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Structural Studies of Vγ2Vδ2 T Cell Phosphoantigens

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Summary

Human γδ T cells containing the Vγ2Vδ2 (Vγ9Vδ2) T cell receptor are stimulated by a broad variety of small, phosphorus-containing antigenic molecules called phosphoantigens. The structures of several species present in both Mycobacteria (TUBags1–4) and in *Escherichia coli* have been reported to contain a formyl-alkyl diphosphate core. Here we report the synthesis of the lead member of the series, 3-formyl-1-butyl diphosphate. This compound has low activity for γδ T cell stimulation, unlike its highly active isomer (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate, necessitating a revision of the structure of TUBag1. Likewise, the structure of the species identified as the pentyl analog (TUBag 2) is revised to 6-phosphogluconate. These results indicate that neither TUBag1 nor the m/e 275 species proposed for TUBag2 are 3-formyl-1-alkyl diphosphates, leading to the conclusion that none of the natural phosphoantigens (TUBags1–4) possess the structures reported previously.

Introduction

Human γδ T cells expressing the Vγ2Vδ2 T cell receptor (TCR) (also known as the Vγ9Vδ2 TCR) are activated by a wide range of nonpeptide antigens, including small organic diphosphates (phosphoantigens) [1, 2], alkylamines [3], and bisphosphonates [4, 5] that are used to treat various bone resorption diseases. Such Vγ2Vδ2 T cells rapidly proliferate on exposure to these antigens during infections or with immunizations, often expanding from ~2%–3% to ~50% of blood T cells [6, 7] (reviewed in [8]) with parallel, rapid elevations in TNF-α and IFN-γ secretion [9]. Extracts of phosphoantigens from many pathogenic organisms, such as *Mycobacterium tuberculosis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Plasmodium falciparum* (one causative agent of malaria), stimulate human Vγ2Vδ2 T cells [8, 10, 11], as do some tumor cell lines [12]. Vγ2Vδ2 T cells also have broad antitumor properties and can lyse tumor cells from a variety of tissue lineages [13]. For this reason, the use of such small-molecule phosphoantigens to activate Vγ2Vδ2 T cells for cancer immunotherapy is currently being intensively studied and has shown clinical efficacy in one study [14].

There are, however, many questions as to the mechanism of action of phosphoantigens, as well as to their chemical structures. For example, phosphoantigens have been proposed to interact directly with the γδ T cell receptor, based on the structural requirements for phosphoantigen bioactivity [15] and on TCR mutagenesis studies [16, 17], but no binding between a potent synthetic phosphoantigen and the Vγ2Vδ2 TCR was observed in a crystallographic investigation [18]. Furthermore, although it is likely that phosphoantigens require a presenting molecule, none has been found. In contrast to the numerous pyrophosphate phosphoantigens which have been studied [19], bisphosphonates [12, 20] such as risedronate, and alkylamines [21], appear to stimulate Vγ2Vδ2 T cells through an indirect process, by inhibiting isoprenoid biosynthesis at the level of farnesyl diphosphate synthase, resulting in the upstream accumulation of isopentenyl diphosphate, but again the molecular basis for this activity has not yet been elucidated in detail. More recently, Scotet et al. [22] reported that Vγ2Vδ2 T cell receptors interact with F1-ATPase and apolipoprotein A-1. This recognition is proposed to be the basis for Vγ2Vδ2 T cell recognition of various tumor cell lines, although we have found examples where Vγ2Vδ2 TCR-mediated tumor recognition does not appear to involve F1-ATPase (unpublished observation). Additionally, this study did not elucidate structural details or any possible connection with γδ T cell activation by phosphoantigens or other small-molecule antigens. Nevertheless, because this work demonstrated the first actual TCR interaction, it is of considerable interest.

In this context, it is of interest to note that γδ T cell antigens can affect lipid metabolism [23], and, in some cases, inhibit ATP↔ADP transporters (the isopentenyl pyrophosphate [IPP] resulting from the action of bisphosphonates reacts with ATP to form the toxic ATP analog, ApppI [24]). Thus, there may be connections between ATPase/TCR signaling events and the small molecules that activate γδ T cells. But because the actual chemical nature of natural phosphoantigens has itself

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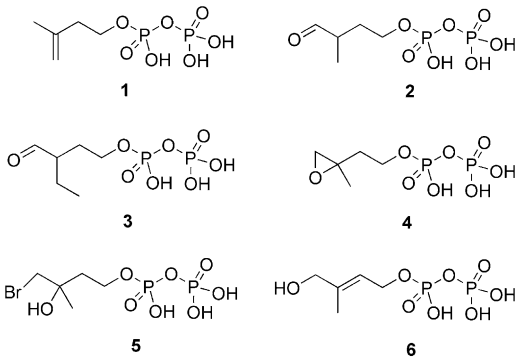

Figure 1. Structures of Phosphoantigens

1 = isopentenyl pyrophosphate (IPP), **2** = 3-formyl-1-butyl pyrophosphate (3-FBPP), **3** = 3-formyl-1-pentyl pyrophosphate, **4** = epoxide of **1**, **5** = bromohydrin pyrophosphate (Phosphostim), **6** = (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP).

been the topic of considerable debate, and because these species are exceptionally active (100 pM), it is essential to clarify their chemical structures in order to aid in the development of these molecules for use in cancer immunotherapy.

Our previous work in *Mycobacterium smegmatis* showed that one phosphoantigen, having a mass/charge (m/e) ratio of 245, corresponded to isopentenyl diphosphate (**1**; Figure 1) and suggested that a second species having m/e = 275 might correspond to a hydroxymethyl derivative of **1** [1]. Studies with *M. tuberculosis* [2, 25], *M. fortuitum* [26], and *E. coli* [27] found evidence for the existence of phosphoantigens termed TUBag1 and TUBag2, with m/e of 261 and 275, respectively. Based on mass spectrometry, NMR, UV-vis, and chemical modification results on these and other related antigens (TUBag3 and TUBag4), TUBag1 was proposed to be 3-formyl-1-butyl diphosphate (3-FBPP; m/e = 261; **2**; Figure 1) [26], while TUBag2 was proposed to be 3-formyl-1-pentyl diphosphate (m/e = 275; **3**; Figure 1) [27]. Based on these results, molecular modeling approaches were then used [28, 29] to design phosphoantigens, such as the epoxide of IPP, **4** (which has the same empirical formula as **2**; Figure 1), as well as the bromohydrin of **1** (**5**). Both are potent activators of Vγ2Vδ2 T cells, and the bromohydrin (**5**) is now under development as a novel immunotherapeutic agent [30, 31]. However, the recent delineation of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway [32–34] found in most Eubacteria (including Mycobacteria and *E. coli*) has brought the assignment of the m/e = 261 species as 3-formyl-1-butyl diphosphate [26] into question, as this intermediate is not in the MEP pathway. Instead, a compound from *E. coli* having m/e = 261 has been shown to be (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP) (**6**; Figure 1) [35], an extremely potent Vγ2Vδ2 T cell antigen with an EC_{50} of ~30–100 pM [36].

These results immediately raise the following questions. Is the 3-formyl-1-butyl diphosphate (3-FBPP, **2**) structure for TUBag1 correct? If not, is it likely that the structure of TUBag2 is 3-formyl-1-pentyl diphosphate (**3**)? Are there other likely possibilities for the m/e 275 ion structure? Does 3-FBPP (**2**) actually activate $\gamma\delta$ T cells? Answering these questions is clearly of interest in the context of the further development of phosphoantigens as cancer vaccines. In this paper, we therefore report the first total synthesis of 3-formyl-1-butyl diphosphate (**2**), its characterization by ^{1}H and ^{31}P NMR spectroscopy, and its efficacy in Vγ2Vδ2 T cell activation. We also report on the chemical nature of the 275 ion species that has been proposed [27] as TUBag2, the homolog **3** of TUBag1, and briefly assess the likelihood that TUBag3 and TUBag4 contain formyl-alkyl diphosphate cores.

Results and Discussion

Synthesis of 3-Formyl-1-Butyl Diphosphate (2)

Our synthetic route to 3-formyl-1-butyl diphosphate (**2**) started from commercially available 2-methylbutyrolactone (**7**), as shown in Figure 2. Ring opening of (**7**) with HBr in ethanol gave the bromoester (**8**) in high yield (94%), and then direct reduction of (**8**) with DIBAL at −80°C afforded 4-bromo-2-methyl-butanal (**9**) in 84% yield. An attempt to obtain 3-formyl-1-butyl diphosphate

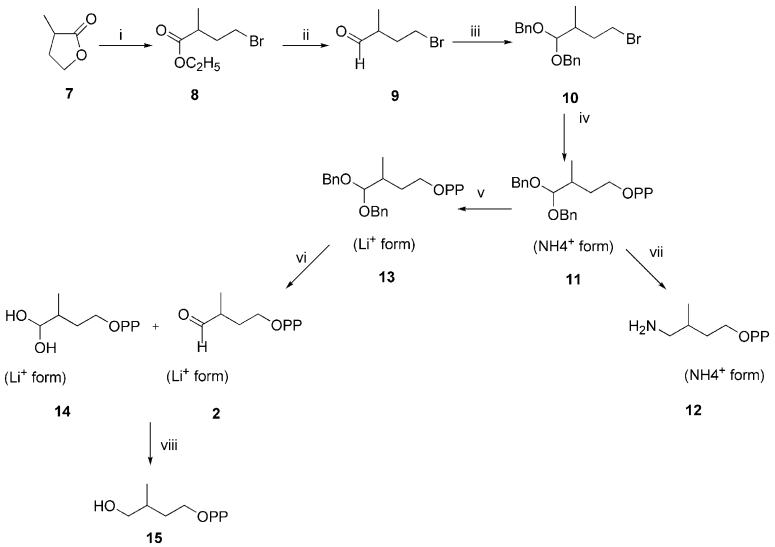

Figure 2. Synthesis of **2** and **12**

(i) HBr/C_2H_5OH, 90%; (ii) DIBAL/ether, 84%; (iii) TMSOBn/$FeCl_3$, 75%; (iv) $(n\text{-}Bu_4N)_3HP_2O_7/CH_3CN$, ion exchange ($NH_4^+$), 70%; (v) ion exchange (Li^+), 80%; (vi) 10% Pd/C/H_2O, 90%; (vii) 10% Pd/C, NH_4Cl-H_2O, 70%; (viii) $NaBH_4$ (in situ, in the NMR tube). PP, pyrophosphate; Bn, benzyl.

(2) via reaction of (9) with $(Bu_4N)_3HP_2O_7$ was unsuccessful, probably due to the instability of the formyl moiety under basic conditions. Protection of the bromo aldehyde (9) with benzyloxyltrimethylsilane (TMSOBn) to form the dibenzyl acetal (10) was therefore carried out, using $FeCl_3$ as a catalyst [37]. The product (10) was then reacted with $(Bu_4N)_3HP_2O_7$ at room temperature overnight and the resulting mixture was subjected to ion exchange purification to give the diphosphate (11) as the triammonium salt. Hydrogenation of (11) using 10% Pd/C gave the reductive amination product 4-amino-3-methyl-1-butyl diphosphate (12), so 11 was ion exchanged to form the trilithium salt (13), after which 3-formyl-1-butyl diphosphate (2), along with the hydrate (14), was obtained, in 70% yield.

NMR Characterization of Synthetic Species

The ^1H and ^{31}P NMR spectra of all intermediates were unremarkable. However, both the ^1H and the ^{31}P NMR spectra of 3-formyl-1-butyl diphosphate (2) were more complex than anticipated and indicated the presence of a mixture of (chiral) species (Figures 3A and 3B; see Figure S1 in Supplemental Data available with this article online). In particular, while the 600 MHz ^1H NMR spectrum of 2 did contain a highly deshielded (δ = 9.5 ppm) feature corresponding to the aldehydic proton, this feature had only ~50% of the expected intensity. Moreover, as can be seen in Figure 3A, the spectrum is clearly not that expected for 2 alone. Likewise, the ^{31}P spectrum of 2 (Figure S1B) consisted of two sets of resonances, with the more shielded feature (at ~−9 ppm) being split into two sets of doublets. These features arise from an equilibrium between the aldehyde (2) and its hydrate, the 4,4-diol (14). Similar hydration behavior has been reported previously by Lawrence and Sutherland [38] for glycolaldehyde diphosphate and glycolaldehyde triphosphate. Upon integration (Figure S1), both the ^1H and ^{31}P NMR spectra correspond to a ~1:1 ratio of the free aldehyde (2) and the hydrate diphosphate (14). These conclusions are supported by inspection of the 600 MHz 1D spectrum (Figure 3A) and the 2D COSY spectrum (Figure 3B). In Figure 3A, the aldehyde peak (in 2) as well as the methine peak in the hydrate (14) are readily observed, with the hydrate methine peak showing a clear ^3J coupling (^3J = 4.5 Hz) to H3 (14), confirming the equilibrium between the aldehyde (2) and its hydrate (14). The 2D COSY spectrum (Figure 3B) also permits a correlation between all protons in the aldehyde subspectrum (illustrated in Figure 3A) and a similar correlation and set of assignments for the hydrate (14) (Figure 3B). These results are further supported by the observation that the reductive amination product (12) had ^1H and ^{31}P NMR spectra consistent with a single species (Figure 2; Figure S1). Moreover, reduction of the 2 (14) mixture (in the Li^+ form) with $NaBH_4$ (in situ, in the NMR tube) gave 4-hydroxy-3-methyl-1-butyl diphosphate (15) (Figures S1E and S1F). The mass spectrum of the 2 (14) mixture yielded, however, only the m/e = 261 ion in its mass spectrum, indicating dehydration in vacuo. The ^1H NMR chemical shifts of 2, 6, and 14 were all consistent with those expected theoretically, but the results observed for 2 differed from those reported for TUBag1, the compound previously assigned this structure [26].

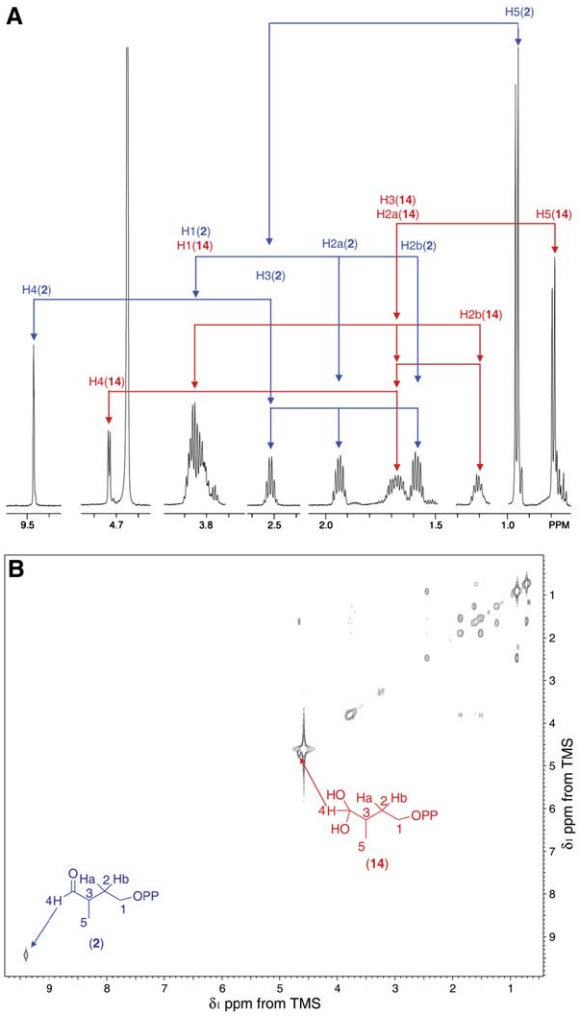

Figure 3. 600 MHz ^1H NMR Spectra of 2 (14)

(A) 1D spectrum showing peaks of interest, connectivities, and assignments from (B).

(B) COSY spectrum showing connectivities among aldehyde (2) and hydrate (14) peaks, also illustrated in (A).

Identification of the 275 m/e Ion in *M. fortuitum* and *M. smegmatis* as 6-Phosphogluconate

We next questioned whether the proposed structure of the m/e = 275 species as the homolog, 3-formyl-1-pentyl diphosphate (3), was correct. We investigated the mass spectrum of the m/e = 275 species previously partially purified from supernatants of *M. smegmatis* and *M. fortuitum* [1]. Using accurate mass matching (FT-ICR MS, negative ion mode), the exact mass of the m/e = 275 species from *M. smegmatis* was 275.017210 amu. Using a 30 ppm mass tolerance and a charge = −1, we obtained the possible elemental composition results shown in Table 1, which strongly support a $C_6H_{12}O_{10}P$ structural formula (with a 0.15 mmu or 0.55 ppm error). In contrast, the ion corresponding to 3-formyl-1-pentyl diphosphate (3) has a 29.4 ppm mass error, the largest among the 12 structures returned (Table 1). The likely chemical structure corresponding to the $C_6H_{13}O_{10}P$ compound is 6-phosphogluconate (16), which is used in the synthesis of ribulose 5-phosphate (17) and 2-dehydro-3-deoxy-6-phosphogluconate (18) (Figure S2)

Table 1. Experimental and Calculated Masses and Structural Formulae for the m/e = 275 Ion

Mass (Expected) (amu)	Mass (Calculated) (amu)	Error (mmu)	Error (ppm)	Formula
275.017210	275.013854	3.36	12.20	$^{12}C_{20}{}^{1}H_{3}{}^{16}O_{2}$
275.017210	275.019729	−2.52	−9.16	$^{12}C_{13}{}^{1}H_{7}{}^{16}O_{7}$
275.017210	275.010349	6.86	24.95	$^{12}C_{2}{}^{1}H_{11}{}^{16}O_{15}$
275.017210	275.011486	5.72	20.81	$^{12}C_{13}{}^{1}H_{8}{}^{16}O_{5}{}^{31}P$
275.017210	**275.017361**	**−0.15**	**−0.55**	$^{12}C_{6}{}^{1}H_{12}{}^{16}O_{10}{}^{31}P$
275.017210	275.018498	−1.29	4.68	$^{12}C_{17}{}^{1}H_{9}{}^{31}P_{2}$
275.017210	275.024373	−7.16	−26.05	$^{12}C_{10}{}^{1}H_{13}{}^{16}O_{5}{}^{31}P_{2}$
275.017210	275.009118	8.09	**29.42**	$^{12}C_{6}{}^{1}H_{13}{}^{16}O_{8}{}^{31}P_{2}$
275.017210	275.016130	1.08	3.93	$^{12}C_{10}{}^{1}H_{14}{}^{16}O_{3}{}^{31}P_{3}$
275.017210	275.022005	−4.80	−17.44	$^{12}C_{3}{}^{1}H_{18}{}^{16}O_{8}{}^{31}P_{3}$
275.017210	275.013762	3.45	12.54	$^{12}C_{3}{}^{1}H_{19}{}^{16}O_{6}{}^{31}P_{4}$
275.017210	275.020774	−3.56	−12.96	$^{12}C_{7}{}^{1}H_{20}{}^{16}O^{31}P_{5}$

[39–41]. The high-resolution mass spectra also enable the assignment of several other ions seen in supernatants of *M. smegmatis* (Figure 1c in [1]) including m/e = 339 as $C_6H_{13}O_{12}P_2$, corresponding to a hexose-diphosphate, and m/e = 277 as $C_5H_{12}O_9P_2$, corresponding to 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate.

To confirm the identity of the 275 ion species as 6-phosphogluconate, daughter ion spectra were obtained from the natural 275 species and synthetic 6-phosphogluconate. The parent/daughter spectrum of the 275 species from *M. smegmatis* was identical to that of 6-phosphogluconate, **16** (Figure S3), and very similar to the reported parent/daughter spectrum for the m/e = 275 species that was proposed as 3-formyl-1-pentyl diphosphate (**3**). Sustained off-resonance collision-induced dissociation gave no evidence for diphosphate (m/e = 159) in the spectrum of the *M. smegmatis* extract. 6-phosphogluconate appears to decompose by loss of H_2O (m/e = 257) and HPO_3, forming a carbanion having m/e = 177. Taken together, these results demonstrate that the m/e = 275 ion species observed in mass spectra of extracts of Mycobacteria spp. and *E. coli* corresponds to 6-phosphogluconate (**16**).

Activity of 2 (14), 12, and 16 in Vγ2Vδ2 T Cell Stimulation

We next assessed the biological activity of the various compounds by their ability to stimulate TNF-α production and proliferation by several Vγ2Vδ2 T cell clones and to expand Vγ2Vδ2 T cells in peripheral blood mononuclear cells (PBMC) from normal donors. The JN.24 and 12G12 Vγ2Vδ2 T cell clones were stimulated with **2** (**14**) and **12**, with **1**, **5**, and **6** as positive controls. One representative data set for JN.24 is shown in Figure 4A and a summary of the EC_{50} results is presented in Table 2. In both the TNF-α release and cell proliferation assays we find the expected activity pattern for the positive controls, with **6** (HMBPP) having extremely high activity, followed by **5** (Phosphostim), and then **1** (IPP). Both the aldehyde (hydrate) **2** (**14**) and the amine (**12**) have low to medium activity. Compared to HMBPP (**6**), the aldehyde **2** (**14**) is ~20,000-fold less active in the JN.24 cell line and ~100,000-fold less active in the 12G12 cell line (Table 2). The results for all four experiments (two assays, two cell lines, five compounds) can be readily compared simply by plotting the TNF-α release EC_{50}s against the cell proliferation EC_{50}s, as there is a shift in EC_{50} for

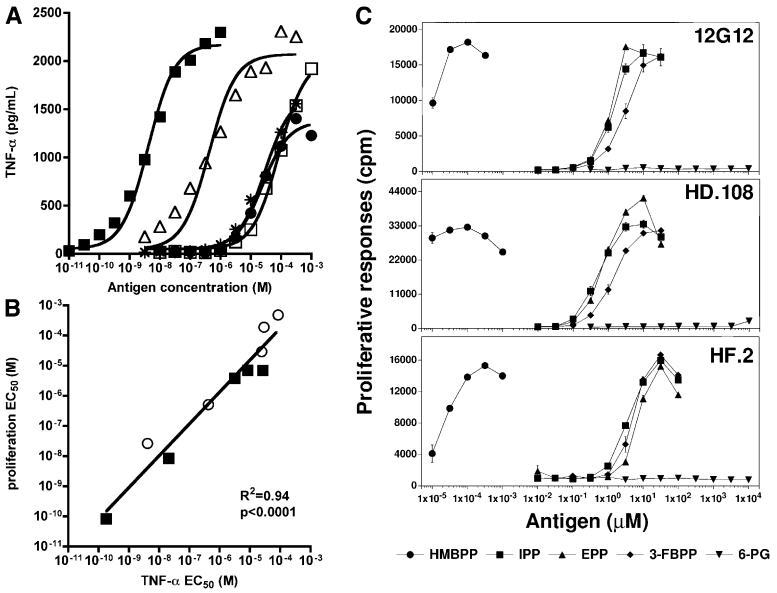

Figure 4. Vγ2Vδ2 T Cell Stimulation by 3-Formyl-1-Butyl Diphosphate (**2**) but Not 6-Phosphogluconate (**16**)

(A) TNF-α release by JN.24 T cells in response to antigen stimulation with **1** (asterisk), **2** (closed circles), **5** (open triangles), **6** (closed squares), and **12** (open squares) (with fitted curves).

(B) Correlation between Vγ2Vδ2 TNF-α release and Vγ2Vδ2 T cell proliferation EC_{50}s. JN.24 cell EC_{50}s are denoted by open circles and 12G12 cell EC_{50}s by closed squares. For the overall correlation, $R^2 = 0.94$ and $p < 0.0001$.

(C) 6-phosphogluconate (**16**) does not stimulate Vγ2Vδ2 T cells. 12G12, HD.108, and HF.2 Vγ2Vδ2 T cell clones were stimulated with various antigens for 2 days in the presence of Va-2 or CP.EBV antigen presenting cells. EPP, ethyl pyrophosphate.

Table 2. EC_{50} Results for γδ T Cell Stimulation Assays

		EC_{50} (μM)				
Assay	Cells	1	2 (14)	5	6	12
TNF-α release	JN.24 + CP.EBV	29	85	0.43	0.004	25
TNF-α release	12G12 + Va-2	3.2	27	0.02	0.0002	8.6
Proliferation	JN.24 + CP.EBV	190	490	0.52	0.027	30
Proliferation	12G12 + Va-2	3.8	7.0	0.008	0.00008	7.1

both TNF-α release and cell proliferation between the two cell lines (open circles are EC_{50}s for JN.24 cells; black squares are EC_{50}s for 12G12 cells; Figure 4B). The single correlation line shown has an $R^2 = 0.94$ with $p < 0.0001$. The low activity of **2** and **14** was not due to competing inhibitory and stimulatory activities, as there was no inhibition upon mixing **2** (**14**) with **6** (HMBPP) (Figure S4). Testing of 6-phosphogluconate (**16**) showed that this compound has essentially no activity for Vγ2Vδ2 T cells for stimulating proliferation (Figure 4C) or TNF-α release (data not shown). There was minimal activity at 10 mM for the HD.108 (Figure 4C, middle panel) and DG.SF68 (data not shown) clones. The ability of these compounds to induce the expansion of Vγ2Vδ2 T cells from the PBMC of two normal donors was similar to the results noted for the T cell clones, with HMBPP (**6**) inducing large expansions (65% and 70%), **2** (**14**) inducing moderate expansions (15% and 51%), and 6-phosphogluconate inducing no expansions (4% and 4%) (Figure S5).

Identification of Natural Phosphoantigens for Vγ2Vδ2 T Cells

The chemical structure of the m/e = 261 ion observed in phosphoantigen extracts of Mycobacterium spp. and *E. coli* has been the topic of debate for many years [1, 26, 27, 35]. In early work, it was proposed that this species was a hydroxy analog of IPP, as IPP itself was known to be a phosphoantigen with m/e = 245 and the novel species had a mass 16 amu larger, corresponding to addition of one oxygen atom [1]. Later, it was proposed that the m/e = 261 species corresponded to the species 3-formyl-1-butyl diphosphate (**2**) [26], and, even later, it was proposed to be another isomer, (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (**6**; HMBPP) [35]. The latter compound has now been prepared by total synthesis and has been found to be a ~30–100 pM activator of Vγ2Vδ2 T cells. HMBPP is required for Vγ2Vδ2 T cell activation because mutant *E. coli* lacking this compound have little bioactivity for Vγ2Vδ2 T cells [42]. Moreover, *E. coli* overproducing HMBPP due to a LytB mutation induce Vγ2Vδ2 T cells to expand in the human peripheral blood mononuclear cells-SCID-beige mouse model (our unpublished observations). Taken together, these data suggest that the assignment of the m/e = 261 species as HMBPP is correct. However, it also seemed possible that HMBPP (**6**) might convert to 3-formyl-1-butyl diphosphate (**2**) under some conditions, for example, via an isopentenyl diphosphate/dimethylallyl diphosphate isomerase (IPPI, *idi* gene)-catalyzed hydrogen shift to form the enol (Figure 5), which could then isomerize to the aldehyde (**2**). Therefore, we had two questions of interest. Does **2** activate Vγ2Vδ2 T cells? And does this putative isomerization actually occur?

In this report, we detail the synthesis and testing of 3-formyl-1-butyl diphosphate (**2**) and find that the aldehyde (**2**) is in equilibrium with its hydrate form (**14**) in aqueous solution. Based on the Vγ2Vδ2 T cell activation EC_{50} results for TNF-α release and cell proliferation in Vγ2Vδ2 T cell clones (Table 2), neither the free aldehyde (**2**) nor its hydrate form (**14**) has strong activity for Vγ2Vδ2 T cell stimulation. Thus, even if present, neither **2** nor **14** is likely to contribute significantly to Vγ2Vδ2 T cell activation by pathogenic bacteria or protozoa using the MEP pathway. Moreover, when HMBPP was incubated with IPPI (wild-type, from *E. coli*), the 600 MHz ^{1}H NMR spectrum of HMBPP (**6**) showed no evidence of an aldehyde peak, and the ^{1}H NMR spectrum of the aldehyde (hydrate), **2** (**14**), showed no evidence of an olefinic double bond (data not shown). Thus, there is no evidence for the conversion of HMBPP (**6**) to 3-formyl-1-butyl diphosphate (**2**), which strongly suggests that no active **6** could be produced from **2** (**14**) by this mechanism.

As noted in the Introduction, a second aldehyde structure, 3-formyl-1-pentyl diphosphate (**3**), has been proposed for the m/e = 275 ion species found in bioactive preparations from *M. smegmatis*, *M. fortuitum*, and *E. coli*. Given the modest activity of 3-formyl-1-butyl diphosphate, we questioned whether this proposed structure was correct. Exact mass measurement and the similarity of fragmentation patterns seen between spectra of m/e = 275 species from *M. smegmatis* and synthetic 6-phosphogluconate (**16**) indicate that the m/e = 275 species corresponds to 6-phosphogluconate (**16**) rather than 3-formyl-1-pentyl diphosphate. Moreover, 6-phosphogluconate (**16**) did not stimulate Vγ2Vδ2 T cells (Figure S5). The monophosphorylated species 6-phosphogluconate likely copurifies with pyrophosphate compounds on anion exchange chromatography because of the third negative charge from the carboxylic acid moiety. These findings suggest that the bioactive compound corresponding to TUBag2 is not the m/e = 275 ion species, but is actually a lower abundance compound with high bioactivity. Further purification and characterization of natural phosphoantigens will be required to identify the actual species responsible for bioactivity.

Significance

Activation of human γδ T cells by small, phosphorus-containing antigenic molecules, phosphoantigens, is of great importance as part of the first line of defense against a broad variety of pathogens. The structures of four major phosphoantigens from Mycobacteria and *E. coli*, TUBag1, TUBag2, TUBag3, and TUBag4, have long been considered as 3-formyl-1-alkyl

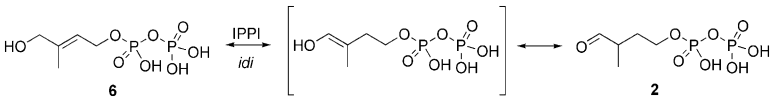

Figure 5. Possible Route for Interconversion of **2** and **6**, Catalyzed by Isopentenyl Pyrophosphate Isomerase (*idi*) via the Enol of **2**

diphosphates, based on which novel nonpeptide antigens have been developed. Here, however, we show by a combination of chemical synthesis, NMR spectroscopy, and mass spectrometry that such formyl species are unlikely to be responsible for human γδ T cell stimulation by pathogens. Our study describes the first total synthesis of 3-formyl-1-butyl diphosphate and demonstrates that this molecule is only a low-potency activator of Vγ2Vδ2 T cells. This finding supports an assignment of TUBag1 as (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBPP). Additionally, mass spectrometry strongly supports the assignment of the m/e = 275 species as 6-phosphogluconate. Based on these results, we conclude that none of the major phosphoantigens (TUBags1–4) responsible for Vγ2Vδ2 T cell activation contains a central 3-formyl-1-alkyl diphosphate core structure.

Experimental Procedures

Reagents

All chemicals for synthesis were reagent grade or better. Anhydrous solvents were dried prior to use. IPP (**1**) was from Sigma, **5** was prepared as described elsewhere [29], and **6** was the kind gift of José-Luis Giner. 6-phosphogluconate (**16**) was from Sigma.

NMR Spectroscopy

^{1}H and ^{31}P NMR spectra were obtained at 400 or 600 MHz (^{1}H resonance frequencies) using Varian Inova spectrometers. ^{1}H chemical shifts were referenced to an external standard of TMS (10% v/v tetramethylsilane, in $CDCl_3$) using the convention that high-frequency, low-field, paramagnetic, or deshielded values are positive (IUPAC δ scale). ^{31}P NMR spectra were referenced to an external standard of 85% (v/v) H_3PO_4, using the same convention.

Mass Spectrometry

Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) was performed using electrospray ionization in the negative mode on the 7-Tesla machine at the Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory. Internal standards of IPP (**1**) or geranyl diphosphate were used for exact mass determinations. Electrospray ionization tandem mass spectrometry (ESI MS/MS) was performed in negative ion mode using an API-III triple-quadrupole mass spectrometer (PE-SCIEX), as described [1].

Synthesis Details

Ethyl 4-Bromo-2-Methylbutyrate (8)

2-methylbutyrolactone (**7**) (2 g, 20 mmol) was added to a freshly prepared saturated solution of dry hydrogen bromide in absolute ethanol (20 ml). The solution was stirred at room temperature for 2 days, then poured into ice water and extracted with ethyl acetate (100 ml). The combined organic extracts were washed with a saturated $NaHCO_3$ solution (30 ml) and water (30 ml), dried (Na_2SO_4), and concentrated under reduced pressure to give ethyl 4-bromo-2-methylbutyrate (**8**) (3.8 g, 94%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.17 (d, *J* = 6.8 Hz, 3H, CH_3CHCOO); 1.25 (t, *J* = 7.2 Hz, 3H, OCH_2CH_3); 1.86–1.94 (m, 1H, $BrCH_2CH_2$); 2.20–2.29 (m, 1H, $BrCH_2CH_2$); 2.68 (m, 1H, $CHCOOCH_2CH_3$); 3.48 (t, 1H, *J* = 6.8 Hz, 2H, $BrCH_2$); 4.16 (q, *J* = 7.2 Hz, 2H, OCH_2CH_3).

4-Bromo-2-Methylbutanal (9)

To a solution of **8** (2.09 g, 10 mmol) in anhydrous dimethyl ether (30 ml) was carefully added DIBAL-H (10.5 ml, 1.0 M in hexane) so that the temperature did not exceed −80°C. After addition, the mixture was kept at −80°C for 1 hr and was then quenched with saturated NH_4Cl. The aqueous layer was extracted (ether, 30 ml) and the combined organic layer dried (Na_2SO_4), evaporated to dryness, then subjected to chromatography to afford **9** (1.39 g, 84%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.16 (d, *J* = 7.6 Hz, 3H, CH_3); 1.80–1.89 (m, 1H, $BrCH_2CH_2$); 2.27–2.35 (m, 1H, $BrCH_2CH_2$); 2.63–2.68 (m, 1H, CH_3CHCHO); 3.40–3.52 (m, 2H, $BrCH_2CH_2$); 9.67 (s, 1H, CHO).

4-Bromo-2-Methylbutanal Dibenzyl Acetal (10)

To a mixture of $FeCl_3$ (80 mg, 0.5 mmol) and **9** (0.83 g, 5 mmol) in anhydrous CH_2Cl_2 (5 ml) was added benzyloxyltrimethylsilane (2.2 g, 12 mmol) at 0°C under argon. The resulting mixture was stirred for 2 hr, quenched with saturated $NaHCO_3$, and extracted with ether. The combined organic layer was dried (Na_2SO_4) and evaporated to dryness to afford crude **10** (2.42 g, 75%) which was used in the next step without further purification.

Triammonium 4,4-Dibenzyloxy-3-Methyl-1-Butyl Diphosphate (11)

Published methods [43] were used with some modifications, including the omission of the cellulose column chromatography step. Tris (tetra *n*-butyl) ammonium hydrogen pyrophosphate (2.7 g, 2.8 mmol) was added to a solution of crude **10** (0.5 mmol) in anhydrous MeCN (3 ml). The suspension was stirred at room temperature for 6 hr. After it was washed with pentane (3 × 10 ml) and concentrated, the residue was dissolved in 2 ml of ion exchange buffer (25 mM NH_4HCO_3 containing 2% 2-propanol). The resulting solution was passed through a column (2 cm × 10 cm) of Dowex AG 50W-X8 (100–200 mesh) cation exchange resin (ammonium form). The column was eluted with two column volumes of ion exchange buffer, and the eluent was lyophilized to dryness. The residual white solid was partially dissolved in 20 ml of 25 mM aqueous NH_4HCO_3:MeCN:isopropanol (3:5:5). The gel-like mixture was vortexed, centrifuged, and the supernatant solution was decanted into another round bottom flask. The extraction was repeated five more times until a white solid formed. The supernatant was concentrated to near dryness by rotary evaporation. The solid was washed with 5 ml of ethanol:diethyl ether (1:2) and then lyophilized to dryness (190 mg, 70%). ^{1}H NMR (D_2O, 400 MHz): δ 0.8 (d, *J* = 7.6 Hz, 3H, CH_3); 1.70–1.80 (m, 1H, $PPOCH_2CH_2$); 1.90–2.01 (m, 1H, $PPOCH_2CH_2$); 3.80–3.92 (m, 2H, $PPOCH_2CH_2$); 4.40–3.70 (m, 5H, $[PhCH_2O]_2CH$); 7.2–7.4 (m, 10H, ArH).

3-Formyl-1-Butyl Diphosphate (2)

11 (190 mg) was dissolved in H_2O (2 ml) and then passed through a column (2 cm × 3 cm) of Dowex AG 50W-X8 (100–200 mesh) cation exchange resin (lithium form). The column was eluted with water, and then the eluent was lyophilized to dryness to afford **13** (140 mg, 80%). Ten percent Pd/C (30 mg) was added to a solution of **13** (140 mg) in H_2O (3 ml) and the mixture was hydrogenated with an H_2 balloon at room temperature. After 5 hr, the mixture was filtered through a short column padded with celite, then lyophilized to give a syrup which was washed with ethanol:diethyl ether (1:1, 5 ml), and lyophilized to afford **2** (61 mg, 70%) along with its hydrate (**14**) (^{1}H NMR spectrum shown in Figure 3).

Purification of the m/e 275 Species

Mycobacterium smegmatis and *M. fortuitum* were grown in Middlebrooks 7H9 media and phosphorylated compounds were purified as detailed in [1]. Mass spectrometry analysis was done on the samples reported in Figures 1c and 2b from [1].

γδ T Cell Assays

Vγ2Vδ2 T cell TNF-α release and proliferation assays were performed as described previously [44]. Briefly, to measure bioactivity for Vγ2Vδ2 T cells, the $CD4^+$ JN.24, $CD4^+$ HF.2, $CD8\alpha\alpha^+$ 12G12, or the $CD4^-8^-$ HD.108 Vγ2Vδ2 T cell clones were stimulated with phosphoantigens in the presence of CP.EBV (an EBV transformed B cell line) for $CD4^+$ clones or Va-2 (a transformed fibroblast) for $CD8\alpha\alpha^+$ and $CD4^-8^-$ clones. CP.EBV and Va-2 were fixed with 0.05% glutaraldehyde (EM grade, Sigma) for use as antigen

presenting cells. Note that although the relative potencies of the phosphoantigens were similar, the NKG2D$^+$ Vγ2Vδ2 clones, 12G12 and HD.108, exhibited higher antigen sensitivity, likely due to costimulation through their NKG2D receptors by their interaction with the NKG2D ligands MICA, ULBP2, and ULBP3 that are expressed by the Va-2 cell line. We have previously shown that the NKG2D-MICA interaction significantly increases antigen sensitivity [45]. Concentrations required to achieve 50% of the observed T cell response (EC_{50}s) were obtained by using the Prism 4.0 program (Graphpad Software), using a sigmoidal dose-response function. Curve-fitting minima for each experiment (e.g., TNF-α release from JN.24 cells) were determined using the global fitting technique, as implemented in Prism 4.0. Curve-fitting maxima were optimized for each individual compound without the use of any constraints.

For expansion of Vγ2Vδ2 T cells by phosphoantigens, PBMC from two normal donors were isolated by centrifugation on Ficoll-Hypaque gradients. PBMC (1×10^5) in 0.2 ml media were incubated in 96-well round bottom wells with the various phosphoantigens. IL-2 was added to 1 nM on day 3. The cells were harvested on days 7 and 14, stained with HIT3a FITC-anti-CD3 (eBioscience) and B6 PE-anti-Vδ2 (BD Pharmingen) monoclonal antibodies, and analyzed by flow cytometry.

Supplemental Data

Supplemental data include five figures and are available at http://www.chembiol.com/cgi/content/full/13/9/985/DC1/.

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