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Chemical Modulators of Mucosal Associated Invariant T Cells

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CONSPECTUS:

Over the past decade, we have contributed to the chemistry of microbial natural products and synthetic ligands, related to riboflavin and uracils, that modulate immune cells called mucosal associated invariant T cells (MAIT cells). These highly abundant T lymphocytes were only discovered in 2003 and have become recognized for their importance in mammalian immunology. Unlike other T cells, MAIT cells are not activated by peptide or lipid antigens. In collaboration with immunology and structural biology research groups, we discovered that they are instead activated by unstable nitrogen-containing heterocycles synthesized by bacteria. The most potent naturally occurring activating compound (antigen) is 5-(2-oxopropylideneamino)-D-ribitylaminouracil (5-OP-RU). This compound is an imine (Schiff base) formed through condensation between an intermediate in the biosynthesis of riboflavin (vitamin B2) and a metabolic byproduct of mammalian and microbial glycolysis. Although it is very unstable in water due to intramolecular ring closure or hydrolysis, we were able to develop a non-enzymatic synthesis that yields a pure kinetically stable compound in a nonaqueous solvent. This compound has revolutionized the study of MAIT cell immunology due to its potent activation (EC_{50} = 2 pM) of MAIT cells and its development into immunological reagents for detecting and

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Notes

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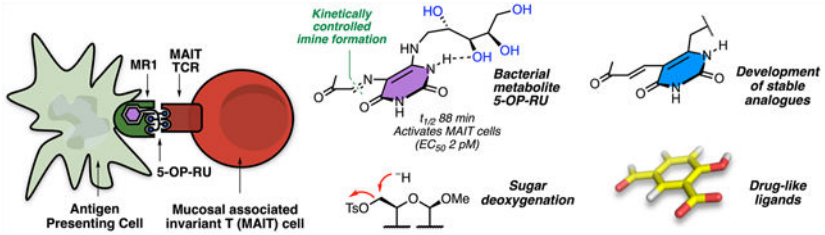
characterizing MAIT cells in tissues. MAIT cells are now linked to key physiological processes and disease, including antibacterial defense, tissue repair, regulation of graft-vs-host disease, gastritis, inflammatory bowel diseases, and cancer. 5-OP-RU activates MAIT cells and, like a vaccine, has been shown to protect mice from bacterial infections and cancers. Mechanistic studies on the binding of 5-OP-RU to its dual protein targets, the major histocompatibility complex class I related protein (MR1) and the MAIT cell receptor (MAIT TCR), have involved synthetic chemistry, 2D ^{1}H NMR spectroscopy, mass spectrometry, computer modeling and molecular dynamics simulations, biochemical, cellular, and immunological assays, and protein structural biology. These combined studies have revealed structural influences for 5-OP-RU in solution on protein binding and antigen presentation and potency; informed the development of potent (EC_{50} = 2 nM) and water stable analogues; led to fluorescent analogues for detecting and tracking binding proteins in and on cells; and enabled discovery of drugs and drug-like molecules that bind MR1 and modulate MAIT cell function. MAIT cells offer new opportunities for chemical synthesis to enhance the stability, potency, selectivity, and bioavailability of small molecule ligands for MR1 or MAIT TCR proteins, and to contribute to the understanding of T cell immunity and the development of prospective new immunomodulating medicines.

Graphical Abstract



1. INTRODUCTION

T cells (T lymphocytes) are key cellular mediators of adaptive immunity and are activated by peptide[5] or lipid[6] antigens (*e.g.*, **1** or **2**; Figure 1A). A new type of highly abundant unconventional T cell discovered in 2003 is the mucosal associated invariant T cell (MAIT cell), which is not activated by peptides or lipids[7] and is becoming progressively more recognized for its importance in mammalian immunology and disease.[8] The riboflavin biosynthesis pathway in bacteria was implicated in 2012 as the source of naturally occurring ligands for mammalian MAIT cells,[7] but the structures of the most potent naturally occurring antigens were not reported until 2014 as water-unstable heterocyclic uracil derivatives, such as 5-(2-oxopropylideneamino)-D-ribitylaminouracil (5-OP-RU, **3**; Figure 1A).[1]

The mechanism by which they activate T cells differs from most ligand−receptor binary complexes (*e.g.*, ligand complexes of G-protein coupled receptors). First, the antigen (peptide, lipid, or uracil) binds to an antigen-presenting protein (MHC, CD1, or MR1, respectively; Figure 1B) expressed on an antigen-presenting cell (APC; Figure 1B), which is usually a dendritic cell, macrophage, or B cell. This antigen−protein complex engages a second protein (T cell receptor) expressed on a T cell, NKT cell, or MAIT cell for

peptide, lipid, or heterocyclic antigens, respectively. This complex mechanism enables sophisticated control over the degree of antigen presentation to the TCR, with both antigen and antigen-presenting protein cooperatively interacting with the TCR to trigger T cell activation. Despite mechanistic similarities, the three antigen classes interact differently with both antigen-presenting proteins and TCRs (Figure 1B). Peptides sandwich between TCR and MHC proteins using their side chains and main chains to interact with both proteins. Lipids such as α-GalCer embed their lipid chains in CD1d and use their cyclic sugar moiety to interact with NKT TCR. By contrast, ribityluracils such as 5-OP-RU use their uracil moiety to interact with MR1 and their ribityl group to interact with both MR1 and MAIT TCR, as discussed ahead.

Discovery of this third type of antigen, exemplified by the nitrogen-containing heterocycle 5-OP-RU, has helped catalyze a recent explosion of MAIT cell biology (MAIT publications/year: 41/2005, 170/2012, 6200/2020). The availability of synthetic 5-OP-RU has enabled potent activation of MAIT cells, and production of antigen−MR1 tetramers for the detection and immunological characterization of mammalian MAIT cells in physiology and disease. In turn, this has led to MAIT cells being implicated in antimicrobial defense (bacterial,[10–12] viral[13]), tissue repair,[14,15] cancer,[16] and inflammatory diseases.[17] Here, we describe the identification of ligands that bind MR1 protein and activate or inhibit MAIT cells.

2. DISCOVERY OF ANTIGENS FOR MAIT CELLS

Nonstimulatory MR1 Ligands Derived from Vitamin B9

The discovery of bacterial ligands that bind MR1 and modulate MAIT cells was the result of an interdisciplinary collaboration between the McCluskey (immunology, University of Melbourne), Rossjohn (structural biology, Monash University), and Fairlie (chemistry, University of Queensland) research groups.[18] It required exchange of materials, protocols, information, and sharing of expertise between laboratories in two cities and three universities to enable efficient identification and characterization of the highly reactive antigens.

The first breakthrough was the discovery that 6-formylpterin (6-FP, **4**), a photodegradation product of folic acid (vitamin B9) in protein buffer, could refold MR1 (Scheme 1).[7,9] A crystal structure of **4** bound to MR1 and MAIT TCR showed that its aldehyde formed a reversible covalent bond (Schiff base) with the Lys43 amine side chain in MR1 (PDB: 4GUP). Although 6-FP could bind, refold, and upregulate MR1 expressed in APCs, it could not stimulate MAIT TCR activation. Instead, it weakly inhibited activation of MAIT TCR by bacterial supernatants. Acetyl-6-formylpterin (Ac-6-FP, **5**) also upregulated MR1, inhibiting MAIT cell activation more potently (IC_{50}, 0.1 μM). Although nonstimulatory, **4** and **5** provided the first clues to the structural requirements for MR1 binding and refolding.

Stimulatory Ligands Derived from Vitamin B2 (Riboflavin)

A second breakthrough came through genetic studies on Gram-positive and Gram-negative bacteria, which showed that only bacteria possessing enzymes for riboflavin biosynthesis could produce ligands that bound MR1 and stimulated MAIT TCR.[1] MR1 could be refolded

by the supernatant of *Salmonella typhimurium*, a riboflavin-producing bacterium. The molecular formulas of ligands that bound and refolded MR1 were established through EI-MS and isotopic mass distribution as $C_{12}H_{18}N_4O_7$ (*m*/*z* 329.1099, negative mode) and $C_{11}H_{16}N_4O_7$ (*m*/*z* 315.0944, negative mode). We examined the riboflavin biosynthetic pathway (Scheme 2) for compounds matching these formulas. The key biosynthetic intermediate 5-A-RU (**6**) condenses with glycolysis product 3,4-dihydroxy-2-butanone-4-phosphate (**7**) to give 5-(1-methyl-2-oxopropylideneamino)-6-D-ribitylaminouracil (5-MOP-RU, **8**), a process catalyzed by lumazine synthase. This intermediate readily undergoes dehydrative ring closure (also catalyzed by lumazine synthase) to form bicyclic 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-DiMe, **9**). Riboflavin synthase then catalyzes the disproportionation of two molecules of **9** to give riboflavin (**10**) and 5-A-RU (**6**), which re-enters the catalytic cycle. However, none of these compounds matched the molecular formula of the bacterial isolate.

Chemical Synthesis of Riboflavin Biosynthetic Intermediates and Metabolites

To explore other compounds in the pathway as putative antigens, we took advantage of the knowledge that key intermediate 5-A-RU (**6**) was also known to non-enzymatically produce RL-6,7-DiMe (**9**; MW 326),[19] which can undergo further benzoquinone-mediated oxidation to metabolite 17 (RL-6-Me-7-OH; MW 328; Scheme 3). A two-electron reduction of **17** could theoretically give **18** (rRL-6-Me-7-OH; MW 330). Alternatively, cyclocondensation of 6 with glycolysis byproduct[20] 1,3-dihydroxyacetone (**14**) could yield **15** (rRL-6-HM; MW 330). Therefore, we undertook chemical syntheses of **9, 15, 17,** and **18** in search of the potent MAIT-activating antigen. Reaction of pH-adjusted aqueous 5-A-RU (**6**) with 1,3-dihydroxyacetone (**14**) gave **15** *in situ,* which was prone to aromatizing oxidation or dehydration to form **16**. Switching the dicarbonyl electrophile to 2,3-butanedione (**13**) gave **9** (RL-6,7-DiMe), which was oxidized to **17** (RL-6-Me-7-OH). However, we could not isolate 18 (rRL-6-Me-7-OH), as it was very susceptible to oxidative rearomatization to RL-6-Me-7-OH (**17**) and only transiently detectable during reduction of 17 or ring closure of acid **19** (Scheme 3). All three synthetic compounds (**9, 15,** and **17**) activated MAIT TCR, measured by upregulation of surface marker CD69, interferon-γ, and tumor necrosis factor, but samples of rRL-6-HM (**15**) were the most potent by orders of magnitude.

Unexpected Capture of 5-OP-RU by MR1

Compound **15** was generated over 5−20 min from pH-adjusted aqueous 5-A-RU (**6**) and 1,3-dihydroxyacetone (**14**), liberated from its commercially available dimer *in situ* by heating to 80 °C. Product **15** was unstable and not isolated completely pure, but it possessed the same MS/MS fragmentation pattern and properties (ability to refold MR1 and activate MAIT TCR) as the antigen in the bacterial supernatant. It was therefore used for cocrystallization with MR1 and MAIT TCR proteins. Instead of **15**, the structure of the ternary complex (Figure 2) showed that MR1 and MAIT TCR had preferentially captured a trace amount of the highly unusual ring-opened imine isomer 5-OP-RU (**3**), one of the possible condensation products of **6** and **11** (Scheme 3). Like **4** and **5**,[7] 5-OP-RU (**3**) was covalently bonded to the side chain amine of Lys43 in MR1, forming an imine (Figure 2). Although ligand-protein interactions forming imines (Schiff bases) are known in biology

(pyridoxal phosphate, fructose 1,6-bisphosphate, retinal),[21] the covalent linkage between the uracil and MR1 (Figure 2) involves two consecutive Schiff bases, creating a 1,2-diimino motif (-NH═CR^1−CR^2═N-) unprecedented in biological chemistry.

In addition to the imine being prone to hydrolysis, 5-OP-RU (**3**) was poised for a 6-*exo*-trig aromatizing cyclocondensationbetween the carbonyl and nucleophilic vinylogous amide nitrogen to give **12**. LCMS indicated that an isomer of **15** was indeed present as a low-level impurity in the sample of **15** used for crystallization (only later confirmed as 5-OP-RU after its successful synthesis, *vide infra*) and that trace amounts of methylglyoxal (**11**) could also be formed *in situ* during synthesis of **15** (Schemes 3 and 4).

Synthesis of 5-OP-RU

We sought to synthesize pure 5-OP-RU (**3**) from 5-A-RU (**6**) and glycolysis byproduct[23] methylglyoxal (**11**; Scheme 3). Several challenges needed to be overcome. First, reaction of diamine **6** with dicarbonyl **11** was known to form bicyclic lumazine **12**,[24] with **3** postulated to be a fleeting intermediate that simultaneously forms and degrades. Second, the condensation could form multiple regioisomers. We attempted to use LCMS and NMR spectroscopy to detect the compound *in situ* during its transient formation from 5-A-RU (**6**) and methylglyoxal (**11**) in water (5-A-RU is insoluble in many solvents). However, we detected only transient formation of trace amounts of 5-OP-RU, and the accumulation of lumazine **12** and its 6-methyl regioisomer (**16**). Varying the temperature, concentration, and pH all changed the reaction rate and regioisomeric distribution, but did not enable isolation of 5-OP-RU from water.

Reexamination of the mechanism of 5-OP-RU (**3**) formation and degradation led to the next breakthrough (Scheme 5). We noted that although 5-OP-RU cyclized readily, the *E*-configuration of the imine held the carbonyl and nucleophilic nitrogen apart. Thus, for 5-OP-RU to cyclize, the imine must first rehydrate to hemiaminal **21**. This enables rotation of both C−N bonds highlighted in **21** (Scheme 5), allowing intramolecular condensation. Therefore, 5-OP-RU was not a reactive intermediate but rather a kinetically stable product that was thermodynamically unstable but potentially isolatable under carefully controlled conditions.

To investigate this possibility, we focused on controlling the fate of hemiaminal **21**, the intermediate from which 5-OP-RU (kinetic product) or the lumazine (thermodynamic product *via* **23** and **24**) would competitively form. We hypothesized that, as imine formation in polar solvents typically proceeds *via* the iminium intermediate (**22**), aprotic polar solvents might stabilize the cation while avoiding solvent attack on iminium **22**, thus diverting the reaction toward 5-OP-RU.

Thus, we condensed methylglyoxal with model compound **6m** in solvents of increasing dielectric constants, namely, nitromethane-d_3, DMF-d_7, and DMSO-d_6 (Scheme 5). While **6m** was sparingly soluble in nitromethane, and DMF gave a mixture of imine **3m** and lumazine **12m**, DMSO exclusively yielded **3m**, providing evidence for our mechanistic proposal. DMSO was then similarly employed to deliver 5-OP-RU (**3a**) as a single product, unambiguously characterized by 2D NMR spectroscopy.

Although 5-OP-RU is reasonably pure and characterizable *in situ* in DMSO-d_6, we purified 5-OP-RU further by rapid reversed phase HPLC using an acetonitrile−water gradient to definitively prove its structure and acquire it in pure solid form (Figure 3A). Speed, neutralization of product fractions, and lyophilization under acid-free conditions were critical to success. The solution structure of 5-OP-RU was established in 2013 by HRMS and 2D-NMR spectroscopy, with the $^{1}H-^{13}C$ HMBC NMR spectrum establishing the structure of the distinctive iminocarbonyl group and its connectivity to the uracil ring (Figure 3B). The material matched the crystal structure of proteinbound 5-OP-RU obtained from the sample of 15, but pure 5-OP-RU was remarkably more potent in activating MAIT cells (EC_{50}, 2 pM),[2] thus securing the identity of the bacterial MAIT cell antigen. This extremely potent antigen is indefinitely stable in DMSO at −20 °C, with a half-life of 88 min under aqueous conditions at 37 °C, pH 5.4.[2] The *m/z* 315 species from the bacterial supernatant was attributed to its aldehyde homologue 5-OE-RU, which was similarly synthesized from 5-A-RU and glyoxal (another glycolysis byproduct[23]) and characterized spectroscopically and crystallographically (PDB 4NQE),[1] but it was much less stable[2] and more difficult to purify.

Impact of 5-OP-RU

Synthetic 5-OP-RU (3) has made an indelible impact on MAIT cell immunology. The MR1−(5-OP-RU) binary complex is recognized by MAIT TCR (and slight variants), which is uniquely expressed on MAIT cells. Synthetic 5-OP-RU has been used to form fluorophoric immunological tetramers,[25,26] consisting of four MR1−(5-OP-RU) complexes tethered together. These reagents selectively bind MAIT TCRs and are regarded as the gold standard[27] for identifying and staining MAIT cells, since even antibodies raised against MAIT TCR exhibit nonspecific binding to other TCRs. These tetramers have been distributed worldwide[8] through licensing of our patents to the U.S. National Institutes of Health (NIH) Tetramer Facility.[28]

While early studies relied on bacteria or supernatant mixtures as the antigen source, availability of pure 5-OP-RU (**3**) allowed MAIT cells to be activated with much greater efficiency and precision. This enabled collaborators to discover the requirement of cofactors or co-stimulants for the expansion of MAIT cell numbers (*e.g.*, toll-like receptor 2 agonists),[29] lung accumulation (CXCL16),[30] augmented effector function (α-ketoglutarate),[31] and vaccination (IL-23).[11] It was used to interrogate the intracellular pathway[32,33] of antigen capture and presentation by MR1, and to study an MR1 mutation in a person without MAIT cells.[34] Other collaborators used 5-OP-RU to study MAIT cell activation in gastritis,[17] graft-vs-host disease,[35] and antibiotic resistsance.[36] Similarly, the ability for 5-OP-RU to selectively activate MAIT cells has been used to detect MAIT cell (or MAIT-like) populations in animals in the absence of species-specific MR1 tetramers.[37–39] Landmark studies found that 5-OP-RU controls MAIT cell development in the thymus,[40–42] imprints MAIT cells in early life, and promotes tissue repair.[14]

Medicinal applications are also being explored. MAIT cells are attractive vaccine targets,[43] and 5-OP-RU (**3**) can confer antibacterial protection against *Legionella longbeachae* and *Francisella tularensis* infections in mice.[10–12] 5-OP-RU used as a treatment during

chronic *Mycobacterium tuberculosis* infection in mice increased MAIT cell expansion and reduced bacterial loads.[44] 5-OP-RU also conferred protection in mouse models of B16F10 (melanoma) and E0771 (breast) metastasized lung cancer,[45] suggesting the potential for 5-OP-RU in cancer immunotherapy. Collectively, these findings highlight the importance of this natural product as a tool for MAIT cell immunology and immunotherapeutics.

3. ROLE OF THE RIBITYL CHAIN

In view of the remarkable immunogenicity of 5-OP-RU (**3**), and the known roles of sugars in mediating cell adhesion and recognition, we sought to explore the immunological importance of the 5-OP-RU ribityl chain, which resides at the interface between MR1 and MAIT TCR. DFT calculations and molecular dynamics (MD) simulations suggested different influences from each hydroxyl group, so we synthesized all four deoxyribityl analogues of 5-OP-RU by merging the corresponding deoxyribitylamines into the synthesis of 5-OP-RU (black, Scheme 6).[3]

The 2-deoxy analogue of 5-OP-RU (25) was accessed from commercial 2-deoxyribose. The 3-deoxy analogue (28) was synthesized from D-xylose (the C3-OH epimer of D-ribose); this epimer's stereochemistry enabled facile regioselective protection (**26**) to allow C3-OH radical deoxygenation *via* iodide **27**. Meanwhile, 4- and 5-deoxy analogues (**31** and **34**) were synthesized from D-ribose *via* orthogonal protection (**29** and **32**) and then radical deoxygenation of **30** or hydride displacement of **33**, respectively. We similarly synthesized four monohydroxylalkyl analogues (blue, **38**−**41**) from commercial aminoalcohols.

Next, we examined the analogues for MR1 binding and MAIT TCR activation through cellular assays and protein−ligand crystal structures (PDB codes in Scheme 6).[46] Loss of the 2′-OH led to considerable reduction of MAIT TCR activation potency. Although it formed the only direct hydrogen bond with MAIT TCR (Scheme 6 inset), its removal surprisingly did not ablate activity. This could be partially due to 3′-OH compensating through a new interaction with MAIT TCR Tyr95α, as observed in MD simulations in water and supported by the solid-state crystal structure. Loss of 3′-OH also significantly reduced potency. Although it does not interact with TCR (Scheme 6 inset),[1] MD simulations suggest it is important for restricting the ribityl conformation and projecting other hydroxyls for interaction with MR1 and MAIT TCR. It does so through an intramolecular H-bond between 3′-OH and a uracil NH, conceptually similar to conformation-stabilizing intramolecular H-bonds in cyclic peptides.[47] Conversely, although 4′- and 5′-OH interact with MR1 R94, Q153, Y152, and TCR G98β (through water; Scheme 6 inset),[1] loss of either 4′- or 5′-OH had little effect on MAIT TCR activation. Using protein crystal structures of **31** and **34**, MD simulations revealed that these hydroxyl groups could attenuate activation potency by hindering protein−protein interactions. Monohydroxylalkyl analogues were significantly less potent than deoxy analogues, but hydroxyethyl (**38**) and hydroxypropyl (**39**) analogues were the most potent within that series, consistent with 2′-OH and 3′-OH interacting with MAIT TCR Tyr95α.

Others have determined activities for other diastereoisomeric[48,49] sugar and alkyl[50] analogues (Scheme 6, purple and blue, respectively). Their reported biological data show

trends similar to our findings, but should be interpreted cautiously. For example, one study systematically inverted stereochemistry at each ribityl hydroxyl group and similarly found that 2′-OH and 3′-OH epimers (**35** and **36**) but not 4′-OH (**37**) lost potency.[49] However, they reported EC_{50} 100 nM for 5-OP-RU (**3**), compared to our EC_{50} 2 pM.[2] Another study on sugar analogues found that the 2′-OH epimer (**35**) was less active than 5-OPRU.[48] However, all of those compounds exhibited comparable activities, including 5-OP-RU (**3**), its 2-deoxy analogue (**25**), and 2′-OH, 3′-OH, and 4′-OH epimers (**35**−**37**), likely because they were all tested at a single high concentration (10 μM). Another study found that hydroxyethyl and hydroxypropyl analogues **38** and **39** were the most potent hydroxyalkyl analogues, but **38** was reported to be equipotent with 5-OP-RU (EC_{50} ~ 1 μM).[50]

We attribute these discrepancies to the very low conversion to 5-OP-RU (or analogues) in water relative to DMSO (Figure 4, Scheme 5).[2] Although we reported early MAIT TCR activation data for 5-OP-RU derived from its synthesis in water (EC_{50} ~10 μM),[1] researchers need to know that aqueous preparations result in extremely low yields and rapid degradation of 5-OP-RU and analogues (Figure 4).[2] We recommend the use of DMSO for substantially higher yields (Figure 4A), purity, and quantitation by NMR spectroscopy,[51] facilitating comparison with true antigen potency.[2] Water exposure should be minimized to limit 5-OP-RU degradation, although small amounts of water in DMSO can be tolerated. We found that NMR quantified[51] 5-OP-RU, generated *in situ* from 5-A-RU[52] and methylglyoxal (1.1 eq, 40% aqueous solution) in dry DMSO-d_6 as described,[2] can be used directly in assays without further purification, with no reduction of activity compared to lyophilized material from HPLC purification, provided that the final DMSO concentration in aqueous media does not affect cell viability. Importantly, once 5-OP-RU binds MR1, it is sequestered, stabilized, and no longer susceptible to degradation.

The literature qualitatively corroborates our findings, emphasizes the importance of MAIT TCR Tyr95α engagement for TCR activation, and demonstrates the exceptional power of MD simulations for rationalizing structure−activity relationships in these dynamic systems. Although protein crystal structures containing deoxyribityl analogues were all very similar, MD simulations in a water environment uniquely correlated MR1-TCR SPR affinity and MAIT TCR activation potencies with ligand and MR1 residue flexibility.[3,46] Together, these newly revealed determinants of potency and use of computer modeling and MD simulations[3,46] to account for flexibility in solution provide important new clues for analogue design.

4. STABLE ANALOGUES OF 5-OP-RU

Instability of 5-OP-RU (**3**) and analogues in water[2] is a significant limitation for immunologists, pharmacologists, and medicinal chemists in probing MAIT cell biology and potential applications of activators as vaccines and immunostimulants, or inhibitors as anti-inflammatory agents.[43] Other groups have addressed the instability of precursor 5-A-RU (**6**, prone to aerial oxidation) by isolating its hydrochloride salt (**42**)[27] or forming a prodrug[53] (**43**) (Figure 5), both of which can very weakly activate the MAIT TCR after reacting with cellular metabolites to form trace amounts of 5-OP-RU and 5-OE-RU *in situ*. However, we focused instead on stabilizing the antigen 5-OP-RU (Scheme 7).

The 5-OP-RU protein crystal structure[1] indicated that the two exocyclic nitrogens, implicated in its degradation (Scheme 7), did not directly contact the proteins (although the vinylogous amide formed a water-bridged hydrogen bond with MR1 Tyr62), which gave logical starting points for modifications. Initially, we methylated the vinylogous amide to block cyclization (**44** and **45**; Scheme 7). Surprisingly, this reduced both stability and antigen potency, which we attributed to sterically induced torsional twisting of the ribityl and carbonyl groups, as supported by calculations.[2]

To solve this problem, we designed JYM72 (**48**) to replace both nitrogen atoms with carbon. Although routes *via* orotic acid (**49**) or ortho-lithiation (**50**) failed, the analogue could be synthesized in 10 steps from D-ribose. The target enone side chain posed a challenge, but a pragmatic approach with excess base and 4-(trimethylsilyl)but-3-yn-2-one was used to construct the all-carbon backbone in moderate yields. This analogue was stable in water and functionally comparable to 5-OP-RU (3), including its ability to bind MR1, activate MAIT TCR, expand MAIT cells in mouse lung, and induce cytokines ex vivo.[2] However, it was 1000-fold less potent than 5-OP-RU *in vitro* and *in vivo*,[2] possibly because the sp^2-nitrogen to sp^3-carbon replacement increased ribityl chain flexibility, affecting its projection in the MR1-MAIT TCR complex (PDB: 6PUK).[46] Nonetheless, JYM72 (**48**) was a water-stable and potent antigen (EC_{50} 2 nM) that illustrates the potential for rational development of stable antigens.

MHC and CD1 antigen-presenting proteins typically present intracellularly derived antigens, but MR1 presents extracellular bacterial antigens. This may be important for restricting MAIT cell activation to when APCs are also present, thereby enabling co-stimulatory responses. However, it was unclear whether extracellular antigens bound to MR1 on the cell surface or after entering antigen-presenting cells and encountering intracellular MR1. To investigate this, we developed a chemically stable fluorescent analogue of **48**, namely, JYM20 (**52**), enabling the cellular location of MR1 binding to be visualized by microscopy.[32] JYM20 lacked the MAIT TCR-binding ribityl chain of JYM72, greatly simplifying its synthesis (*via* **51**) and permitting stepwise installation of the enone side chain. This side chain doubly activates the uracil ring for 1,6-addition by amines, so we introduced it after the glycyl linker, which incorporates two polar amides to increase water solubility.

Despite the simplifications, JYM20 (**52**) behaved like 5-OP-RU (3) in cell uptake kinetics, MR1 binding, and appearance at the cell surface.[32] Without the ribityl hydroxyls, it did not activate MAIT TCR but did inhibit 5-OP-RU-mediated activation, consistent with competitive binding to MR1.[32] Microscopy and biochemical experiments indicated that JYM20 (**52**) first enters cells to bind MR1 in the endoplasmic reticulum (Figure 6),[32] refolding MR1 to enable translocation of the MR1−ligand complex to the APC surface for MAIT TCR engagement.[32] This illustrates the distinctiveness of the MAIT cell activation process, although the mechanism of cell entry and which specific APCs present antigens to MAIT cells in different tissues are not yet established. Nevertheless, we envisage that JYM20 (**52**) would find general utility for characterizing other aspects of MR1 biology, and as an archetype of other tagged MR1-binding ligands incorporating bespoke payloads.

In summary, more stable potent activators of MAIT cells are desired with a longer duration of action, ease of handling, storage, and distribution. In view of the potent immunostimulating properties of 5-OP-RU (**3**), but lack of aqueous stability, an equipotent stable analogue could represent a key advance, but the challenge is to confer stability without sacrificing potency. Our success in developing stable ligands equipotent with proteins that bind to other cell surface receptors[54] gives us confidence this goal can be reached.

5. DRUG-LIKE LIGANDS

MR1 has multiple tyrosines that form a hydrophobic cleft in which uracils and pterins bind, so we probed whether drugs and drug-like molecules could also bind MR1. We used *in silico* virtual screening to examine molecular fragments similar to 5-OP-RU from a library of 6000 commercial compounds (Figure 7).[4] We also used a library of FDA drugs to perform a shapebased screen on Ac-6-FP (**5**) and a receptor-based screen on the 5-OP-RU binding site in MR1. Of 81 compounds assayed for binding MR1 and modulating MAIT TCR, some hit compounds were studied using protein crystallography (PDB codes in Figure 7).

A diverse range of putative MR1-binding ligands, including salicylates, diclofenac, and flavonoids weakly modulated MAIT cells (Figure 7).[4] New ligands that upregulated MR1 expression included **55**−**58**. Compounds **57** and **58** also inhibited 5-OP-RU-mediated activation of the MAIT TCR. MR1 upregulating ligand **55** modestly activated MAIT TCR, while noncovalent anti-inflammatory drug diclofenac **53** and major metabolite **54** activated the TCR more potently without triggering MR1 upregulation. Interestingly, these ligands activated distinct MAIT TCR variants derived from different MAIT cell subsets, suggesting the potential for future subset-specific chemical modulation.

This study was the first to show that drugs and drug-like molecules could activate MR1 and the MAIT TCR. As expected from unoptimized hits, most of these ligands lacked potency. For example, Ac-6-FP (**5**; Scheme 1) was a more potent upregulator of MR1 and inhibitor of MAIT TCR, while **55** was ~10^7 times less potent than 5-OP-RU. Nonetheless, ligands such as 55 (activator; EC_{50} ~ 20 μM) and **58** (inhibitor; IC_{50} ~ 10 μM) could be drug-like leads for chemical elaboration. Despite their low potencies, some drug concentrations might be clinically relevant and help explain side effects. For example, MR1 upregulator **56** is a known photodegradation product of chemotherapeutic drugs aminopterin and methotrexate. The extent of formation of **56** *in vivo* is unknown, but a chemotherapeutic dose of methotrexate can result in higher drug concentrations in plasma than required for MR1 upregulation. Also, oral **53** can produce drug concentrations that would be sufficient for activating the MAIT TCR *in vitro*, which may reconcile some drug complications such as hepatotoxicity. These preliminary studies provide a glimpse into the future for MR1 and MAIT cell modulation with drug-like compounds.

6. CONCLUSION AND PERSPECTIVE

The seminal discovery of potent antigens for MAIT cells has heralded a new class of T lymphocyte modulators and, along with MR1-antigen tetramers developed therefrom,

is enabling the activation and detection of MAIT cells, respectively. These heterocyclic pyrimidine antigens raise potential opportunities for developing new drugs, vaccines, and immunological tools for interrogating a new area of T cell immunity.

New efficacious and functionally distinct ligands are still needed to characterize MR1 and MAIT cell biology and assess the therapeutic potential of modulating them. However, ligand potency is extremely sensitive to subtle chemical modifications, making development of superior compounds challenging. Structure−activity modeling has suggested that very small changes to bond lengths and dihedral angles can dramatically affect potency.[2,46] Furthermore, protein−ligand crystal structures are often extremely similar for ligands with disparate activities, so computationally expensive and complex MD simulations might be necessary for deeper insights into dynamic protein−antigen interactions. This sensitivity is expected, given that T cell activation must be highly regulated to prevent hyperreactivity, and reflects complex processes controlling cell uptake, intracellular ligand−protein trafficking, protein refolding, and cell surface expression.[32,33] The densely decorated 5OP-RU presents synthetic challenges with selective functionalization often requiring circuitous routes. Removing or masking reactive groups can confer stability, but electrophiles that covalently bond to MR1 Lys43 have been required so far for potent antigenicity and are not easily incorporated.

Despite the challenges, organic synthesis will surely deliver new ligands that can significantly shape the future of MAIT cell immunology. It is not yet clear which diseases require activators or inhibitors of MAIT cells, but both will be useful investigative tools. Compounds **5**, **25**, and **58** lack potency, but have been patented for treating skin conditions.[55] MAIT cell activation has also been associated with COVID-19 disease severity.[56] Activation seems unlikely to be *via* MR1, although SARS-CoV-2-infected macrophages induce MAIT cell cytotoxicity in an MR1-dependent manner.[57] One strategy toward inhibitors is to first develop potent stable 5-OP-RU analogues. Not only might these be potentially useful vaccine adjuvants[43] and cancer immunotherapeutics,[45] but they are potentially also tight-binding ligands that offer clues to more drug-like scaffolds, tagging[58] of 5-OP-RU for monitoring cellular uptake, and structure−activity relationships that lead to stable and effective modulators of MR1 and MAIT TCR proteins.

Lastly, a better understanding of MR1-binding requirements might inform the discovery of other naturally occurring antigens of MAIT cells or other MR1-restricted T cells.[59,60] For example, MR1-mediated activation of MC7.G.5 T cells are able to kill most cancer cell lines without affecting healthy cells,[61] indicating promise for cancer immunotherapy[62] if specific antigens can be identified. In conclusion, MAIT cells offer new opportunities for chemical synthesis to contribute to the understanding of T cell immunity and the development of new immunomodulating medicines.

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Biographies

Jeffrey Mak completed his Ph.D. in the total synthesis of caged diterpenoid natural products with Prof. Craig Williams in 2012 before joining the Fairlie group at the Institute for Molecular Bioscience. His research interests are focused on the application of organic synthesis and chemical principles in the solution of diverse biological problems, including in immunity, drug development, and the synthesis of bioactive natural products. He has lectured undergraduate synthetic chemistry at UQ since 2017 and was promoted to Research Fellow in 2020.

Ligong Liu pursued undergraduate studies at Nankai University, China, postgraduate research at Beijing Medical University, China and Central Queensland University, Australia, and postdoctoral research at Central Queensland University and the Australian National University in Canberra (1997−2001). He spent 7 years in the Australian biotechnology industry (Alchemia, Progen Pharmaceuticals) specializing in carbohydrate and oligosaccharide-based therapeutics. From 2008, he was Senior Research Officer at the Institute for Molecular Bioscience, University of Queensland, Australia. Research interests are in chemical synthesis, medicinal chemistry, drug design and development, combinatorial chemistry, and modulators of enzymes/growth factors/GPCRs associated with inflammatory disorders and cancers.

David Fairlie is Senior Principal Research Fellow of the National Health and Medical Research Council. His undergraduate, postgraduate, and postdoctoral studies were at Adelaide, Australian National, New South Wales, and Stanford and Toronto Universities. At University of Queensland, he led the Chemistry Group in the Centre for Drug Design and Development and is Professor and Head of the Division of Chemistry and Structural Biology at the Institute for Molecular Bioscience. His research in medicinal/organic chemistry, drug discovery, protein/peptide mimics and biochemistry/pharmacology of GPCRs, enzymes, and protein−protein interactions has permitted mechanistic studies of chemical, immunological, and biological reactions and of disease development and drug action, particularly in inflammation, infection, immunity, and cancer.

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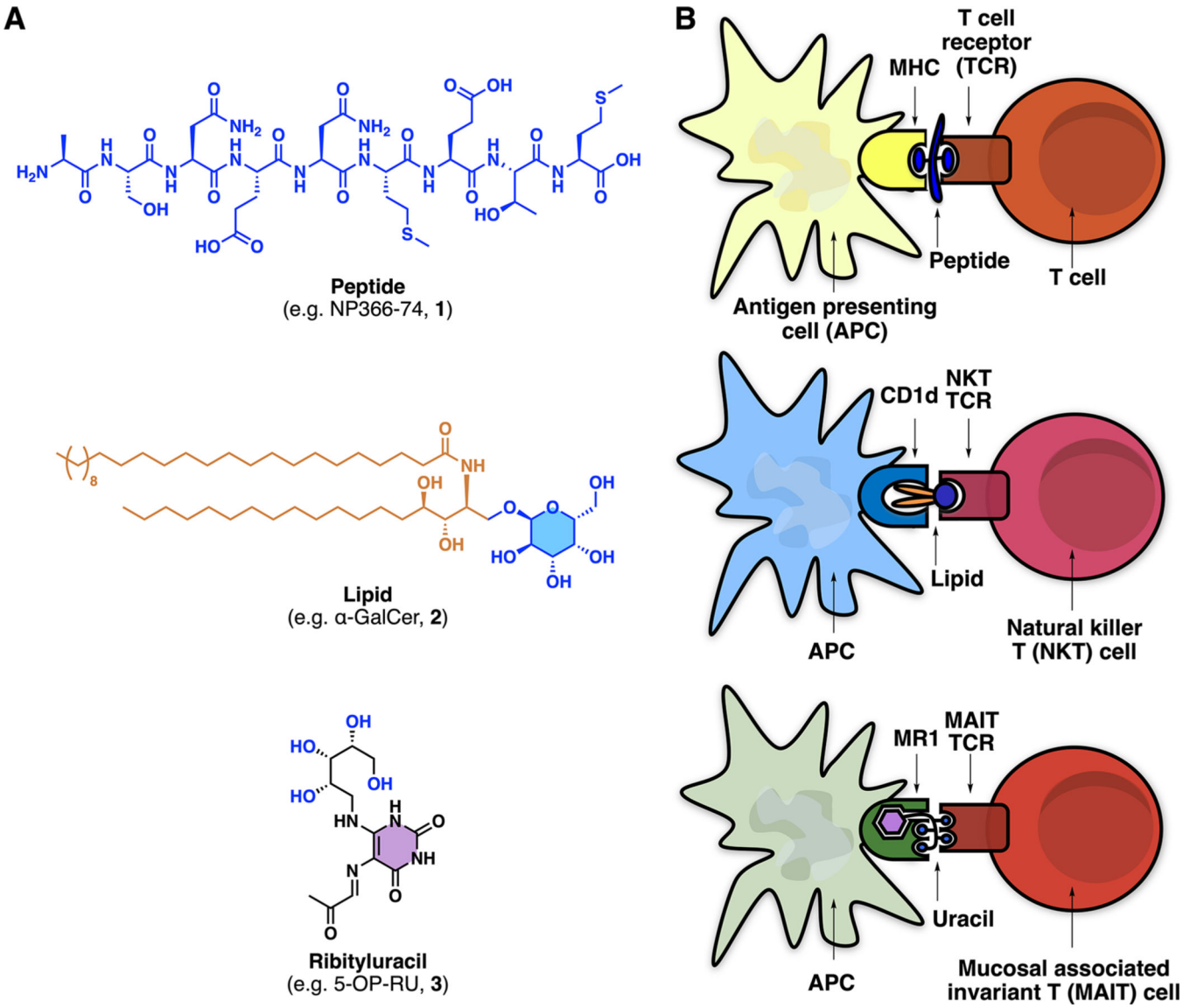


Figure 1.

Three compound classes that activate T lymphocytes. (A) Examples of peptide, lipid, and ribityluracil antigens that activate T cells. (B) A peptide, lipid, or ribityluracil antigen first binds to an antigen-presenting protein (MHC, CD1, or MR1, respectively) expressed on an antigen-presenting cell (APC) and then interacts with the T cell receptor (TCR) expressed on a T cell, NKT cell or MAIT cell, respectively. Adapted with permission from ref 9. Copyright 2012 Springer Nature.

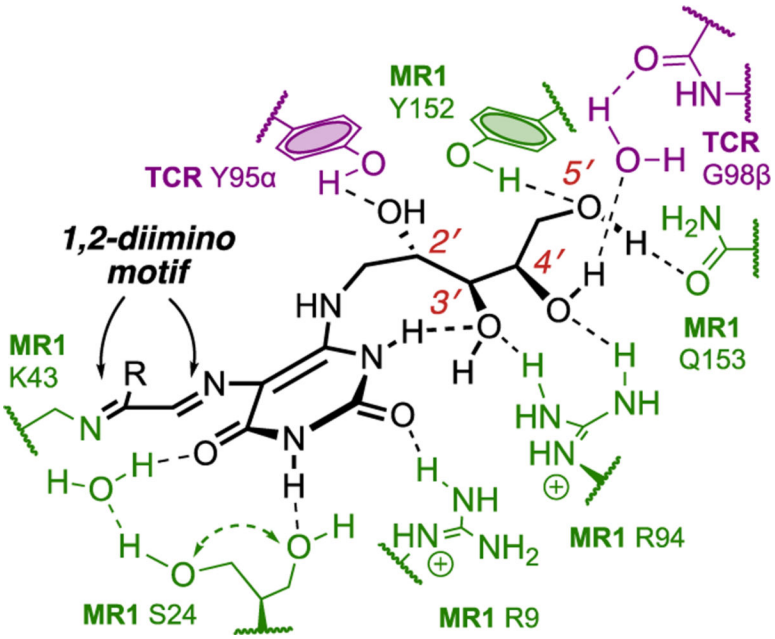


Figure 2.

Hydrogen bonds between 5-OP-RU (black) and protein residues of MAIT TCR (violet) and MR1 (green) as revealed by the crystal structure (PDB: 4NQC). Data from ref 1.

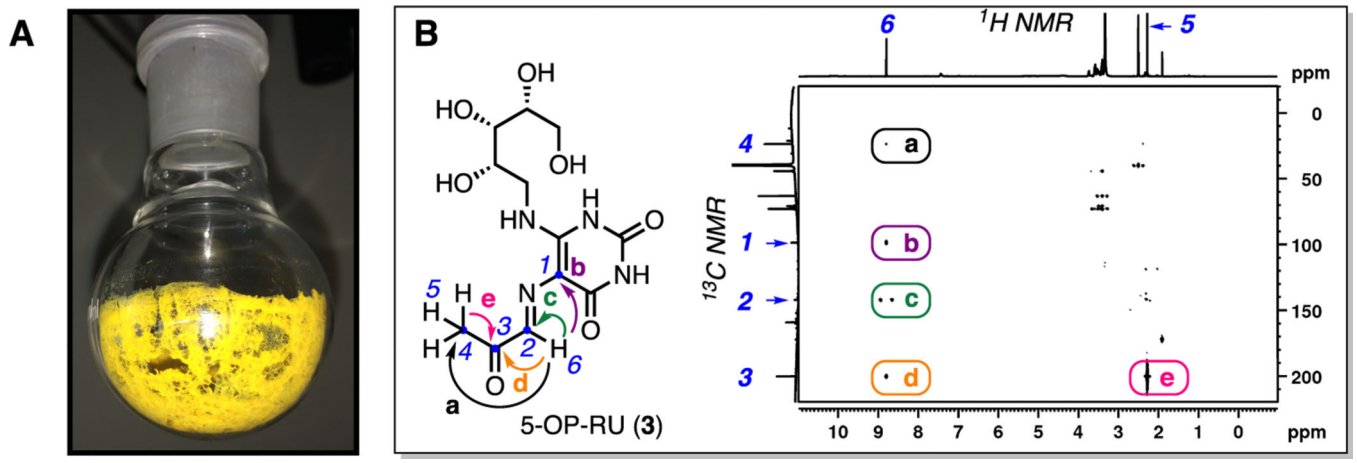


Figure 3.

(A) Kinetically stable 5-OP-RU after HPLC purification, and (B) characterization of 5-OP-RU (3) by ^{1}H−^{13}C HMBC NMR spectroscopy in DMSO-d_6. Key correlations (colored arrows and round boxes, labeled a−e) established the connectivity of atoms (numbered 1−6) in the iminocarbonyl group. Data from ref 2.

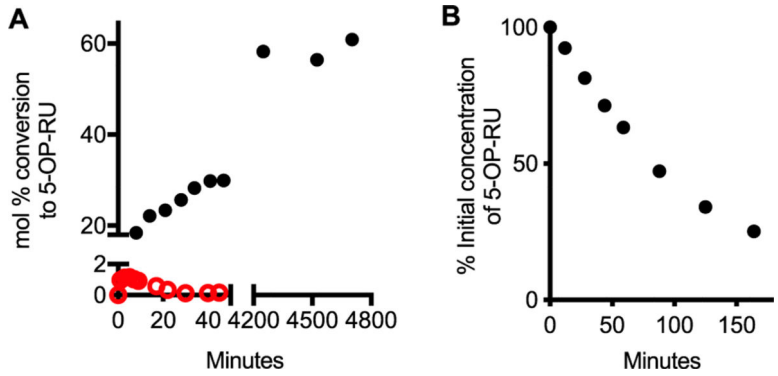


Figure 4.

(A) Formation of 5-OP-RU in DMSO at 22 °C (black) *vs* PBS at 37 °C (red). (B) Degradation of 5-OP-RU (pH 7.4 PBS, 37 °C). Data from ref 2.

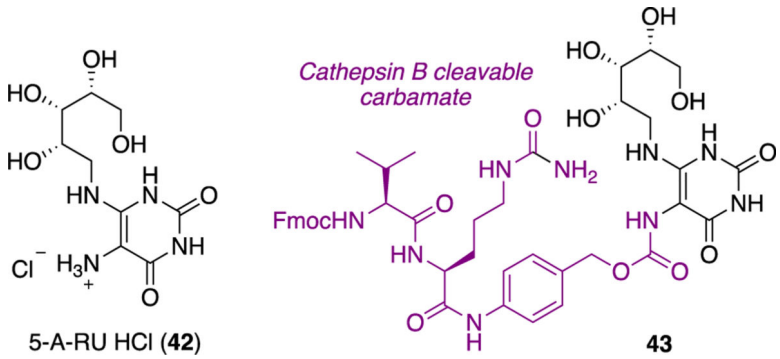


Figure 5.
Stabilizing 5-A-RU *via* its HCl salt (**42**) or carbamate prodrug (**43**).

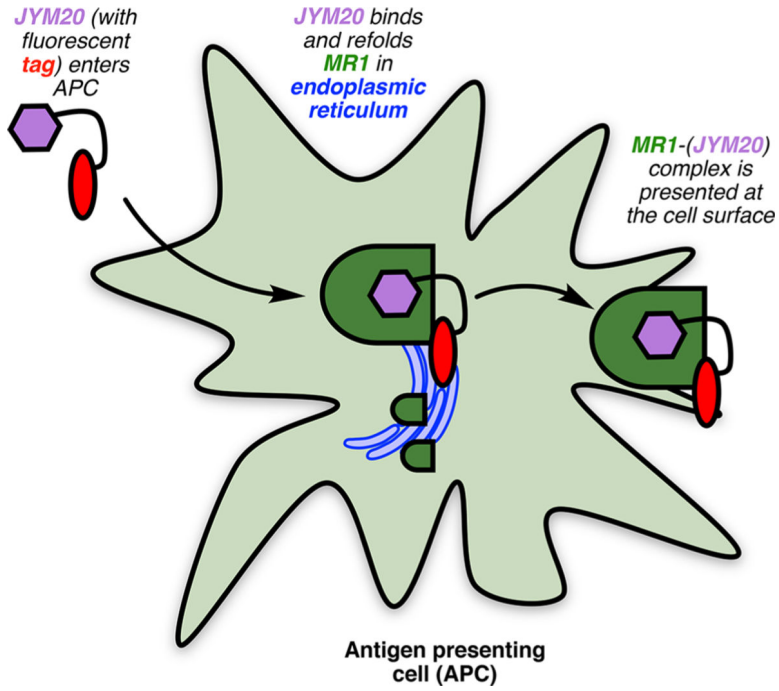


Figure 6.
Cellular pathway of MAIT cell ligand presentation.

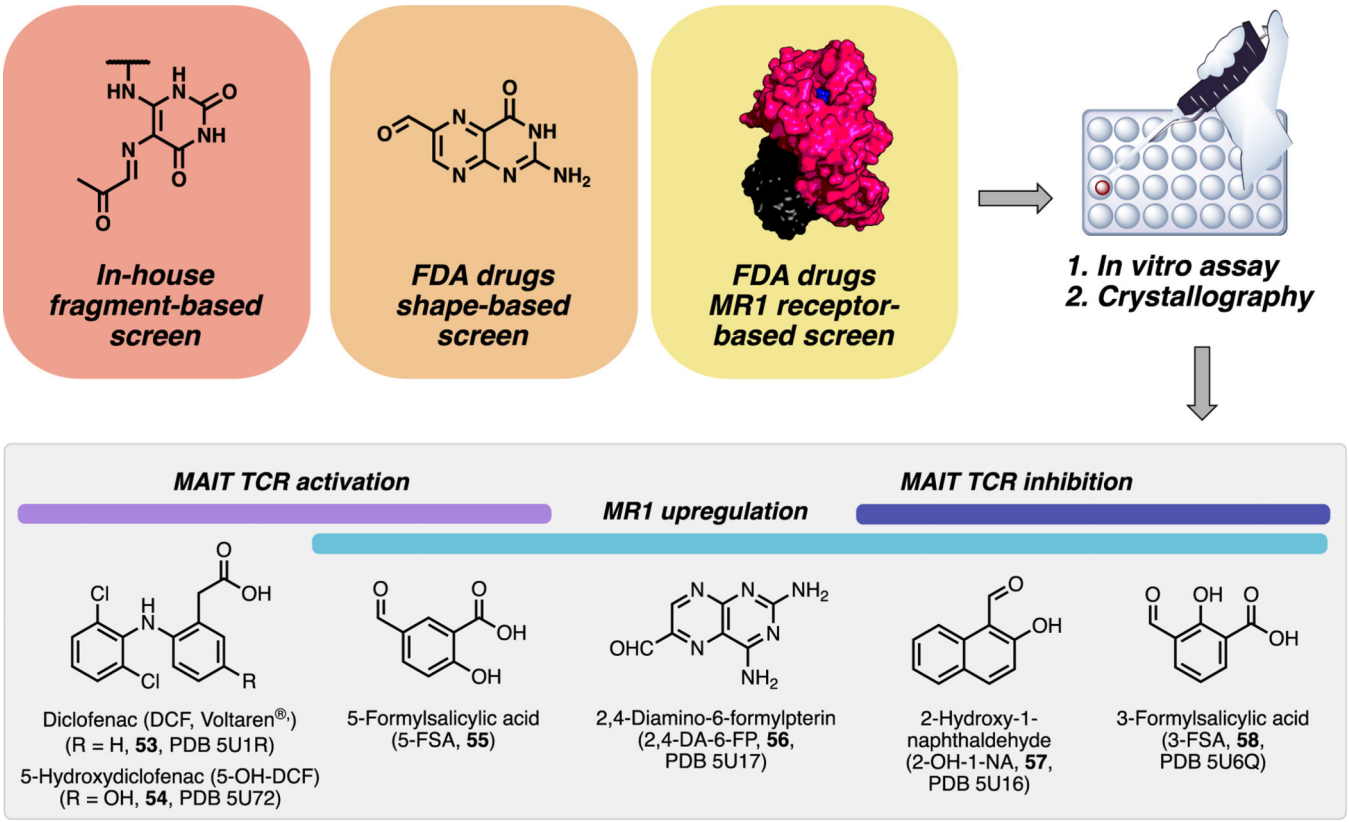
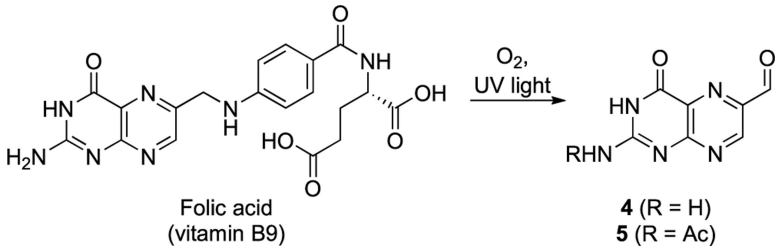
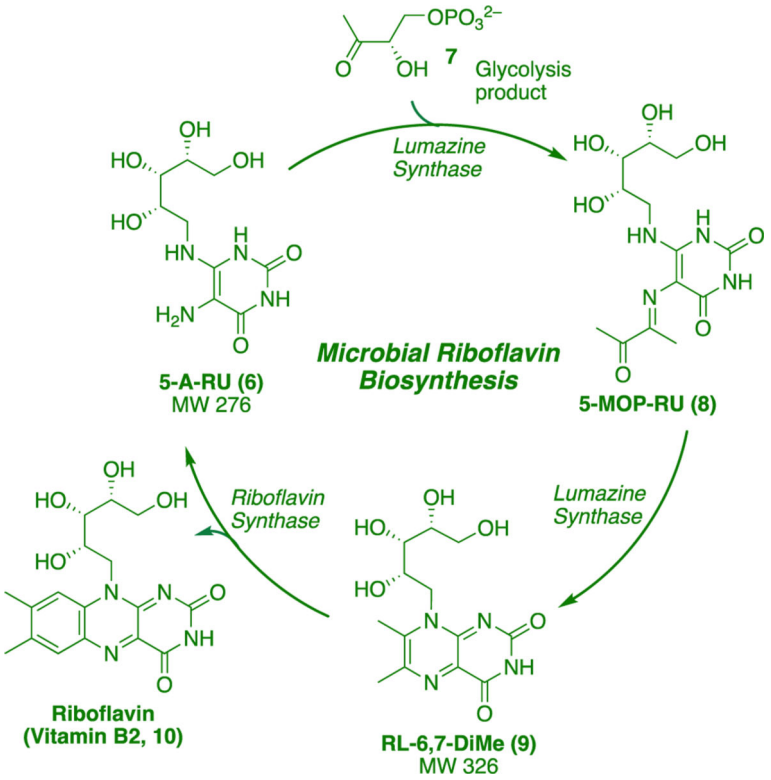


Figure 7.

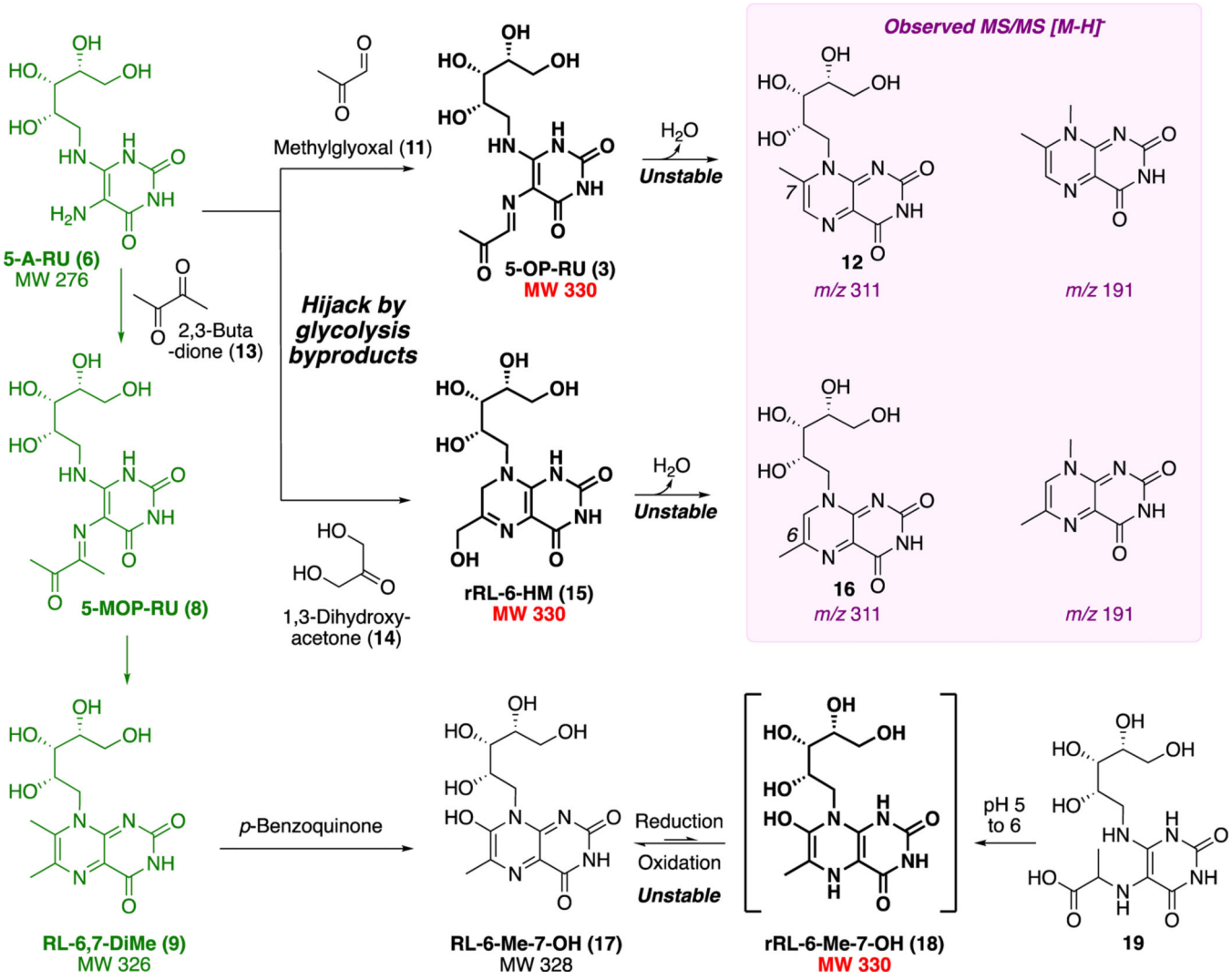
Drugs and drug-like compounds that bind MR1 and activate or inhibit MAIT TCR or upregulate MR1 expression in antigen-presenting cells, and PDB codes for ligand−protein crystal structures (PDB 5U1R, 5U72, 5U17, 5U16, and 5U6Q). Data from ref 4.



Scheme 1. MR1 Ligands 6-Formylpterin (4) and Acetyl-6-formylpterin (5) That Weakly Inhibit MAIT Cell Activation

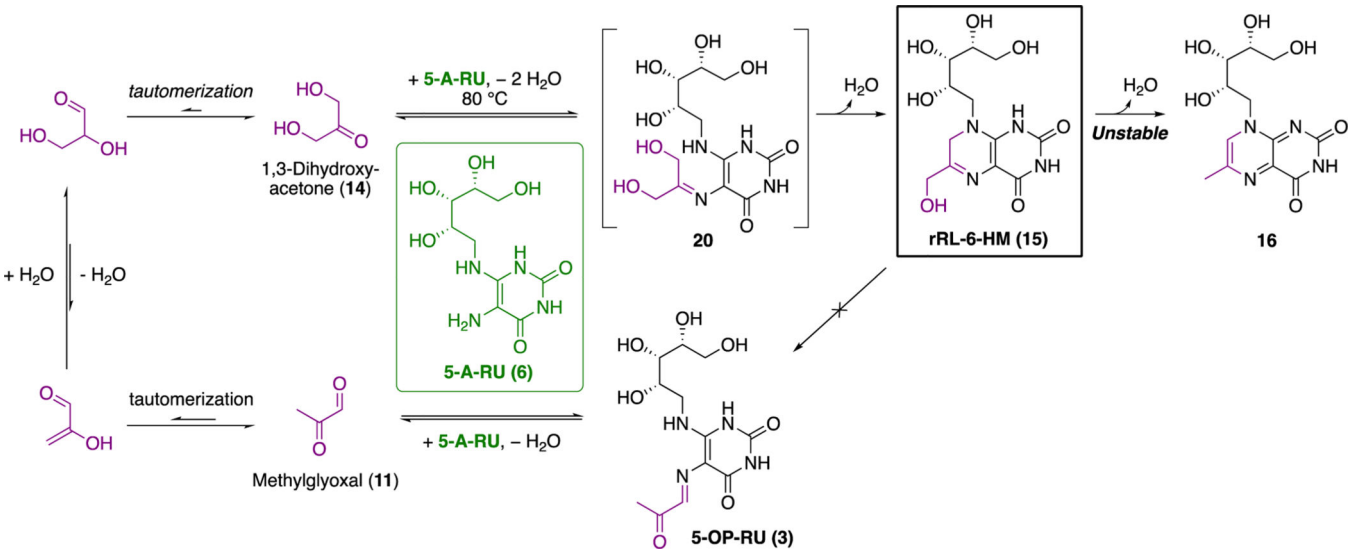


Scheme 2. Biosynthesis of Riboflavin (10)



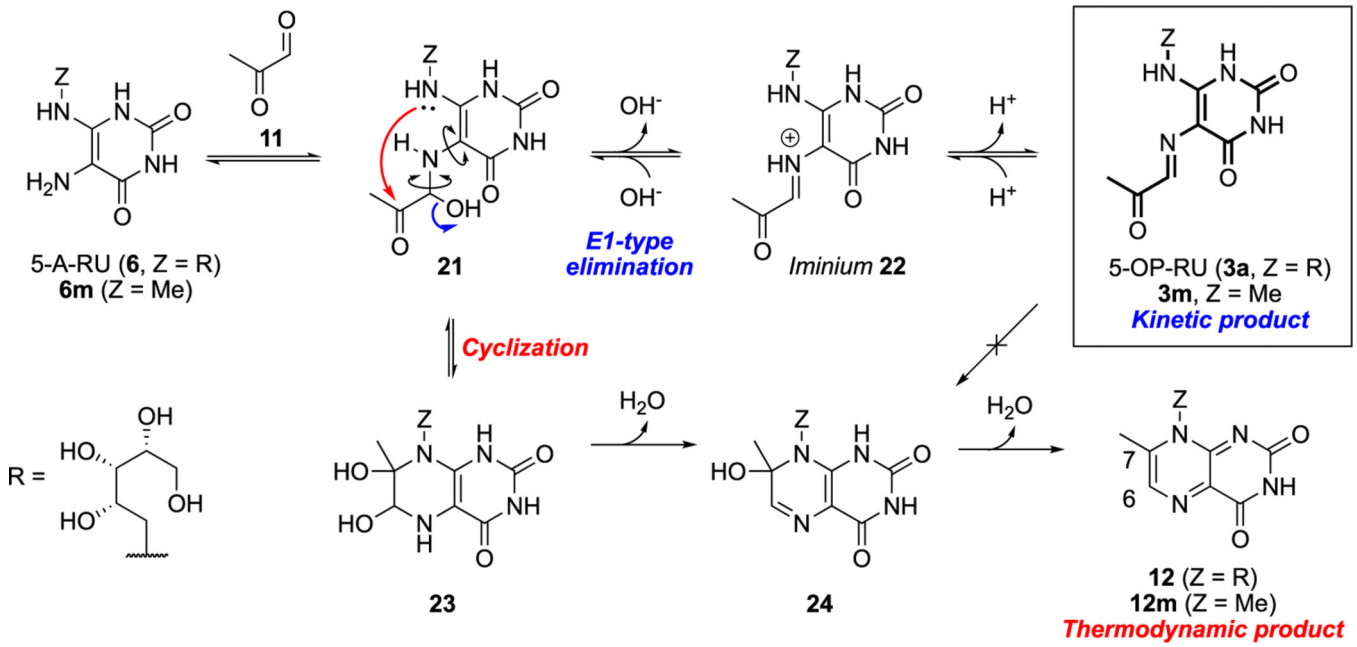
Scheme 3. Putative Antigens Derived from Riboflavin Biosynthetic Intermediates[a]

[a]Unstable **3**, **15**, and **18** have molecular formulas matching the stimulatory ligand in the bacterial supernatant; **3** and **15** have near identical MS/MS fragments (violet). Data from ref 7. and ref 1.



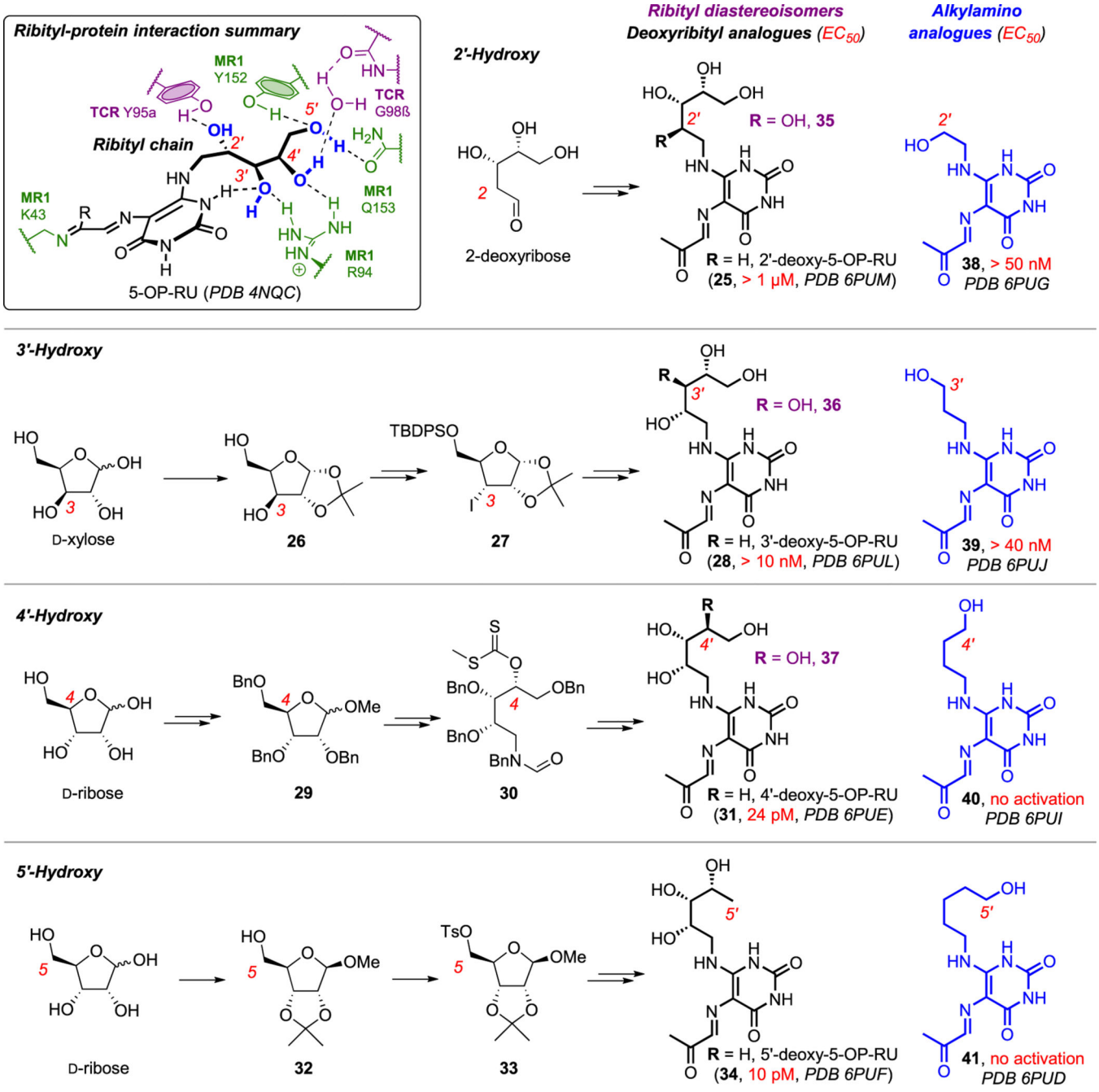
Scheme 4. Proposed Origin of 5-OP-RU (3) During the Synthesis of 15[a]

[a]Tautomerization[22] and *in situ* dehydration of 1,3-dihydroxyacetone (**14**) to trace methylglyoxal (**11**) at 80 °C could produce 5-OP-RU (**3**) during the synthesis of rRL-6-HM (**15**).



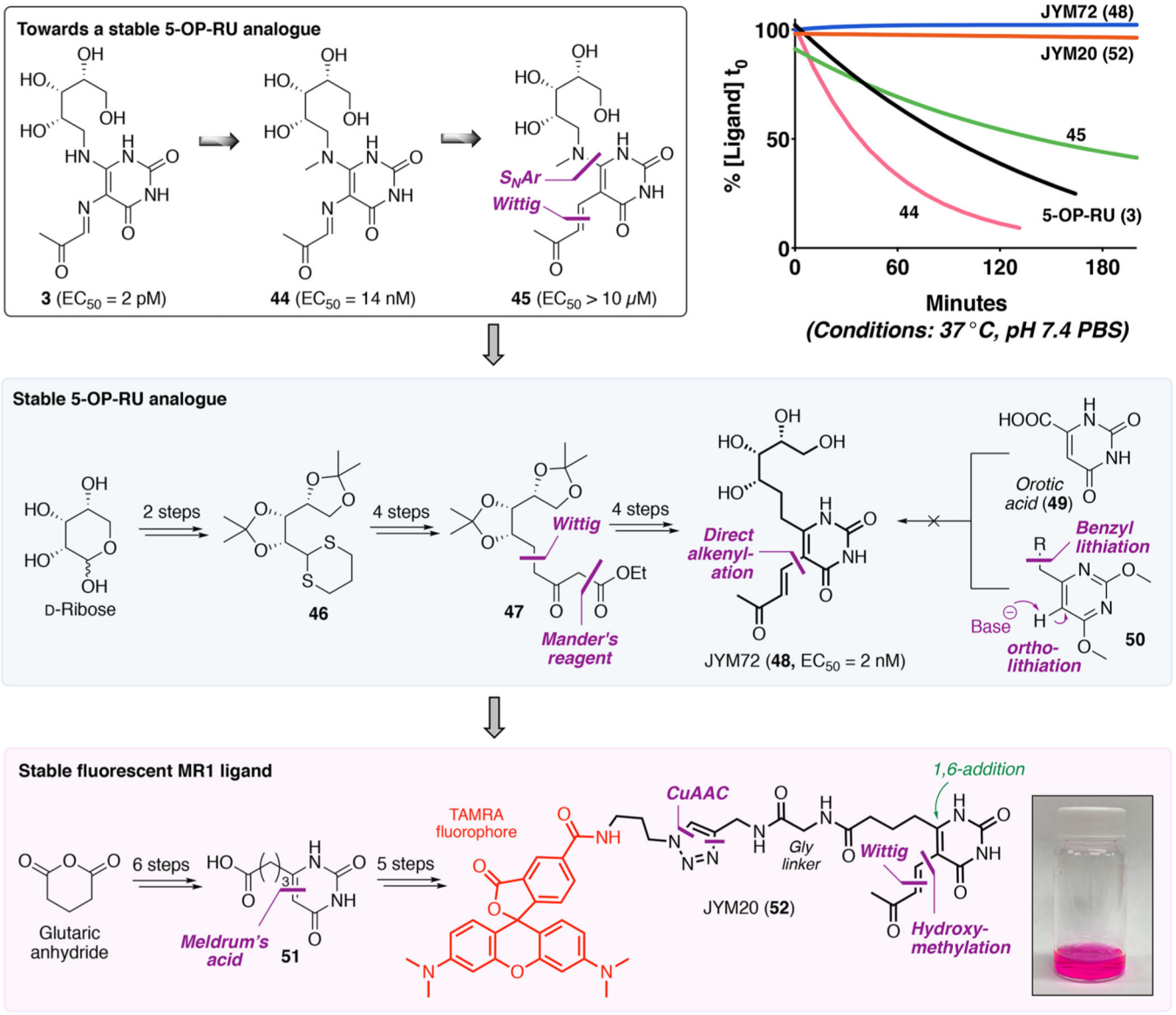
Scheme 5. Mechanism of Formation (Blue) and Degradation (Red) of 5-OP-RU (3a) and Model Compound 3m[a]

[a]Data from ref 2.



Scheme 6. Summary of Interactions between the Ribityl Chain of 5-OP-RU with MR1 and MAIT TCR (Inset) [1] and Synthetic Analogues of 5-OP-RU for Investigating the Roles of Ribityl Hydroxyl Groups in MAIT Cell Activation *via* Cellular Assays and Protein−Ligand Crystal Structures[a]

[a]PDB codes: 4NQC, 6PUM, 6PUG, 6PUL, 6PUJ, 6PUE, 6PUI, 6PUF, and 6PUD. Data from ref 3 and ref 46.



Scheme 7. Development of Stable 5-OP-RU Analogue JYM72 (48) and Stable Fluorescent MR1 Ligand JYM20 (52)[a]

[a]Data from ref 2 and ref 32.