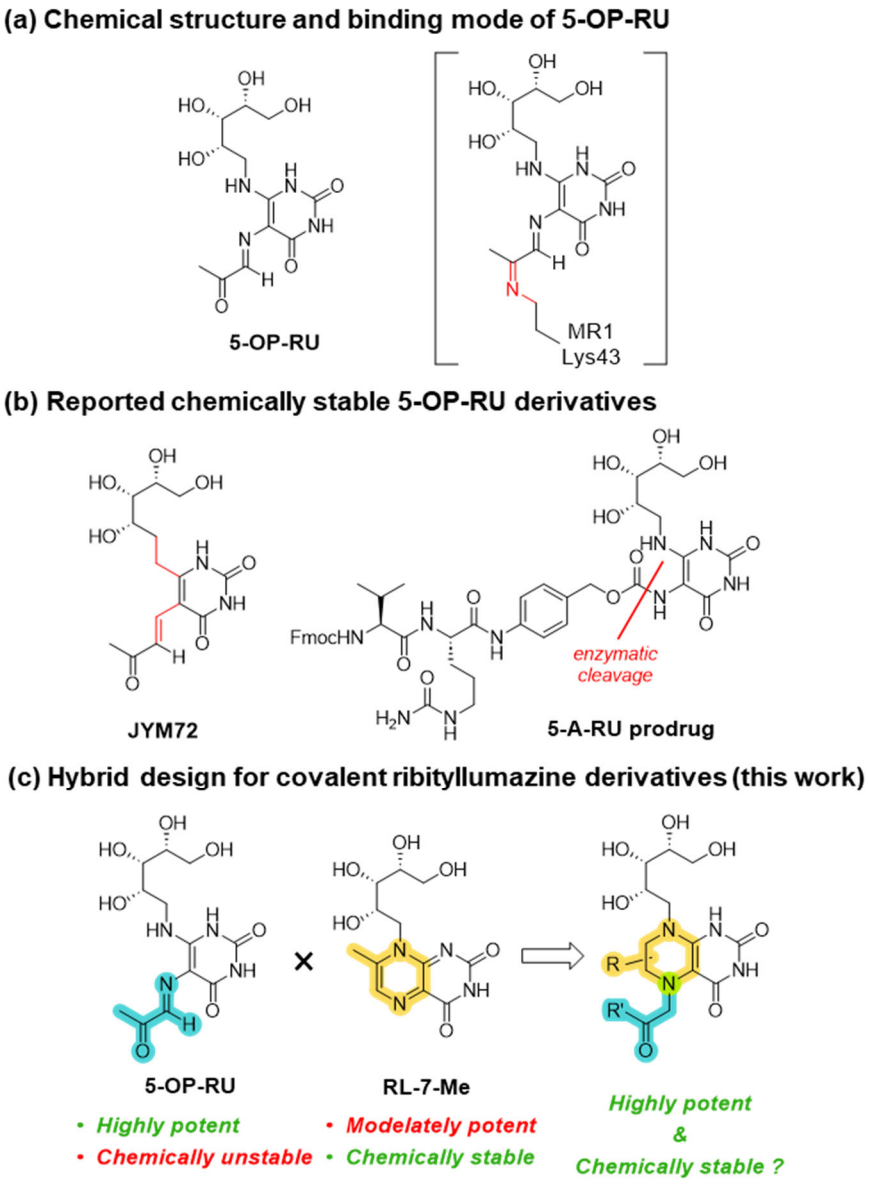


Figure 2. Our design of chemically stable MAIT cell activators. (a) Chemical structure and binding mode of 5-OP-RU. (b) Pioneering designs of chemically stable 5-OP-RU analogs. (c) Our ligand design strategy.

RESULTS AND DISCUSSION

Ligand Design.

We focused on the bicyclic ribityllumazine scaffold found in natural MR1 ligands to design chemically stable and potent MAIT cell activators. For example, 7-methyl-8-D-ribityllumazine (RL-7-Me, Figure 2c), a thermodynamically stable compound generated by intramolecular cyclization of 5-OP-RU, moderately activates MAIT cells (EC_{50}: 120 nM) despite lacking the reactive moiety for covalent binding to MR1 Lys43.[36,37] We planned to synthesize and evaluate

a series of hybrid derivatives of 5-OP-RU and RL-7-Me, which possess both rigid bicyclic parent structures and Lys-reactive groups (Figure 2c). This hybrid strategy should improve bioactivity and chemical stability by locking the conformation close to the active pose and suppressing the deactivation of the Lys-reactive group. No such class of derivatives has been synthesized, potentially expanding the chemical space of MR1 ligands.

Our designed derivatives **1**–**6** bearing fused bicyclic structures are shown in Figure 3. Previous structure-activity relationship studies of 5-OP-RU revealed that the existence of hydroxy groups with the appropriate stereochemistry of the ribityl fragment contributes significantly to the potency of 5-OP-RU.[36–39] Thus, retaining the ribityl moiety, we modified the uracil ring with a fused ring as an RL-7-Me mimic and introduced a carbonyl group as the Lys-reactive group.[40,41]

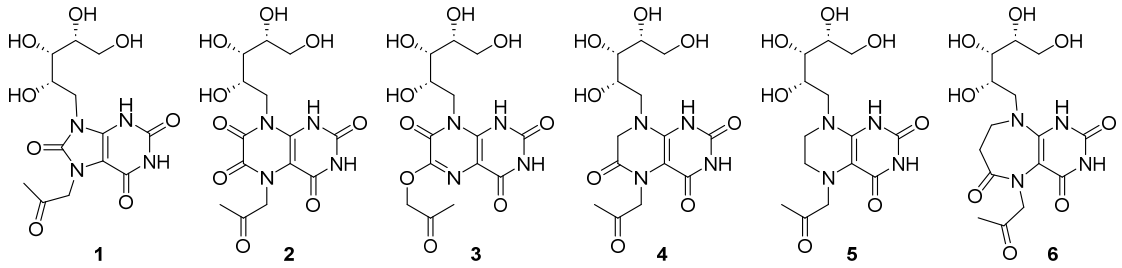
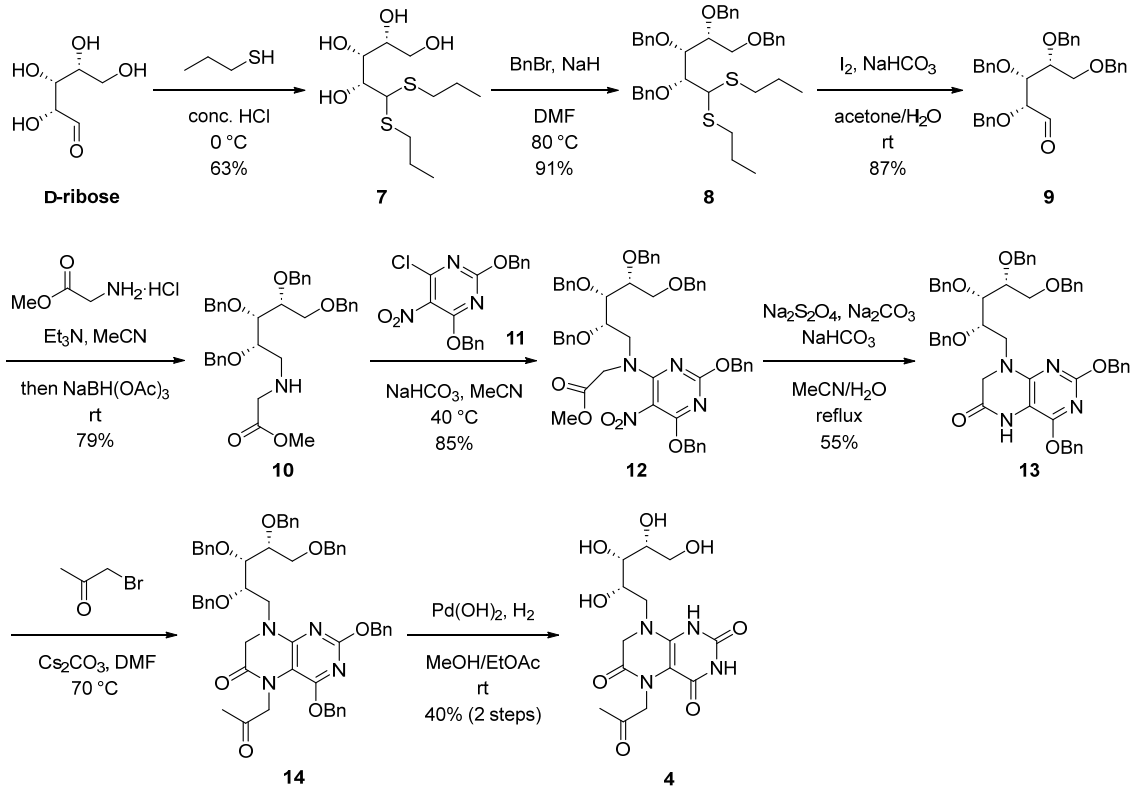


Figure 3. Structure of bicyclic derivatives **1**–**6**.

Chemistry.

We next investigated an efficient synthetic route to bicyclic analogs for robust derivatization and simple operation. The synthesis of ribityllumazine derivative **4** is shown in Scheme 1. Hydroxy groups and pyrimidine oxygen atoms were protected throughout the synthesis with stable benzyl groups, which can be globally removed at the final step. The carbonyl group (for covalent bond formation) was introduced in the latter step to facilitate our diversity-oriented synthesis. Following a literature procedure,[42] D-ribose was converted to benzyl-protected aldehyde **9**. The reductive amination of **9** furnished amine **10** bearing the ester structure, which served as a component for the bicyclic scaffold construction. Amine **10** was connected to benzyl-protected pyrimidine **11** to afford **12**, and the subsequent reduction of the nitro group and intramolecular cyclization gave bicyclic compound **13**. The carbonyl group for covalent bonding with MR1 was then introduced, and the removal of all benzyl groups gave the target compound **4**. Other derivatives were synthesized by a similar synthetic method (See Supporting Information).



Scheme 1. Synthesis of ribityllumazine derivative **4**.

Structure-Activity Relationship Study of Ribityllumazine Derivatives.

We then evaluated the MAIT cell activity of the synthesized derivatives based on a co-culture assay. Human MR1-expressing HeLa (HeLa.MR1) cells and TG40 cells transfected with MAIT TCR (TG40.MAIT-TCR) cells were used as model cell lines of APCs and MAIT cells. Co-cultures were incubated in the presence of the derivatives for 24 h, and the surface expression of CD69, a T cell activation marker, was detected by flow cytometry (Figure S1). The results are shown in Figure 4. Uric acid analog **1** reduced stimulatory activity compared to the parent compound RL-7-Me. Derivatives **2**–**5** bearing a fused six-membered ring displayed different responses. Derivative **2**, with a 1,2-dicarbonyl structure in the left ring, and its *O*-alkylated congener **3** both showed no activity. In contrast, derivative **4** with one carbonyl group in the left ring induced significant activation that was superior to that of RL-7-Me. Further deletion of the carbonyl group had a negative effect on the potency (derivative **5**). The seven-membered ring analog **6** also slightly activated MAIT cells similarly to **5**.

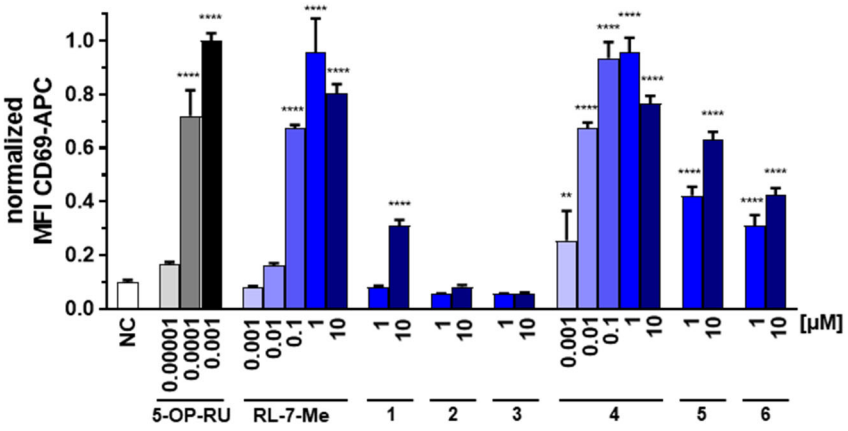


Figure 4. T cells activation assay. CD69 surface expression on TG40.MAIT-TCR cells detected by flow cytometry after 24 h incubation in co-culture with HeLa.MR1 cells in the presence of each ligand, followed by labeling with an APC (allophycocyanin)-conjugated anti-CD69 antibody. The graph shows the mean ± SD of triplicate measurements, and the result shown is representative of three independent experiments. NC = negative control (without stimulation of ligands). $^{**}P < 0.01$, $^{****}P < 0.0001$ vs NC, one-way ANOVA and Dunnett's test.

We then optimized the structure of the promising ligand **4**. Considering the significant influence of the bicyclic scaffold on potency, as described above, we introduced substituents onto the methylene group (7-position) of the pteridine core of **4** to tune activity (Figure 5). Introducing a methyl group (**15**, **16**) or cyclopropyl ring[43] (**17**) resulted in a slight decrease in activity compared to the parent ligand **4**. A bulkier dimethyl substitution (**18**) showed no detectable response. These results indicated that introducing a substituent onto the 7-position of the pteridine core of **4** was undesirable for MAIT cell activation.

The MR1-ligand complexes on the cell membrane are endocytosed and degraded, with some recycled back to the cell surface. In this process, the acidic conditions in the endosome promote hydrolysis of the Schiff base of the complex, causing dissociation or ligand replacement.[24,44] Therefore, we next investigated the effect of modifying the steric environment around the Lys-reactive group, which should affect the dynamics of imine formation and its retro process. As shown in Figure 5, the terminal methyl group can be replaced with an ethyl group (**19**) with retention of activity. The *n*-propyl analog **20** showed decreased but moderate potency. Branched substitutions were not tolerated except for the cyclopropyl substituted derivative **21**, whereas isopropyl (**22**) and *t*-butyl (**23**) replacements exhibited no cell activation. We also investigated the importance of the carbonyl group. One carbon extended derivative **24** and the carbonyl deletion analog **25** had marginal effects on MAIT cell activation. Stimulating with alcohol **26**, which may interact with the MR1 Lys non-covalently, caused only modest activation. These results highlighted the significance of the carbonyl group in **4** for forming a covalent bond with Lys43 of MR1.

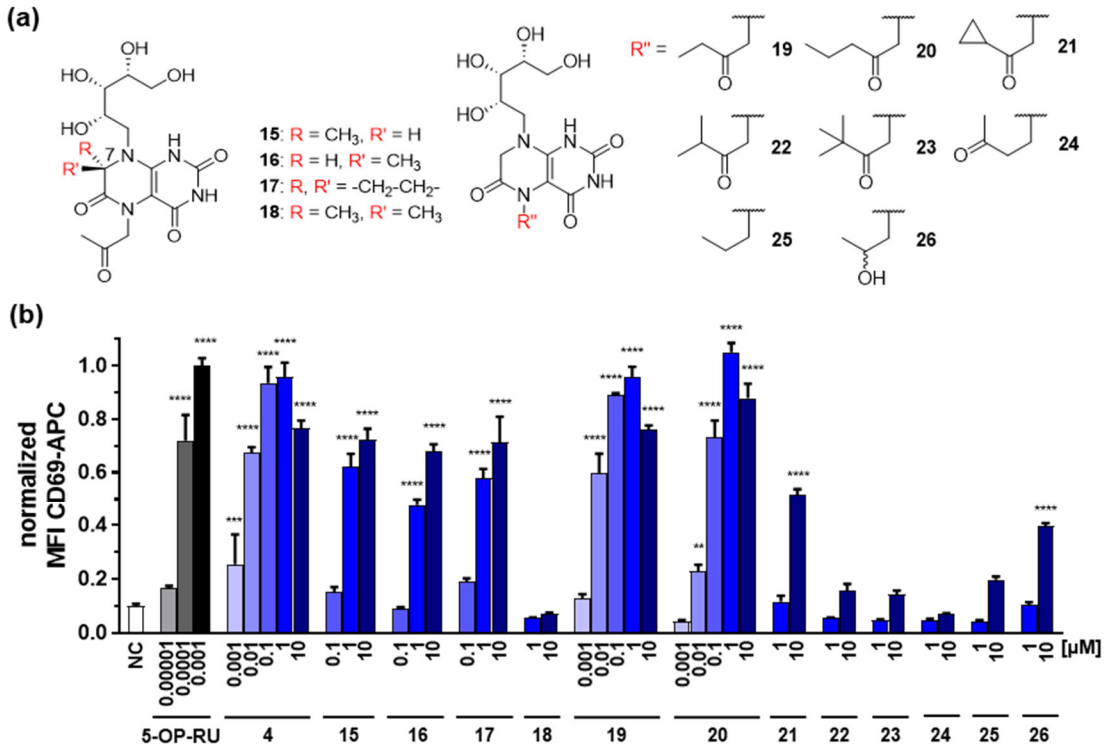


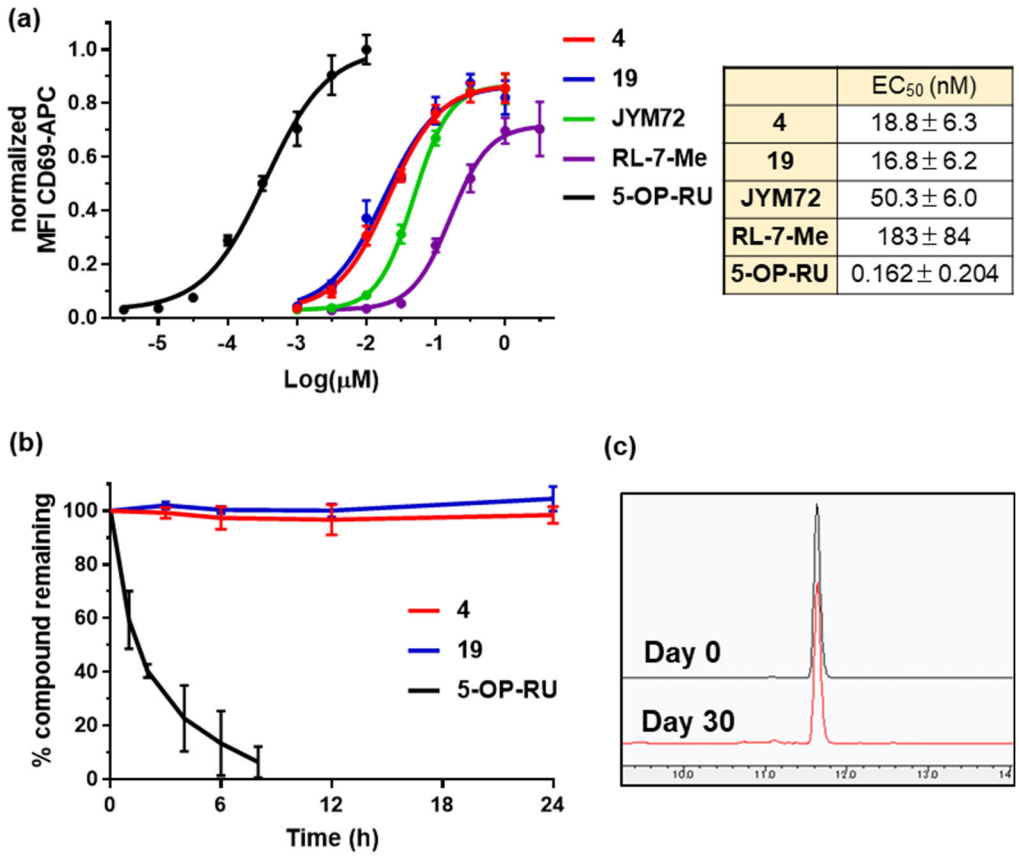
Figure 5. Substituent effects of bicyclic scaffold and carbonyl group on MAIT cell activation. (a) Structure of derivatives **15**–**26**. (b) CD69 surface expression on TG40.MAIT-TCR cells detected by flow cytometry after 24 h incubation in co-culture with HeLa.MR1 cells in the presence of each ligand, followed by labeling with an APC-conjugated anti-CD69 antibody. The graph shows the mean ± SD of triplicate measurements, and the result shown is representative of three independent experiments. NC = negative control (without stimulation of ligands). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs NC, one-way ANOVA and Dunnett's test.

Functional Analysis of the Developed Derivatives.

The functional properties of the derivatives were investigated in detail. Initially, we calculated the EC_{50} values in MAIT cell activation by the co-culture assay (Figures 6a and S2). The EC_{50} values for ligands **4** and **19** were 18.8 and 16.8 nM, respectively, which are significantly more potent than RL-7-Me (EC_{50}: 183 nM), the parent compound in our ligand design. These EC_{50} values were comparable to that of JYM72 (EC_{50}: 50.3 nM), which is the most potent ligand among reported chemically stable MAIT cell activators. We also measured interleukin-2 (IL-2) release into the supernatant of the co-culture system as another indicator of T cell activation using an enzyme-linked immunosorbent assay (ELISA). Consistent with the above results for MAIT cell activation, ligands **4** and **19** showed considerable potency that exceeded the potency of RL-7-Me (Figure S3).

Next, the chemical stability of **4** and **19** was assessed. A 100 μM phosphate-buffered saline (PBS) solution of each derivative was incubated at 37 °C, and then aliquots were analyzed using analytical LC-MS. A percentage of the remaining compound was calculated by

measuring the peak area relative to that of the 0 h time point (Figure 6b). As a result, neither derivative was noticeably degraded after 24 h incubation, indicating improved chemical stability compared to 5-OP-RU. In addition, almost no decomposition was observed (> 95% of **4** remained) when the PBS solution of **4** was stored for one month at 4 °C (Figure 6c). This increased stability can be attributed to the suppressed intramolecular reaction of the Lys-reactive carbonyl group, resulting from the introduction of the rigid bicyclic scaffold. Moreover, attaching an electron-withdrawing carbonyl group onto the nitrogen at the 5-position of the uracil ring would also contribute to inhibiting the oxidation of the electron-rich diaminouracil ring, as mentioned in the design of the 5-A-RU prodrug.[26]



	EC_{50} (nM)
4	18.8 ± 6.3
19	16.8 ± 6.2
JYM72	50.3 ± 6.0
RL-7-Me	183 ± 84
5-OP-RU	0.162 ± 0.204

Figure 6. Ligand properties. (a) Investigation of the dose-dependency of MAIT cell activation. CD69 surface expression on TG40.MAIT-TCR cells detected by flow cytometry after 24 h incubation in co-culture with HeLa.MR1 cells in the presence of each ligand, followed by labeling with an APC-conjugated anti-CD69 antibody. The graph and table show the mean ± SD of triplicate measurements. (b) Chemical stability analysis. Each ligand was incubated (37 ℃, 100 μM in PBS), and the remaining compounds were quantified by measuring the peak area detected by absorbance of 254 or 365 nm lamp using analytical LC-MS. The graph shows the mean ± SD of triplicate measurements. (c) LC chromatograms of before and after one month preservation of PBS solution of **4** at 4 ℃.

We then carried out inhibition experiments with acetyl-6-formylpterin (Ac-6-FP), a compound that covalently binds to the same binding site (MR1 Lys43) as 5-OP-RU and interrupts MAIT cell activation by 5-OP-RU (Figure S4).[45] MAIT cell activation by derivatives **4** and **19** was inhibited in a dose-dependent manner by the addition of Ac-6-FP, indicating that derivatives **4** and **19** activated MAIT cells in an MR1-dependent manner. Additionally, competition experiments of the less potent derivatives (**2**, **3**, **18**, **22**–**25**) with 5-OP-RU were conducted (Figure S5). HeLa.MR1 cells were pretreated with these derivatives using the co-culture system, and then 5-OP-RU was added. Each derivative inhibited stimulation of 5-OP-RU slightly at high concentrations (10 μM), suggesting that these derivatives interact with MR1; however, their binding potency is not significant.

As mentioned above, the binding event of a ligand to MR1, especially forming a covalent bond, facilitates further folding of MR1 and subsequent translocation from the endoplasmic reticulum to the cell surface.[24] Therefore, we examined the effect of ligand stimulation on the surface expression of MR1. HeLa.MR1 cells were treated with the derivatives, and the cell surface level of MR1 was detected by flow cytometry (Figure S6a). Consistent with a previous report, Ac-6-FP, 5-OP-RU and JYM72 upregulated MR1 surface expression.[22,45] Compounds **4**, **19** and **20**, agonistic ligands toward MAIT cells, showed slightly increased MR1 surface expression (Figure S6b). Interestingly, the MR1 surface levels were significantly lower than cells treated with JYM72, even though derivatives **4**, **19** and JYM72 exhibited comparable MAIT cell activation potency. For other derivatives, no noticeable increase in MR1 surface expression was observed. Taken together, the differences in MAIT cell activation among the ribityllumazine derivatives may be partially caused by the ligand properties, including the MR1 translocation ability and MR1-binding dynamics. Detailed studies of the relationship between MR1 surface expression levels and MAIT cell activation are now under investigation. Note that derivatives **4** and **19** showed no apparent inhibitory effect on the growth of either HeLa.MR1 cells or TG40.MAIT-TCR cells, even at the high concentration of 100 μM (Figure S7).

X-ray Crystallography.

The detailed binding mode of ligand **4** to MR1 and MAIT TCR was investigated by X-ray crystallography of the human MR1 (hMR1), ligand **4** and human MAIT TCR (A-F7) ternary complex. During the course of this work, we found that **4** promoted the refolding of hMR1 as well as known MR1 ligands such as 5-OP-RU and 6-FP,[20,21] clearly indicating that **4** functions as an MR1 ligand. The crystal structure of the hMR1–**4**–A-F7 ternary complex was determined at 3.4 Å resolution (Figure 7a). As expected, derivative **4** bound to the A' pocket of MR1 and formed a covalent bond (Schiff base) with Lys43. The hydroxy groups of the ribityl moiety interacted with MR1 Arg94 and Gln153 via hydrogen bonds, and the bicyclic scaffold interacted with MR1 Arg9, Ser24, Tyr62 and Arg94 (Figure 7b). In particular, the hydroxy group of MR1 Tyr62 weakly interacted with the carbonyl group of the bridged moiety of **4**. However, given that removing of this carbonyl group decreased potency (derivative **5**, Figure

4), this interaction would be critical for the potency of **4**. Moreover, the ring structure forms a π-stacking interaction with MR1 Tyr7. Regarding the recognition of MAIT TCR, Tyr95 of the A-F7 entered the A' pocket of MR1 and formed hydrogen bonds with the ribityl group of **4** and MR1 Tyr152 (Figure 7c). This interaction triad has been observed in other MAIT cell activators and appears to be essential for sufficient MAIT cell activity.[36] Compared to the known 5-OP-RU conformation in the ternary complex, the configuration and recognition of the ribityl fragment and the cyclic scaffold were generally similar, whereas the Schiff base was positioned out of the plane of the ring because this moiety was not conjugated to the aromatic ring (Figure S8).

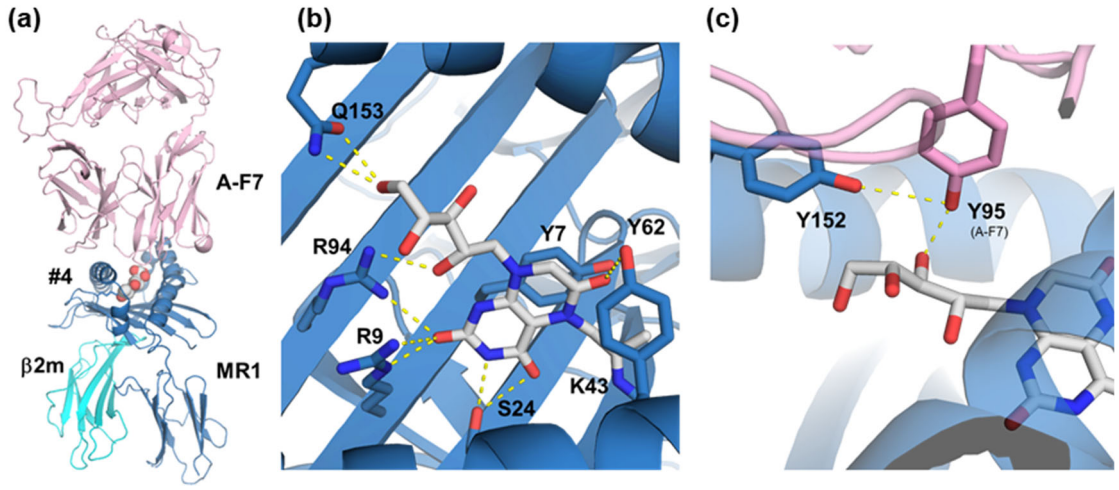


Figure 7. Crystal structure of human MR1–**4**–MAIT TCR (A-F7) ternary complex. (a) Overall structure of ternary complex. Protein and **4** are shown as ribbon and sphere models, respectively. (b) Close-up view of **4**. Amino acid residues directly interacting with **4** are shown as stick models. Polar interactions between **4** and MR1 are indicated with yellow dotted lines. (c) Recognition of A-F7. The polar contacts with Tyr95 of CDR3α are indicated with yellow dotted lines.

Stimulation of Human PBMCs with a MAIT Cell Activator.

Next, we investigated whether the developed ligand activates human MAIT cells in PBMCs. Cell proliferation of MAIT cells was measured as an indicator of activation.[46,47] PBMCs stained with Cell Trace Violet (CTV) reagent were incubated with 5-OP-RU or derivative **4** for seven days, and CTV dilution of MAIT cells (defined as $CD3^+CD161^+$MR1–5-OP-RU $tetramer^+$ cells) was assessed by flow cytometry.

As shown in Figures 8a and 8b, similar to 5-OP-RU as a positive control, the ligand-treated group had a higher percentage of CTV^{low} MAIT cells. In contrast, untreated cells showed minimal proliferation. Supporting this result, the population of MAIT cells gated on live $CD3^+$ cells increased when treated with 5-OP-RU or **4**, reflecting MAIT cell proliferation (Figures 8c and 8d). Conversely, there were few CTV^{low} cells in conventional $CD3^+$ T cells, excluding MAIT cells (< 1% in all treatment groups, Figure S9). Overall, these results indicated that derivative **4** specifically activated human MAIT cells.

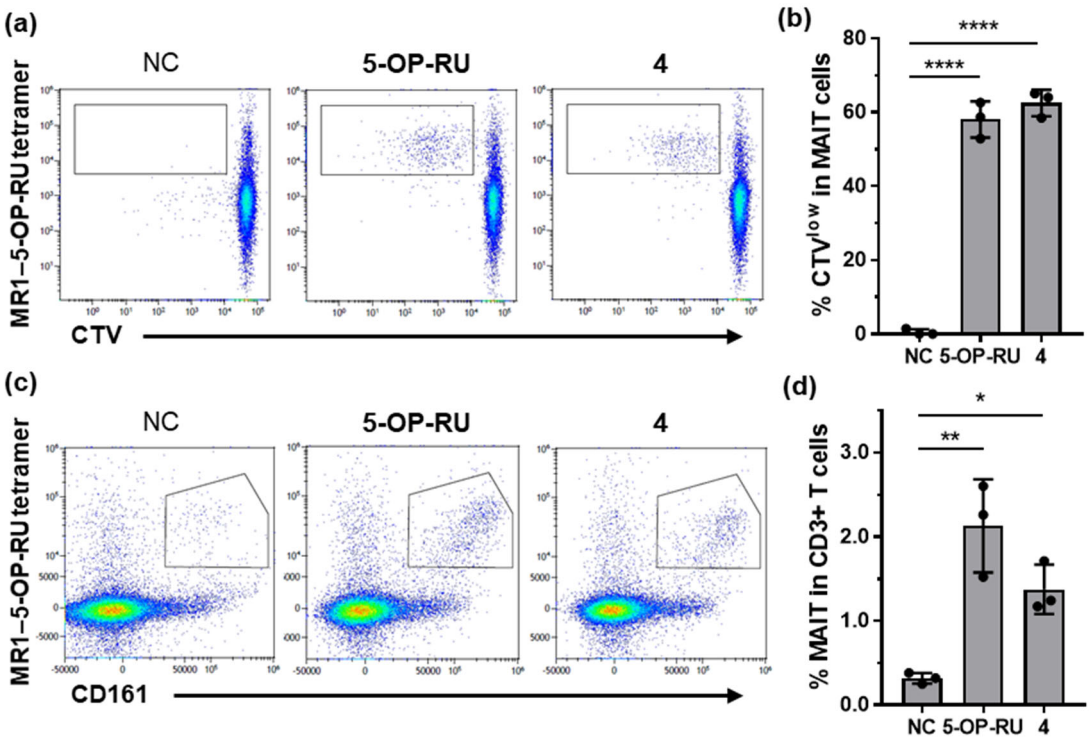


Figure 8. Proliferation experiment of human PBMCs. CTV (CellTrace™ Violet) stained human PBMCs were incubated with each ligand (5-OP-RU: 10 μM, derivative **4**: 100 μM) for seven days, followed by co-staining with PI (propidium iodide), Tet (MR1–5-OP-RU tetramer), and CD3 and CD161 antibodies. (a) Live $CD3^+$ lymphocytes are shown, and the gating indicates the proliferated (CTV^{low}) Tet^+ cells. (b) Percentage of proliferated MAIT cells in total MAIT cells ($CD3^+CD161^+Tet^+$). (c) Live $CD3^+$ lymphocytes are shown, and the gating indicates $CD161^+Tet^+$ MAIT cells. (d) The population of MAIT cells in live $CD3^+$ cells. The data are from one representative donor of three. The graphs show the mean ± SD of triplicate measurements. NC = negative control (without stimulation of ligands). $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ vs NC, one-way ANOVA and Dunnett's test.

Antigen and Ligand Co-Administration Experiments in Mice.

Finally, we examined the immune responses in mice when co-stimulated with derivative **4** and the antigen. Pankhurst et al. reported that co-administration of 5-OP-RU with antigens enhanced antigen-specific antibody production owing to the activation of dendritic cells in a MAIT cell-dependent manner.[48] This study suggested that the MAIT cell activator functions as a vaccine adjuvant. This observation encouraged us to investigate whether derivative **4** shows an adjuvant effect. As shown in Figure 9a, C57BL/6J mice were immunized by intradermal (i.d.) injection with ovalbumin (2.5 mg/kg), either alone or in combination with derivative **4** (30 mg/kg) once a week (days 0, 7 and 14), and serum was collected on days 14 and 21. The production of OVA (ovalbumin)-specific antibodies in serum was evaluated by ELISA. As shown in Figures 9b and S10, a significant enhancement in total IgG levels in the ligand-treated group was observed compared to the non-treated control group. To gain a

detailed understanding of this effect, we also analyzed OVA-specific IgG1, IgG2a and IgM levels, resulting in similarly increased antibody production for all classes. Taken together, these results demonstrated that derivative **4** activates the immune system and promotes an antigen-specific response in mice, suggesting derivative **4** acts as a vaccine adjuvant.

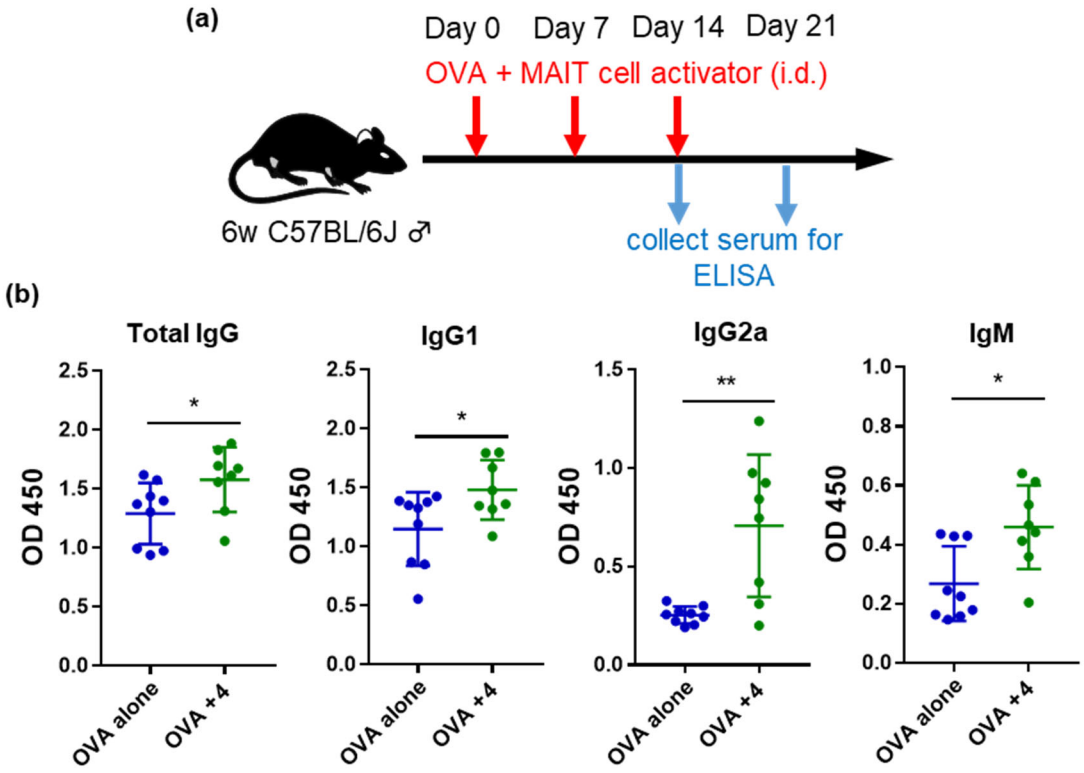


Figure 9. Investigation of the capability of promoting immune response in mice. (a) C57BL/6J mice received intradermal (i.d.) injection of 2.5 mg/kg OVA alone or in combination with derivative **4** (30 mg/kg) at days 0, 7 and 14. Serum was collected at days 14 and 21 and then analyzed using ELISA. (b) Optical density (OD) at 450 nm from serum OVA-specific total IgG, IgG1, IgG2a, and IgM ELISA at day 21. N = 9 (OVA alone) and n = 8 (OVA + **4**) mice per group.*$P < 0.05$, **$P < 0.01$ vs OVA alone, student's t test.

CONCLUSIONS

MAIT cells, a class of innate-like T cells, are attractive therapeutic targets because of their extensive involvement in various disorders. MAIT cells are regulated by TCR-mediated recognition of small-molecule compounds, presented by MR1, which are typically microbial vitamin metabolites. The diversity of MR1 ligands has increased over the years and includes drug molecules,[49] dietary components,[50] and host-derived sulfated bile acid.[47] We recently established a MR1 presentation reporter assay system and identified that coniferyl aldehyde derivatives, ingredients derived from herbal medicines, were recognized by MR1.[51] Research groups have designed artificially modified MR1 ligands for biological assays.[52,53] Despite expanding the number and variety of MR1 binders, practical ligands and their rational design

remain limited.

To address this deficiency, we designed ribityllumazine analogs that combine a Lys-reactive group and rigid bicyclic structures. Based on the established synthetic scheme with simple operation and derivatization, we conducted a structure-activity relationship study of this new class of compounds. We found that derivatives **4** and **19**, with a pteridine scaffold as a key structural element, exhibited good MAIT cell activation potency and chemical stability. Results from the MR1 translocation assay showed that potent ligands **4**, **19** and **20** upregulated the MR1 surface expression in contrast to the other derivatives. Structural data from X-ray crystallography supported these results, revealing that the constructed bicyclic substructure of derivative **4** interacts with MR1, and the interaction triad was formed through hydrogen bonding between MR1, ligand and MAIT TCR, which would be critical for MAIT cell activation.

Taking advantage of the extensive functions of MAIT cells, both agonistic and antagonistic MAIT cell modulators have been used in several therapeutic approaches for defense against infection,[48,54,55] cancer,[25,56] tissue repair[57] and autoimmune diseases.[58–60] In this work, we demonstrated that the chemically stable ligand **4** induced the production of antigen-specific antibodies in mice, highlighting its potential as a vaccine adjuvant. Furthermore, we confirmed that human MAIT cells in PBMCs were activated by derivative **4**. Given the large population of MAIT cells in humans, their modulation with optimized MR1 ligands represents promising therapeutic approaches.

In conclusion, we performed structure-activity relationship studies of ribityllumazine analogs, resulting in the development of chemically stable and potent MAIT cell activators. We believe our results will furnish new insights into the rational design of MAIT cell modulators and accelerate therapeutic applications of attractive but undeveloped MAIT cells.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge at https://

Additional synthetic schemes and biological evaluation data, experimental procedures, and characterization data of all new compounds (PDF).

Notes

The authors declare no competing financial interest.

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