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An overview on the identification of MAIT cell antigens

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MAIT cell antigens

Abstract

Mucosal Associated Invariant T (MAIT) cells are restricted by the monomorphic MHC class I-like molecule, MHC-related protein-1 (MR1). Until 2012, the origin of the MAIT cell antigens (Ags) was unknown, although it was established that MAIT cells could be activated by a broad range of bacteria and yeasts, possibly suggesting a conserved Ag. Using a combination of protein chemistry, mass spectrometry, cellular biology, structural biology and chemistry, we discovered MAIT cell ligands derived from folic acid (vitamin B9) and from an intermediate in the microbial biosynthesis of riboflavin (vitamin B2). While the folate derivative 6-formylpterin (6-FP) generally inhibited MAIT cell activation, two riboflavin pathway derivatives, 5-(2-oxopropylideneamino)-6-D-

ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU), were potent MAIT cell agonists. Other intermediates and derivatives of riboflavin synthesis displayed weak or no MAIT cell activation. Collectively, these studies revealed that in addition to peptide and lipid-based Ags, small molecule natural product metabolites are also ligands that can activate T cells expressing αβ T cell receptors, and here we recount this discovery.

Introduction

Mucosal associated invariant T (MAIT) cells first came to attention because of their semi-invariant TCR α-chain rearrangement observed by Porcelli *et al.* [1]. A rearrangement of the TRAV1-2 element juxtaposed to TRAJ33 was enriched among the $CD4^-CD8^-$ (double negative, DN) T cell population in healthy blood donors [1]. Less frequent usage of TRAJ12 and TRAJ20 segments in combination with TRAV1-2 are also present in MAIT cells [2-5]. Homologous versions were found in DN T cells in mice, cows [6] and sheep [7]. The Vα-Jα rearrangement did allow for two variable amino acids encoded at the V-J junction. In addition, the repertoire of TCR β-chains paired with the semi-invariant α-chain was skewed [1, 6], being dominated by TRBV6 and TRBV20 in humans and the murine orthologous segments of TRBV6 in mice [6]. Notably, this oligoclonality of MAIT TCR β-chains noted by Tilloy *et al.* [6] was later confirmed by Lepore *et al.* [4], who also observed a bias in the length of 11 to 14 amino acids.

The limited TCR repertoire of MAIT cells suggested that they might be restricted by a monomorphic, non-classical major histocompatibility complex (MHC) molecule [1]. Lantz's team proposed the MHC class I-like molecule, MHC-related protein-1 (MR1) [8, 9], as a candidate [6] and later formally demonstrated MR1 as the selective and restrictive element [10]. MR1 is monomorphic [9, 11] and represents the most highly conserved MHC-I like gene across diverse mammalian species, with 90% sequence identity between mouse and human as compared to 70% for classical MHC-I genes in the ligand binding site [8, 9, 12]. Perhaps, it is not surprising therefore, that MR1 selects a restricted TCR repertoire. Lantz's team furthermore showed that while MR1 is present in most mammals, including marsupials, it is absent from several clades of eutherians (e.g. cat, dog, panda, ferret, rabbit, pika, and armadillo) [12]. In species where it has been studied, MR1 co-exists with a TRAV1 α-chain, with a high inter-species conservation of the CDR1 and CDR2 regions of the MR1-restricted TCR α-chain [12] and associated cross-reactivity between certain species such as human, non-human primates,

bovine, rat and mouse [13-16], indicating the phylogenetic conservation of this T cell subset in mammals and suggesting an important physiological function.

Treiner *et al.* observed preferential location of MAIT cells at mucosal sites such as the gut lamina propria (and, to a lesser extent, the lung) of humans and mice. Hence, they named this T cell subset mucosal-associated invariant T (MAIT) cells [10]. Recent studies also showed MAIT cells to be present at non-mucosal sites (e.g. liver) [17, 18] and they appear to be widely distributed among tissues and somewhat more prevalent in the lung than the gut, at least in C57BL/6 mice [19, 20]. This tendency for mature MAIT cells to take up widespread tissue residency, not limited to mucosal localisation, as well as circulating in the blood where they can represent a high percentage of T cells in humans, suggests the moniker of MAIT cells adds to the famously confusing jargon of immunology.

Le Bourhis *et al.* and Gold *et al.* later demonstrated that a wide variety of microbes (bacteria, yeasts) (Table 1), but not viruses, cause MR1-restricted MAIT cell responses. The bacterial response was dependent on the interaction between the MAIT TCR and an unknown antigen (Ag) presented by MR1, presumably either derived from bacteria or a bacteria-induced endogenous Ag [2, 21]. Following these important studies, Lantz's team speculated that 'this ligand could be of multiple compositions, but the MR1-invariant TCR interaction would be non-discriminative, or it could be an extremely conserved compound among microbes' [21]; the latter mirroring the finding of a single universal Ag for type I NKT cells [22].

Based on sequence comparisons by Miley *et al.* and a structure-based computational analysis by Hansen *et al.*, it was suggested that MR1 adopted an 'MHC-I fold' [23, 24]. Indeed, it was known that MR1 associates with β2-microglobulin (β2m) [24, 25], further implying MR1 possessed an MHC-I-like structure. Whilst lipid-based Ags had been proposed to bind to MR1 [26, 27], the residues lining the putative ligand binding groove were noted to be markedly different from either MHC-I or CD1d [23, 24] suggesting that MR1 did not bind peptide or glycolipid Ags. This observation led to the idea that MR1 'may bind a unique ligand with an anchoring scheme unlike that of other MHC class Ia or class Ib proteins' [23].

The nature of the Ag(s) that activated MAIT cells was a major stumbling block in the field of MAIT cell biology. We considered that an understanding of these Ag(s) would help unlock the biology of MAIT cells. In 2009, more than 15 years after the discovery of MAIT cells, our laboratories

embarked on the challenge of identifying MAIT cell-stimulating MR1-bound Ag(s). This question was addressed by a multidisciplinary and highly collaborative approach that utilised a combination of methodologies, including protein chemistry, mass spectrometry, cellular biology, structural biology and chemistry. This review recounts how a team of immunologists, structural biologists and chemists discovered vitamin B-related compounds that modulate MAIT cell function.

Main body

Establishment of experimental models providing key insights into the nature of MR1 bound antigens

As a first step in identifying MR1 ligands that activate MAIT cells, it was key to establish a reliable experimental model to assess MR1-dependent MAIT cell activation. For this purpose, genes encoding MAIT TCR α- and β-chains were introduced into a Jurkat T cell line (Jurkat.MAIT). These expressed the MAIT TCR very well and stained with a TRAV1-2-specific monoclonal antibody (mAb) [28].

Jurkat.MAIT cells upregulated CD69 when co-incubated with bacterially infected C1R cells expressing endogenous levels of MR1. Jurkat.MAIT cell activation was augmented when C1R cells were supertransfected with the MR1 gene and overexpressed MR1 at the cell surface. Jurkat.MAIT cell activity could be blocked by the MR1-specific mAb 26.5 [29], verifying MR1 dependency of the observed activity. As an ongoing source of Ag(s), the bacterium *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) was investigated. *S.* Typhimurium infected C1R cells led to activation of Jurkat.MAIT cells in the co-culture assay. An advance was the discovery that culture supernatant of *S.* Typhimurium passed through a 0.2 μm filter also activated the Jurkat.MAIT cell line in an MR1-dependent manner. The fact that the infection of an Ag-presenting cell (APC) was not required for the presentation of MR1 bound Ag(s) was important as it meant that the Ag(s) of interest were likely to be secreted or shed *in vitro* and hence they were probably soluble. This key observation suggested that biochemically homogeneous and potentially soluble Ag(s) could be recovered from bacterial supernatant rather than working with highly complex bacterial lysates where the solubility of the Ag(s) was unknown.

Given the apparent solubility of the MR1 ligands in bacterial supernatant, we opted to identify their chemical nature *via* biochemical/mass spectrometry approaches. MHC and MHC-I like proteins are generally stable only in the presence of a given Ag, and thus we reasoned that the ability to sufficiently fold MR1 would only be possible in the presence of an MR1 ligand. Using Garboczi's original protocol of MHC-I folding [30], we found that filtered supernatant from *S.* Typhimurium sponsored *in vitro* folding of denatured, soluble MR1, expressed in *Escherichia coli*, in the presence of β2m (Figure 1). Intriguingly, we noticed that for some samples of MR1, folded in the presence of *S.* Typhimurium supernatant, the content of the tube was slightly yellow.

An important control at this point was to test if RPMI medium, used to culture *S.* Typhimurium prior to harvesting the supernatant, also supported folding of MR1. Surprisingly, small amounts of MR1 also folded in the presence of RPMI medium. This finding suggested that RPMI medium also furnished a ligand that could bind to MR1. The native conformation of MR1 folded in the presence of *S.* Typhimurium culture supernatant or RPMI was confirmed by its ability to bind to the anti-MR1 mAb 26.5 in an enzyme-linked immunosorbent assay (ELISA). The size exclusion chromatography retention time was almost overlapping with classical MHC molecules bound to peptide. Moreover, the stoichiometry with associated β2m was 1:1 based on SDS-PAGE, exactly as observed for classical MHC molecules and β2m [31]. Based on these insights, we considered that mass spectrometry of folded MR1 might be a suitable approach to identify the nature of the ligands captured by MR1 for presentation to MAIT cells.

Discovery of MR1 ligands that act as competitive inhibitors of MAIT cell activation

Given that RPMI medium contains defined components and supported folding of small amounts of MR1 in the absence of bacterial culture, we initially turned to this source of material to identify MR1-captured ligands. Among the components in RPMI are vitamin B family members, which are yellow in colour. Thus, MR1 was folded in the presence of a solution of vitamin B tablets obtained from a local pharmacy. Indeed, we recovered native MR1 from this folding experiment. When we then tested individual vitamin components in MR1 folding experiments, we observed that folic acid (vitamin B9) yielded significant amounts of native MR1. MR1 folded in the presence of folic acid or RPMI medium was then analysed by mass spectrometry, in collaboration with the laboratories of Purcell, McConville and O'Hair. In negative ion mode, this analysis revealed two mass-to-charge

(*m/z*) species of 190.03 and 147.03 that were absent in analyses of control blank and classical peptide-MHC-I samples. Puzzlingly, the *m/z* 440.13 expected for folic acid ($C_{19}H_{19}N_7O_6$) by mass spectrometry, was not detected in material captured by MR1 even in the presence of folic acid. A search of the Scripps Metlin data bank revealed that the two identified species matched fragments of biopterin, which shares a pterin moiety with folic acid. It was known that folic acid photodegrades in ultraviolet light to 6-formylpterin (6-FP, $C_7H_5N_5O_2$, *m/z* 190.04) and *p*-aminobenzoyl-L-glutamic acid ($C_{12}H_{14}N_2O_5$, *m/z* 265.08) [32] (Figure 2a). Subsequent collision-induced dissociation (CID) fragmentation of 6-FP revealed that the *m/z* 147.03 species was a product ion of the parent *m/z* 190.03 species. MR1 was previously observed to be expressed at low levels on the surface of cells under basal conditions [33]. Notably, 6-FP upregulated MR1 surface expression, reminiscent of the rescue of surface expression of MHC-I in cells deficient in peptide transporter (*tap-2*) [34]. This observation suggested that 6-FP was a *bona fide* MR1 ligand. We learnt recently that degradation of folic acid to 6-FP also occurs *in vivo*, in skin exposed to ultraviolet radiation [35].

That 6-FP was bound to MR1 was consistent with the ability to fold MR1 in the presence of pure 6-FP purchased from Schircks Laboratories. The purified product was indistinguishable from MR1 folded with folic acid, based on chromatography profiles (e.g. retention time), SDS-PAGE of purified MR1, and identical *m/z* 190.03 species. What was unknown, however, was the molecular mechanism by which MR1 captured 6-FP. Was 6-FP merely bound to the surface of MR1 in a hapten-like manner? Was there a defined Ag-binding pocket? Were there other (unidentified) ligands captured by MR1 in this process that had not been detected by mass spectrometry? The crystal structure of the MR1-6-FP complex began to resolve some of these central questions. It revealed that MR1 indeed adopted an MHC-I-fold, within which 6-FP was sequestered deep within the base of the MR1 Ag-binding cleft. Here, within the A'-pocket, 6-FP was surrounded by a large number of aromatic residues. The structure of the MR1 Ag-binding cleft was distinct from the Ag-binding clefts of MHC and CD1, which capture peptides and lipids respectively, and demonstrated the versatility of the MHC fold to capture three classes of Ags. Indeed, the binding pocket of MR1 appeared to be ideally suited to capture small aromatic molecules. An additional surprise, revealed by the crystal structure, was that 6-FP was covalently bonded to MR1 through a Schiff base linkage (chemically known as an imine, a carbon-nitrogen double bond), formed between the formyl group of 6-FP and the side chain ε-amine of lysine 43 of MR1. Such a covalent interaction between an Ag and Ag-presenting molecule had not been observed previously, and has subsequently been shown to act as a 'molecular switch' that enables MR1 to egress from the endoplasmic reticulum [36].

Whilst 6-FP was presented by MR1, it did not activate MAIT cells, and neither did other ligands from the folate pathway. This suggested that MAIT cells must be activated by a different ligand. It was also observed that MAIT cell activation was inhibited when both non-activating 6-FP and activating *S.* Typhimurium supernatant or synthetic ligands were added together to Jurkat.MAIT and C1R.MR1 cells [37, 38]. The inhibitory effect was specific to 6-FP and the MAIT-MR1 interaction and not observed with other pteridine analogues of folate, such as 6,7-dimethylpterin, 6-hydroxymethylpterin, or 6-hydroxymethyl-7,8-dihydropterin.

In later experiments, we identified an analogue of 6-FP, *N*-acetyl-6-formylpterin (Ac-6-FP) (Figure 2b), which also engendered efficient folding of MR1. Ac-6-FP was more potent than 6-FP in inhibiting activation of Jurkat.MAIT cells by *S.* Typhimurium supernatant in the presence of C1R.MR1 cells (unpublished). Subsequently, we showed that repeated intranasal delivery of Ac-6-FP inhibited MAIT cell activation by stimulatory Ags in the lungs of C57BL/6 mice [39]. Thus, we had identified 6-FP and Ac-6-FP as small molecule antagonists, capable of blocking activation of MAIT cells by ligands from bacteria via competitive inhibition. However, the activating ligand in *Salmonella* supernatant remained elusive.

Discovery of MR1 bound antigens that activate MAIT cells

Identification of the riboflavin biosynthesis pathway as the source of MAIT cell antigens

The discovery that a vitamin B9 degradation product, 6-FP, was captured from RPMI and acted as a non-stimulatory MAIT cell Ag, led us to hypothesise that the active MR1-bound ligand is competed from MR1-binding by the abundance of 6-FP in culture supernatants. Thus, we reasoned that the capture and identification of activating Ag(s) by MR1 might be more efficient if culture conditions minimized the levels of folate in the medium. This was achieved by growing *S.* Typhimurium in a minimal medium (M9), that lacked vitamin B9. Indeed, supernatant of *S.* Typhimurium grown in M9 media, but not the control M9 media alone, activated Jurkat.MAIT cells in the presence of C1R.MR1 cells and engendered folding of MR1 with β2m. High resolution mass spectrometry of the ligand, subsequently eluted from MR1, allowed determination of the *m/z* 329.11 in negative ion mode. Upon examining isotopic mass distribution in the mass spectrometry experiments, the ligand could be unambiguously assigned to the formula $C_{12}H_{18}N_4O_7$. A database search of compounds with this

composition and *m/z* yielded no match, but some ribityllumazine compounds from the riboflavin (vitamin B2) biosynthetic pathway were close, such as 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH, $C_{12}H_{16}N_4O_7$, *m/z* 327.0946 in negative ion mode) and 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe, $C_{13}H_{18}N_4O_6$, *m/z* 325.1153 in negative ion mode) (Figure 3). It was noted from two key studies [2, 21] that only microbes that activated MAIT cells possessed a complete riboflavin biosynthetic pathway (Table 1) [31]. Thus, it became apparent that a riboflavin metabolite may be an activating MAIT cell ligand.

The gold standard in assigning a given Ag to T cell activation is the ability to recapitulate candidate Ag activity with pure, structurally well-defined compounds produced by chemical synthesis. Whilst RL-6-Me-7-OH and RL-6,7-diMe were unstable and challenging to synthesize, they were obtained in sufficient quantities for functional assays. Both compounds specifically activated Jurkat.MAIT cells in the presence of C1R.MR1 cells in an MR1-dependent manner, although the levels of activation were very modest, and much lower than the activity observed for *S.* Typhimurium supernatant [31].

Structural basis of MAIT TCR recognition of MR1-RL-6-Me-7-OH versus MR1-6-FP

The molecular basis for the lack of activation of MAIT cells by 6-FP in the face of activation by riboflavin-derived ligands was resolved in crystal structures of the MAIT TCR-MR1-Ag ternary complexes with 6-FP and RL-6-Me-7-OH. The high resolution to which these MAIT TCR ternary complexes were resolved allowed for unambiguous assignment of the small molecule Ags within these complexes. [37]. These ternary structures revealed that MR1 could capture RL-6-Me-7-OH and 6-FP within the MR1 cleft. Although 6-FP formed a covalent Schiff base adduct with MR1, whereas RL-6-Me-7-OH cannot and was instead non-covalently bound, the overall docking topologies of the TCRs onto MR1 bound to these ligands were very similar. The ribityl moiety of RL-6-Me-7-OH was directly contacted via a hydrogen bond with the highly conserved Tyr 95 of the MAIT TCR α-chain. In contrast, there were no direct TCR contacts with 6-FP, which lacks the ribityl moiety. This observation provided a mechanism for the activation of MAIT cells by RL-6-Me-7-OH, as compared to the inhibition by 6-FP. It also suggested a basis for the dominant selection of TRAJ33 by MAIT TCRs as this was one of only three TRAJ segments that contained a Tyr 95α. Together with the increased affinity of MAIT TCR for MR1 presenting the riboflavin metabolite over the folate ligand,

this provided the first molecular insights into MAIT cell agonism, as well as definitive insights into the nature of the invariant MAIT TCR usage [37].

rRL-6-CH_2OH as a candidate MAIT cell antigen

RL-6-Me-7-OH and RL-6,7-diMe did not match the formula $C_{12}H_{18}N_4O_7$, unambiguously assigned to the Ag derived from *S.* Typhimurium through isotopic mass distribution in mass spectra. Three new compounds could be attributed to this formula, but were not previously described in riboflavin biosynthesis (Figure 4). They were a dihydrogen-reduced form of the key vitamin B2 biosynthesis metabolite RL-6-Me-7-OH (abbreviated rRL-6-Me-7-OH); a dihydrogen-reduced form of 6-hydroxymethyl-8-D-ribityllumazine (called rRL-6-CH_2OH or rRL-6HM); or 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (abbreviated 5-OP-RU) that was expected to be far too unstable to survive in water. Attempts to preserve synthetic rRL-6-Me-7-OH failed due to its extremely facile oxidation to RL-6-Me-7-OH, but rRL-6-CH_2OH could be synthesized from the key intermediate 5-amino-6-D-ribitylaminouracil (5-A-RU) by condensation with 1,3-dihydroxyacetone. The rRL-6-CH_2OH synthesis product, matching the *m/z* species of 329.11 identified from *Salmonella* supernatant, potently activated MAIT cells in an MR1-dependent manner [30]. We aimed to determine the crystal structure of MR1 loaded with synthetic rRL-6-CH_2OH and complexed to a MAIT TCR, however the amounts of rRL-6-CH_2OH synthesis product were insufficient to create complexes with MR1. In order to improve the inefficient folding of MR1 with our synthetic ligands, we made use of an unexpected finding, namely that the lysine to alanine (K43A) mutant, MR1-K43A, could be folded with β2m in the absence of ligand (designated "empty" MR1-K43A). We were able to load rRL-6-CH_2OH synthesis product into "empty" MR1-K43A and determined the crystal structure in complex with a MAIT TCR [40]. To our astonishment, instead of the expected two-ring structure of rRL-6-CH_2OH, the electron density indicated that a one-ring structure was present. Importantly, fluorochrome-coupled tetramers of MR1-K43A loaded with rRL-6-CH_2OH synthesis product stained human MAIT cells permitting their characterisation [5], thus further cementing the notion that we were on the right track in unlocking the ligand biology of MAIT cells. However, we remained puzzled at how this ligand was derived from riboflavin biosynthesis.

A genetic approach showing that the riboflavin pathway was required for MAIT cell activation

Given the challenges of biochemically nailing the riboflavin synthesis pathway as furnishing the precursor of physiological activating MAIT cell ligands, we turned to a genetic approach to understand the origin of MAIT cell activating ligands from microbial riboflavin synthesis (see [41] and Figure 3). Most of the work defining this pathway was performed using *Bacillus* spp.. In these, and other Gram-positive bacteria, including Lactococcus spp., the *Rib* genes are grouped into a single, 4-gene operon (ribGBAH), which is under transcriptional regulation through a riboswitch, such that riboflavin or flavin mononucleotides (FMN) downregulate the further production of riboflavin [41, 42].

Two recent papers demonstrate MAIT cell activation by *Streptococcus pneumoniae* and show that the riboflavin biosynthesis pathway is highly conserved amongst pneumococci and also present in other Streptococcus spp. [43, 44], but lacking from 2400 *S. pyogenes* strains [44], consistent with a previously reported lack of MAIT cell activation [21]. In 571 pneumococci the genes ("*rib* DEAH", equivalent to "*rib* GBAH") were found clustered together in a highly conserved operon [44].

Other bacteria, such as the Gram-negative *E. coli* and *Salmonella* spp., have their riboflavin synthesis enzyme genes dispersed throughout the genome. Production of riboflavin is also regulated in these species, through a number of mechanisms [42]. Furthermore, although an essential pathway for many organisms, evolution has resulted in many gene variants (genetic solutions), including in some bacteria and plants where *ribA* and *ribB* are combined into a bifunctional enzyme, *ribAB*, and in yeasts and plants whereby the pathway differs in this reaction step [45-47]. A further complication is that the enzyme nomenclature also differs among bacteria. The details of riboflavin synthesis and its regulation are complex and still emerging, with the recent discovery of the 5-A-RU 5′-phosphate phosphatase in *Arabidopsis thaliana* [48], completing the pathway.

Adding riboflavin to bacterial cultures of *S.* Typhimurium inhibited the activation of Jurkat.MAIT cells by these culture supernatants [40]. Consistent with our findings, exogenous riboflavin has also recently been shown to decrease activation of MAIT cells by *Streptococcus pneumoniae* [43, 44]. In the *S.* Typhimurium genome, *ribD* and *ribH* are grouped together – thus initial experiments demonstrating the requirement for the riboflavin pathway in the production of MAIT cell stimulatory Ags used a mutant lacking both genes. *S.* Typhimurium SL1344 Δ*ribDH* [40], and later *S.* Typhimurium BRD509 Δ*ribDH* [19], to demonstrate markedly decreased MAIT cell activation, and this activity could be reconstituted by addition to the mutants of *ribDH* expressed from a plasmid.

To pinpoint the specific step in the pathway leading to production of MAIT cell-stimulating Ag(s), individual gene-deficient strains were needed. Thus, the *Lactococcus lactis* CB013 roseoflavin resistant mutant [49], which constitutively expresses riboflavin and lacks the feedback mechanisms that complicated earlier attempts, was used to construct a set of gene-deficient bacteria lacking individual *rib* genes (generated by our collaborators, Jennifer Mahoney and Van Sinderen). In these experiments [40], *ribA* and *ribG* mutant strains (*ribD* in *Salmonella*) clearly abrogated MAIT cell activation, whereas *ribB* and *ribH* did not. Additionally, alongside results from wildtype *S.* Typhimurium (+ve for 329.11 species) and *Enterococcus faecalis* (-ve), the ability of supernatant from these mutants to drive MR1 folding and identification of the *m/z* 329.11 species by mass spectrometry correlated with activity in Jurkat.MAIT cell activation assays [40]. Taken together, these experiments definitively pinpointed the key metabolite necessary to generate activating MAIT cell ligands as 5-A-RU. These studies have now been recapitulated for *E. coli* and *S. pneumoniae* by other groups, and also demonstrate the requirement for *ribD/G* in these bacteria [44, 50].

Multiple strands of evidence came together in an overlapping sequence of discoveries to identify the MAIT cell Ags formed from 5-A-RU. The collective evidence leveraged the contributions of all three laboratories collaborating on the project. These included insights from the old biochemical literature and associated data, X-ray crystallographic structures of MR1-Ag complexes bound to a MAIT TCR and fundamental chemistry considerations.

Clues from the biochemical and chemical literature

While 5-A-RU alone appeared to stimulate Jurkat.MAIT cells, its *m/z* in negative ion mode ($C_9H_{16}N_4O_6$, *m/z* 275.09) was about 54 units smaller than the *m/z* of the Ag derived from *S.* Typhimurium, 329.11. Also, we could readily fold MR1 in the presence of 5-A-RU, the folded product of which was intensely yellow, matching the faint yellow colour occasionally observed when MR1 was folded in the presence of *Salmonella* supernatant. However, mass spectrometry of the resulting purified MR1-Ag, gave an *m/z* 315.09 (negative ion mode), or 40 units larger than the *m/z* of 5-A-RU and 14 units smaller than the *m/z* of the Ag derived from *S.* Typhimurium, 329.11. Thus, we were left with a biochemical puzzle.

Further molecular insights came from reviewing the literature on 5-A-RU as part of the riboflavin synthesis. A key publication from Bacher's team predicted that *en route* to producing the ribityllumazine RL-6,7-diMe during riboflavin synthesis, 5-A-RU forms a Schiff base with 3,4-dihydroxy-2-butanone-4-phosphate (3,4-DH-2-B-4-P) to yield an unstable ring-opened pyrimidine

intermediate, 5-(1-methyl-2-oxopropylideneamino)-6-D-ribitylaminouracil (5-MOP-RU, $C_{13}H_{20}N_4O_7$, *m/z* 343.13) [51]. This resembled the one-ring ligand seen in the crystal structure containing MR1-K43A loaded with rRL-6-CH_2OH synthesis product[40]. Bacher's team also showed that the reaction of the riboflavin pathway catalysed by the lumazine synthase (RibH) could occur spontaneously, in the absence of any enzymatic catalysis. Hence, both 3,4-dihydroxy-2-butanone-4-phosphate and upon dephosphorylation, 2,3-butanedione, could form a Schiff base with 5-A-RU [41, 52]. This suggested that other small molecules might spontaneously undergo similar reactions during *in vitro* folding, one of which might generate the dominant *m/z* 329.11 product derived from *S.* Typhimurium culture supernatant and another the *m/z* 315.09 species observed following folding of MR1 in the presence of 5-A-RU. Indeed, methylglyoxal, lacking a methyl group compared to 2,3-butanedione, would be expected to form a Schiff base with 5-A-RU to yield 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU, $C_{12}H_{18}N_4O_7$), matching the *m/z* of 329.11 in negative ion mode, a compound theoretically generated *en route* to creating 7-methyl-8-D-ribityllumazine (RL-7-Me) (Figure 5). Similarly, we reasoned that it was glyoxal, lacking two methyl groups compared to 2,3-butanedione, that had formed a Schiff base with 5-A-RU to yield 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU, $C_{11}H_{16}N_4O_7$). By analogy with 5-OP-RU, 5-OE-RU was theoretically generated *en route* to creating 8-D-ribityllumazine (RL) (Figure 5).

Aiming to generate MR1 loaded with 5-OP-RU, we initially folded MR1 in the presence of equimolar amounts of 5-A-RU and methylglyoxal. Whilst the dominant species remained *m/z* 315.09, we now observed a minor *m/z* 329.11 species. Using a molar excess of methylglyoxal (an arbitrary molar excess of over 300-fold was chosen), we then solely identifed *m/z* 329.11. Importantly, MR1 tetramers with both species effectively stained MAIT cells [40], and unpublished. Moreover, when re-examining mass spectrometry of MR1 folded in the presence of *Salmonella* supernatant, we observed a minor *m/z* 315.09 next to the dominant *m/z* of 329.11. Also, folding of MR1 in the presence of *E. coli* (DH5α) supernatant gave a distinct, more abundant, *m/z* 315.09 in comparison to a *m/z* 329.11 species [40], consistent with the findings of others [50]. Thus, we reasoned that both the *m/z* 315.09 and 329.11 species were likely acting as potent, natural MAIT cell Ags. Notably, both glyoxal and methylglyoxal are formed from a number of metabolic pathways, including mammalian and bacterial glycolysis [53] and glyoxal is widely abundant as it is produced industrially as a precursor to many products [54]. The almost ubiquitous presence of glyoxal explained the presence of the *m/z* 315.09 species in refolds of MR1 with pure 5-A-RU alone, whereby folding yields could be increased by adding additional glyoxal.

The structural basis for ligand selectivity by MR1

To understand the basis for ligand selectivity by MR1, we determined high resolution crystal structures of the MAIT TCR in complex with MR1 folded in the presence of 5-A-RU and methylglyoxal or glyoxal[40]. In both structures, the electron density at the site of the Ag-binding pocket was unambiguously matching 5-OP-RU and 5-OE-RU, respectively. Analogous to 6-FP, the carbonyl groups derived from methylglyoxal and glyoxal when forming 5-OP-RU and 5-OE-RU, respectively, extended to Lysine 43 of MR1 to form a Schiff base. The Ags were further stabilized in MR1 through an extensive hydrogen-bonding network. As with RL-6-Me-7-OH, the ribityl chains of 5-OP-RU and 5-OE-RU were also directly contacted by the Tyr 95 of the MAIT TCR α-chain and there was an additional water-mediated hydrogen bond by the CDR3β loop of the TCR. The affinities of MR1-5-OP-RU or MR1-5-OE-RU complexes with MAIT TCRs, measured by surface plasmon resonance, were comparable to those with conventional TCRs recognizing MHC-peptide complexes (Kdeq ~ 0.5–10 μM) [40, 55].

When we determined the crystal structure of MAIT TCR complexed with the mutant MR1-K43A loaded with rRL-6-CH_2OH synthesis product, we found that the Ag bound to MR1 was instead 5-OP-RU[40], consistent with this being present in the rRL-6-CH_2OH synthesis product[56] and entirely responsible for the folding of MR1. In subsequent experiments (unpublished) we showed that the retention time of 5-OP-RU matched that of the Ag derived from *S.* Typhimurium, under the same chromatographic conditions. Given that Schiff base formation was not possible with MR1-K43A, it became clear that a Schiff base was not obligatory for ligation of 5-OP-RU. Other interactions with MR1-K43A were evidently sufficient to ligate the Ag and prevent its cyclisation to a ribityllumazine. However, the lack of a covalent Schiff base with MR1 impacted profoundly on the stability of the Ag-MR1-K43A complex, which was markedly reduced (17°C lower half-maximum melting temperature by thermostability) [38]. Unlike 5-OP-RU, 5-A-RU alone, however, could not be loaded into MR1-K43A (unpublished).

Neither 5-OP-RU nor 5-OE-RU were previously known to exist naturally, and they are the first examples of organic heterocycles as T cell Ags. However, they are also chemically very unstable in water where they cyclise to ribityllumazines in minutes at 37°C. A synthesis was therefore devised to produce 5-OP-RU and 5-OE-RU in dimethylsulfoxide, where they were indefinitely stable and could

be extensively characterized in solution by NMR spectroscopy [40]. Later this method was further optimised to provide a reliable source of pure MAIT cell Ags for studying their chemical reactivity [57] and MAIT cell activation *in vitro* and *in vivo* [19].

In summary, instead of capturing the thermodynamically more stable ribityllumazines, MR1 prefers to sequester thermodynamically unstable pyrimidines formed transiently through condensation of compounds arising from two distinct metabolic pathways, 5-A-RU from the bacterial biosynthesis of riboflavin and methylglyoxal/glyoxal from bacterial or mammalian glycolysis. This confirmed our hypothesis that the way MR1 Ags are created and captured is unique amongst T cell Ags [40].

Reflections on MR1 antigen diversity

Classical MHC-I molecules have evolved under strong positive, diversifying, selection [58] causing them to be highly polymorphic. Whilst not formally investigated, MR1 appears monomorphic [9, 11] and the high conservation of the MR1 Ag binding site across species indicates that it has evolved under strong negative, purifying, selection [23]. Early on, this, together with the semi-invariant MAIT TCR usage composed of a nearly invariant TCR α-chain and a limited array of TCR β-chains, led to the hypothesis that MAIT cells recognise a single Ag or a limited number of Ags [1, 6, 10]. Following the finding that MAIT cells recognise a broad array of both bacteria and yeast species, the hypothesis was further refined: 'MAIT cell Ags were either extremely conserved between microbial species or MAIT TCRs would be non-discriminative' [21], contrasting with the exquisite fine-specificity of conventional TCRs recognising structurally related peptide Ags [59].

Indeed, we discovered several activating MAIT cell Ags and they fit both hypotheses: There is substantial evidence that MR1 focuses on a metabolic pathway that is conserved between many microbial species, namely the riboflavin pathway ribityllumazine Ags [30, 36] and the riboflavin pathway-derived pyrimidine [39] Ags. Given the various versions of the riboflavin pathway, and their modifications, variants of 5-A-RU and ribityllumazines might exist. Also, physiological by-products other than methylglyoxal and glyoxal might be capable of forming a Schiff base with both MR1 and 5-A-RU, thus forming Ags around a generic scaffold that could differ based on the microbe as previously described for 5-OP-RU versus 5-OE-RU [40]. Nonetheless, despite their structural

differences, pyrimidines and ribityllumazines are both recognised by the same MAIT TCRs in a pattern-recognition like manner [38].

Whilst MR1 likely presents a limited number of Ags, this is matched with a large precursor frequency of responding MAIT cells, that exceeds the number of T cells available to recognise any peptide-MHC Ag [22], allowing for a rapid powerful response. Notably, there is appreciable variation in the MAIT TCR β-chain repertoire, especially in the CDR3β loop, which also displays remarkable structural plasticity when fine-tuning MR1 recognition in an Ag-dependent manner [38]. Furthermore, the TCR α-chain can feature diversity beyond the canonical TRAV1-2-TRAJ33 rearrangement, where frequently also TRAJ12 and 20 are used [4, 5, 56] but also other TRAJ segments including some that lack the Tyr 95 residue [60], that was shown to be pivotal in MAIT cell activation, hydrogen bonding with the ribityl moiety of activating Ags [4, 5, 28, 37, 38, 40, 56, 61]. Furthermore diverse TRAV1-2$^-$ TCRs with various reactivity patterns have been described: (i) some reacted like classical MAIT TCRs with MR1-5-OP-RU tetramers only [56]; (ii) others reacted with both tetramers with folic acid-derived ligands and 5-OP-RU [56], or in one case responded to both riboflavin pathway-derived Ags and a riboflavin-deficient microbe (*S. pyogenes*) [62] and as such overlapped with the classical MAIT cell reactivity; (iii) a third type reacted with tetramers with folic acid-derived ligands only [56] or responded to self-Ags only [63] and as such was not overlapping with the classical MR1-5-OP-RU-reactive T cell subset. Gold *et al.* [60] also observed distinct MAIT TCR repertoire mobilization in response to diverse pathogens that could reflect the existence of discrete pathogen-associated Ags presented by MR1. TRAV1-2$^-$ MR1-restricted T cells, as investigated by Gherardin *et al.*, also lacked the MAIT cell lineage-specific transcription factors PLZF and RORγt and were heterogeneous for T-bet, suggesting developmental and functional differences compared to the TRAV1-2$^+$ MAIT cells [56].

Thus, additional natural Ags presented by MR1 might exist and be recognised by TCRs in a subset-specific manner. This is also further supported by our observation that MR1 has the versatility and plasticity to accommodate structurally diverse drugs, drug metabolites and drug analogues in the A'-pocket [39]. In addition, Ags might bind in the F'-pocket of MR1, mirroring our observation of crystallisation buffer molecules in the F'-pocket of TCR-MR1-Ag crystal structures [55]. It will be important to identify the chemical structures, metabolic origin and physiological relevance of any newly identified natural Ags, requiring careful functional, biochemical and structural characterisation.

Where to next

The discovery of 5-OP-RU and 5-OE-RU as very potent Ags and development of MR1-tetramers [5, 40], now available from the National Institutes of Health, USA, have opened up the field to start elucidating the roles of MAIT cells in anti-microbial immunity [19, 64-66], in sensing of metabolic changes such as in cancer, stress and cell damage [63, 67], as well as in immunopathology [68-70]. The tetramers have also allowed further characterisation of the phenotypic identity of MAIT cells and their development, with individual- and age-specific differences now being investigated [71-73]. There is still much to be understood about the identity and functions of MAIT cells, including their roles in immunity and disease, which in our experience seem much harder to ascertain.

Figure legends

Figure 1

Schematic of 'Ag-fishing' or bioprospecting, whereby denatured MR1 and β_2m serve as 'bait' to 'fish' potential Ags, such as 5-OP-RU and 6-FP, from complex metabolic mixtures in bacterial supernatants.

Figure 2

MR1 ligands that act as competitive inhibitors of MAIT cell activation. a) Photodegradation of folic acid in ultraviolet light to 6-formylpterin and *p*-aminobenzoyl-L-glutamic acid. b) Acetyl-6-formylpterin, a synthetic analogue of 6-formylpterin. Chemical structures, formulas and *m/z* in negative ion mode are indicated.

Figure 3

Riboflavin biosynthesis pathway. *ribH*, lumazine synthase; X, hypothetical phosphatase. Chemical structures are indicated.

Figure 4

Structures of three regioisomers (*m/z* 329.11, negative ion mode) that have the same molecular formula $C_{12}H_{18}N_4O_7$: rRL-6-Me-7-OH, rRL-6-CH_2OH, and 5-OP-RU and that can be derived non-enzymatically from the key vitamin B2 biosynthesis intermediate 5-A-RU.

Figure 5

Formation of pyrimidines and ribityllumazines from condensation of small metabolites with 5-A-RU. Chemical structures are indicated.

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Conflict of interest

Lars Kjer-Nielsen, Alexandra J. Corbett, Zhenjun Chen, Ligong Liu, Jeffrey Y. W. Mak, Jamie Rossjohn, David P. Fairlie, James McCluskey, and Sidonia B. G. Eckle are inventors on patents describing MR1 antigens and MR1 tetramers. The other authors have no financial conflicts of interest.

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Table 1a

Microbes that activate MAIT cells	Expansion or activation in vitro (and MR1 dependency)	Expansion or activation in vivo	Riboflavin pathway based on KEGG pathway database (and knock-outs)	References
Bacteroides thetaiotaomicron	ND	X	X	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701.
Bifidobacterium animalis	ND	X	No*	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701.
Candida albicans	X(X)	X	X	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701. Dias J, et al. Proc Natl Acad Sci U S A. 2017;**114**:E5434. Gold MC, et al. J Exp Med. 2014;**211**:1601. Meermeier EW, et al. Nat Commun. 2016;**7**:12506.
Candida glabrata	X(X)	ND	X	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701.
Corynebacterium striatum	X(X)	ND	X	Liuzzi AR, et al. J Immunol. 2016;**197**:2195.
Escherichia coli	X (X)	X†	X(X)	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701. Dias J, et al. Proc Natl Acad Sci U S A. 2017;**114**:E5434. Liuzzi AR, et al. J Immunol. 2016;**197**:2195. Corbett AJ, et al. Nature. 2014;**509**:361.

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Enterobacter cloacae	ND	X	X	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701. Dias J, et al. Proc Natl Acad Sci U S A. 2017;**114**:E5434.
Francisella tularensis	X(X)	X††	X	Meierovics A, et al. Proc Natl Acad Sci U S A. 2013;**110**:E3119. Meierovics AI, et al. J Exp Med. 2016;**213**:2793.
Helicobacter pylori	X(X)	X††	X	Booth JS, et al. Front Immunol. 2015;**6**:466. D'Souza C, et al. J Immunol. 2018;**200**:1901.
Klebsiella pneumoniae	X(X)	X	X	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701. Liuzzi AR, et al. J Immunol. 2016;**197**:2195. Reantragoon R, et al. J Exp Med. 2012;**209**:761. Shaler CR, et al. PLoS Biol. 2017;**15**:e2001930. Georgel P, et al. Mol Immunol. 2011;**48**:769.
Lactobacillus acidophilus	X	X	No*	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701.

Lactobacillus casei	ND	X	No*	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701.
Lactococcus lactis	X(X)	ND	X(X)	Corbett AJ, et al. Nature. 2014;**509**:361.
Mycobacterium abscessus	X(X)	X	X	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701.
Mycobacterium avium	X	ND	X	Meermeier EW, et al. Nat Commun. 2016;**7**:12506.
Mycobacterium bovis	X(X)	X†	X	Greene JM, et al. Mucosal Immunol. 2017;**10**:802. Jiang J, et al. Am J Respir Crit Care Med. 2014;**190**:329. Chua WJ, et al. Infect Immun. 2012;**80**:3256.
Mycobacterium smegmatis	X(X)	ND	X	Gold MC, et al. J Exp Med. 2014;**211**:1601. Meermeier EW, et al. Nat Commun. 2016;**7**:12506. Sharma PK, et al. Immunology. 2015;**145**:443.
Mycobacterium tuberculosis	X(X)	ND	X	Meermeier EW, et al. Nat Commun. 2016;**7**:12506. Jiang J, et al. Am J Respir Crit Care Med. 2014;**190**:329. Gold MC, et al. PLoS Biol. 2010;**8**:e1000407. Gold MC, et al. Mucosal Immunol. 2013;**6**:35. Harriff MJ, et al. PLoS One. 2014;**9**:e97515. Jiang J, et al. J Infect. 2016;**72**:338.
Neisseria gonorrhoeae	X	ND	X	Meermeier EW, et al. Nat Commun. 2016;**7**:12506.
Nocardia asteroides	X	ND	X**	Meermeier EW, et al. Nat Commun. 2016;**7**:12506.
Pseudomonas aeruginosa	X (X)	X	X	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701.

				Meermeier EW, et al. Nat Commun. 2016;**7**:12506. Liuzzi AR, et al. J Immunol. 2016;**197**:2195. Jo J, et al. PLoS Pathog. 2014;**10**:e1004210. Reantragoon R, et al. J Exp Med. 2012;**209**:761. Shaler CR, et al. PLoS Biol. 2017;**15**:e2001930. Tang XZ, et al. J Immunol. 2013;**190**:3142.
Saccharomyces cerevisiae	X(X)	ND	X	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701.
Salmonella enterica serovar Typhimurium	X(X)	X[††]	X(X)	Gold MC, et al. J Exp Med. 2014;**211**:1601. Meermeier EW, et al. Nat Commun. 2016;**7**:12506. Corbett AJ, et al. Nature. 2014;**509**:361. Le Bourhis L, PLoS Pathog. 2013;**9**:e1003681. Reantragoon R, et al. J Exp Med. 2012;**209**:761. Salerno-Goncalves R, et al. Front Immunol. 2014;**4**:511. Shaler CR, et al. PLoS Biol. 2017;**15**:e2001930. Gold MC, et al. PLoS Biol. 2010;**8**:e1000407. Chen Z, et al. Mucosal Immunol. 2017;**10**:58. Kjer-Nielsen L, et al. Nature. 2012;**491**:717. Reantragoon R, et al. J Exp Med. 2013;**210**:2305. Salerno-Goncalves R, et al. Front Immunol. 2017;**8**:398.
Salmonella enterica serovar Paratyphi A	X(X)	X	X	Howson LJ, et al. Nat Commun. 2018;**9**:253.

Shigella flexneri	X(X)	ND	X	Meermeier EW, et al. Nat Commun. 2016;**7**:12506. Le Bourhis L, PLoS Pathog. 2013;**9**:e1003681.
Staphylococcus aureus	X	X	X	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701. Meermeier EW, et al. Nat Commun. 2016;**7**:12506. Liuzzi AR, et al. J Immunol. 2016;**197**:2195. Lepore M, et al. Nat Commun. 2014;**5**:3866. Gold MC, et al. PLoS Biol. 2010;**8**:e1000407.
Staphylococcus epidermidis	X(X)	X	X	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701. Reantragoon R, et al. J Exp Med. 2012;**209**:761.
Streptococcus pneumoniae	X(X)	X	X	Brutinel ED, et al. J Bacteriol. 2013;**195**:5479. Kurioka A, et al. J Infect Dis. 2018;**217**:988.
Streptococcus pyogenes	X(X)	ND	No	Meermeier EW, et al. Nat Commun. 2016;**7**:12506.
Vibrio parahemoliticus	X	ND	X	Meermeier EW, et al. Nat Commun. 2016;**7**:12506.
Yersinia enterocolitica	X	ND	X	Meermeier EW, et al. Nat Commun. 2016;**7**:12506.

*Intact KEGG riboflavin synthesis pathways have not been documented in the strains tested to date.

**Species not listed in the KEGG pathway database but other species of the same genus feature the riboflavin pathway.

†Mild MAIT cell accumulation in vivo.

††Strong MAIT cell accumulation in vivo.

ND No data available.

Table 1b

Microbes that do not activate MAIT cells	References
Streptococcus group A	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701.
Enterococcus faecalis	Le Bourhis L, et al. Nat Immunol. 2010;**11**:701. Meermeier EW, et al. Nat Commun. 2016;**7**:12506. Liuzzi AR, et al. J Immunol. 2016;**197**:2195. Corbett AJ, et al. Nature. 2014;**509**:361. Jo J, et al. PLoS Pathog. 2014;**10**:e1004210. Ussher JE, et al. Eur J Immunol. 2014;**44**:195. Ussher JE, et al. Eur J Immunol. 2016; **46**: 1600.
Listeria monocytogenes	Liuzzi AR, et al. J Immunol. 2016;**197**:2195. Lepore M, et al. Nat Commun. 2014;**5**:3866. Salerno-Goncalves R, et al. Front Immunol. 2014;**4**:511. Gold MC, et al. PLoS Biol. 2010;**8**:e1000407.

Figure 1

imcb_12057_f1-5.pdf

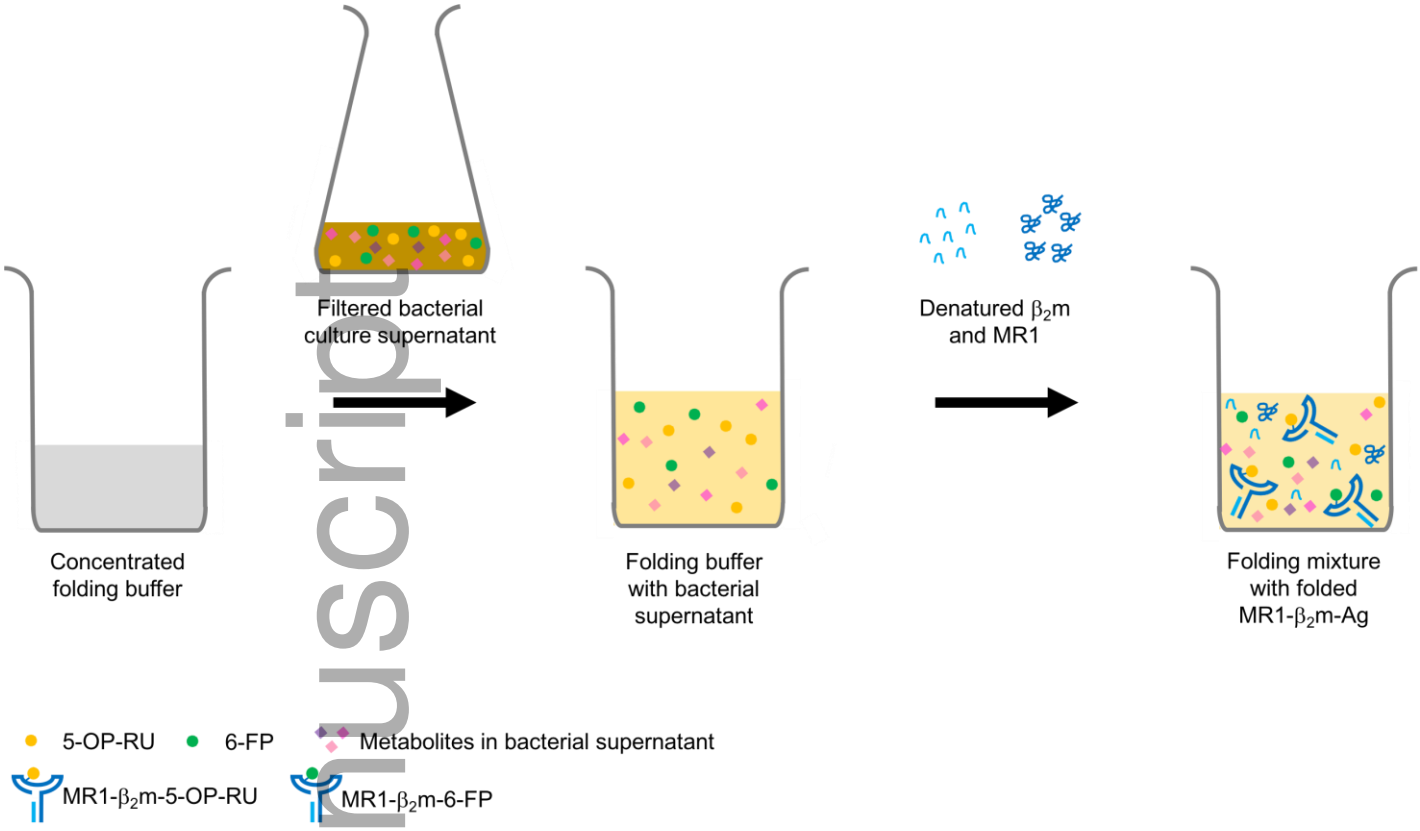
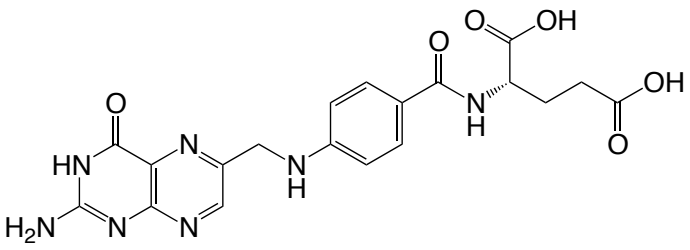


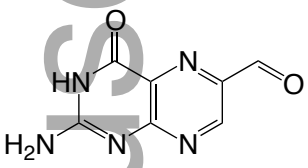
Figure 2

a



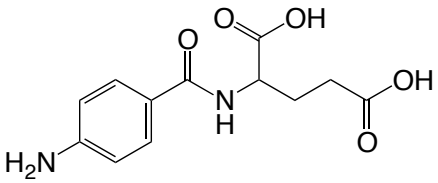
folic acid
($C_{19}H_{19}N_7O_6$, *m/z* 440.13)

Ultraviolet light



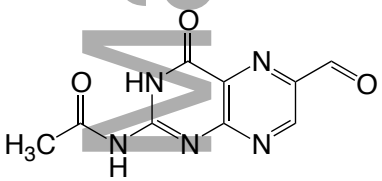
6-formylpterin
(6-FP, $C_7H_5N_5O_2$, *m/z* 190.04)

+



p-aminobenzoyl-L-glutamic acid
($C_{12}H_{14}N_2O_5$, *m/z* 265.08)

b



acetyl-6-formylpterin
(Ac-6-FP, $C_9H_7N_5O_3$, *m/z* 232.05)

Figure 3

a

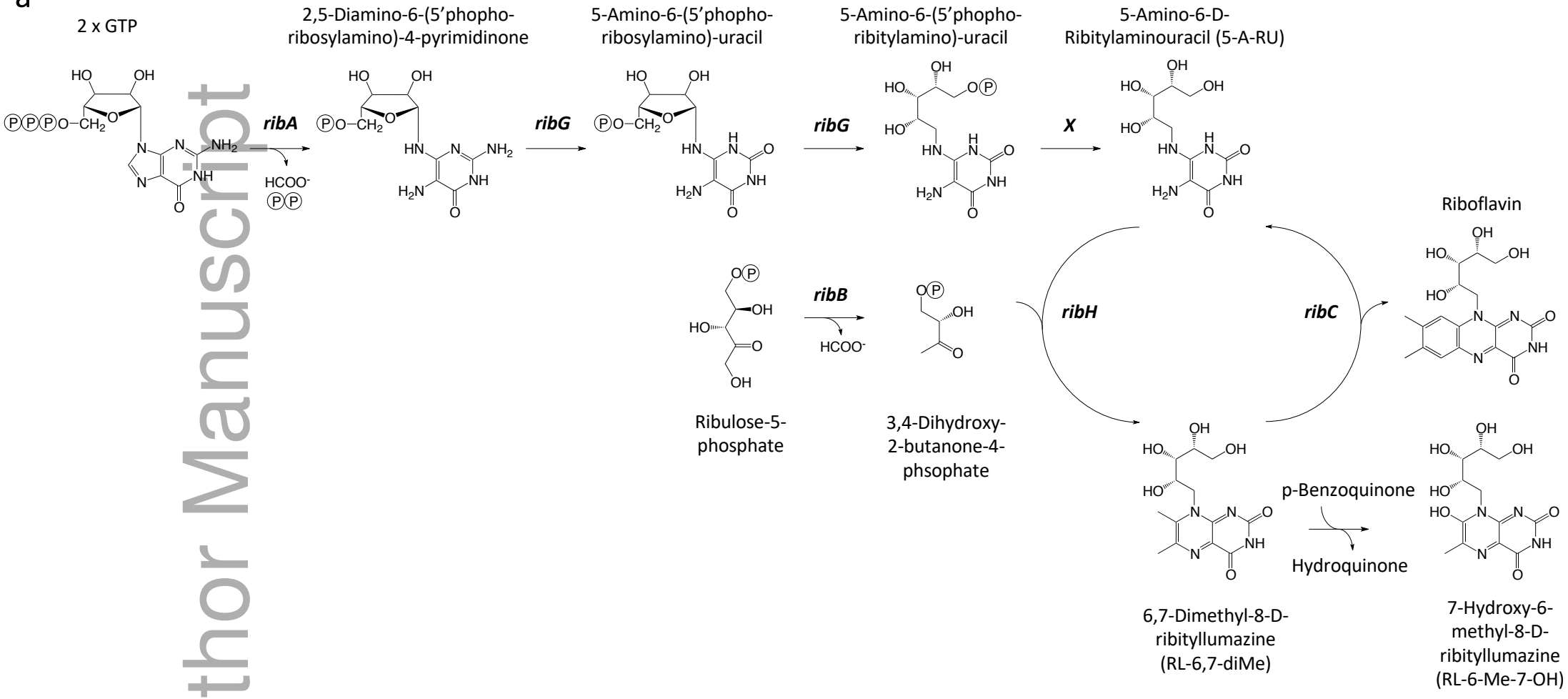


Figure 4

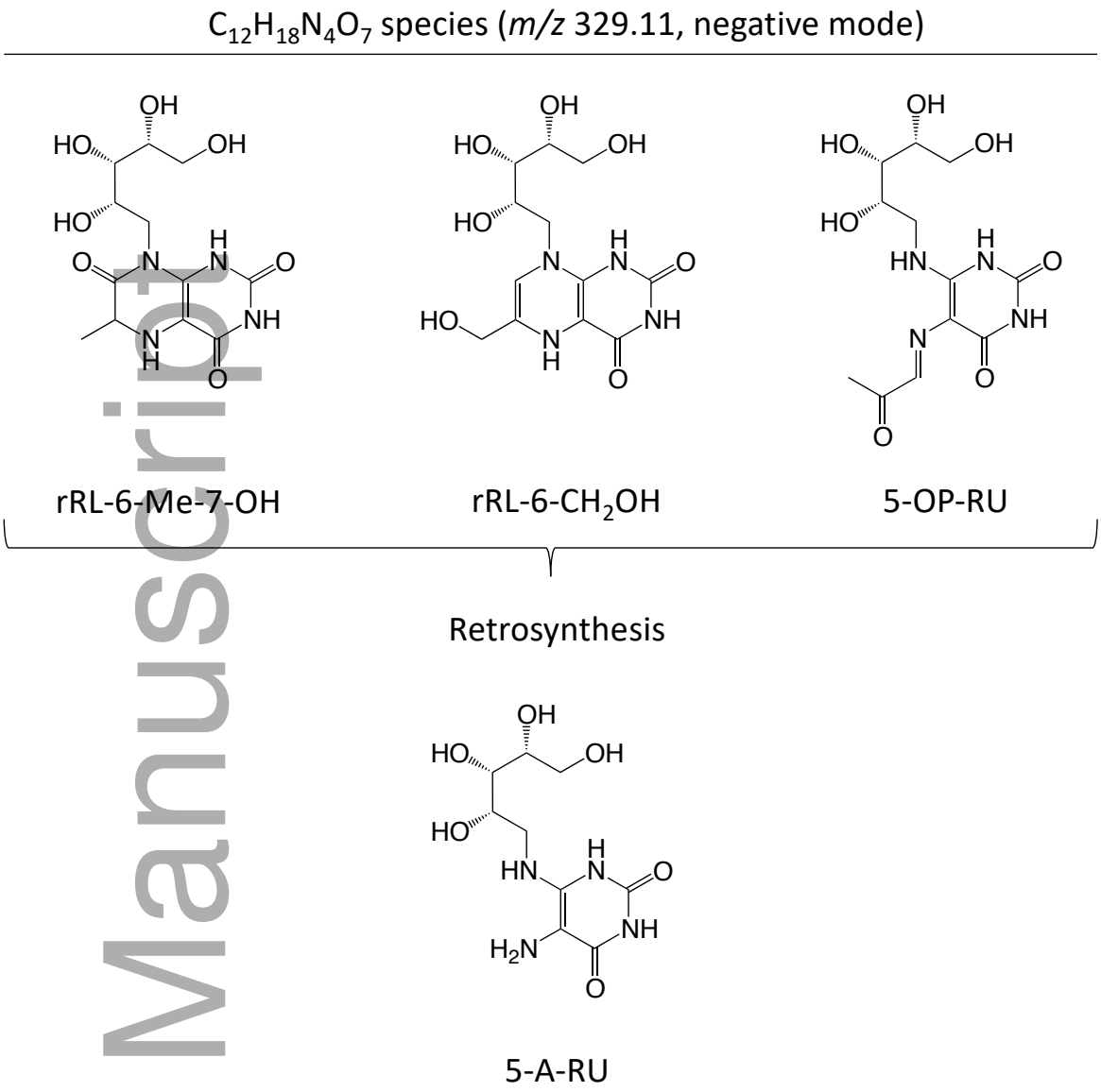


Figure 5