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Replacing the Pyrophosphate Group of HMB-PP by a Diphosphonate Function Abrogates Its Potential to Activate Human γδ T Cells but does not Lead to Competitive Antagonism

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Abstract—The immunological characterization of (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), and its methylenediphosphonate analogue, HMB-PCP, is described. With an EC_{50} of 0.1–0.2 nM, HMB-PP is significantly more potent in stimulating human Vγ9/Vδ2 T cells than any other compound described so far. However, replacing the pyrophosphate by a P–CH_2–P function abrogates the bioactivity drastically, with HMB-PCP having a EC_{50} of only 5.3 μM.

Introduction

Recognition of low-molecular weight non-peptide antigens by human γδ T cells is in striking opposition to the conventional recognition of small antigenic peptides by αβ T cells. Although the reactivity of γδ T cells towards these natural and synthetic metabolites depends on the expression of a functional Vγ9/Vδ2 T cell receptor (TCR) on the surface,[1] the exact molecular mechanism involved still remains to be elucidated, as it does not require classical antigen presentation in the context of the major histocompatibility complex. Yet, more than 70 compounds have already been shown to stimulate Vγ9/Vδ2 T cells,[2] the most potent of which is (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), a recently identified intermediate of the bacterial non-mevalonate pathway of isoprenoid biosynthesis.[3]

While direct contact of an antigen with the TCR has not been demonstrated so far, the crystal structure of the Vγ9/Vδ2 TCR insinuates a putative antigen-binding motif.[4] However, in contrast to immunogenic epitopes of protein antigens, the bioactivity of phosphorylated and pyrophosphorylated low-molecular weight antigens seems to reside, at least in part, in the chemical nature of the molecule. Thus, hydrolysis of the pyrophosphate moiety was proposed to contribute critically to bioactivity, with dephosphorylation-resistant diphosphonate analogues having drastically increased EC_{50} values.[5]

Here, the chemical synthesis and immunological characterization of HMB-PCP, a methylenediphosphonate analogue of HMB-PP, is presented.

Chemical Synthesis

HMB-PCP was synthesized from *tert*-butyldimethylsilyloxypropanone **1**,[6] as depicted in Figure 1. A Wittig–Horner reaction of **1** and P,P-diethylphosphonoacetate yielded a mixture of the ethylesters (*E*)- and (*Z*)-**2**. After separation of the isomers, reduction of pure (*E*)-**2** led to (*E*)-4-(*tert*-butyldimethylsilyl)oxy-3-methyl-but-2-en-1-ol **3**. The alcohol **3** was then transformed into the corresponding bromide **4**, and the P–CH_2–P moiety was added by reaction with Tris(tetra-*n*-butylammonium) hydrogen methylenediphosphonate.[7] Finally, the protecting group of **5** was set free by tetra-*n*-butylammo-

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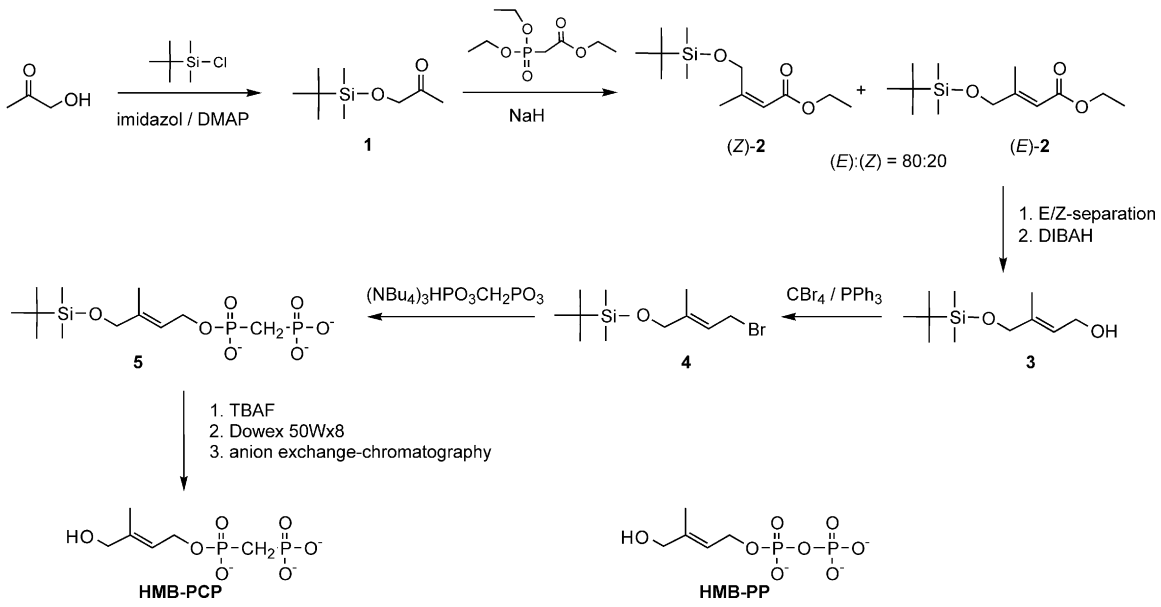

Figure 1. Chemical synthesis of HMB-PCP.

nium fluoride. However, apart from the tetra-*n*-butyl ammonium salt of HMB-PCP, the crude product obtained also contained considerable amounts of Br^- and F^- ions, as well as an excess of methylenediphosphonate. For subsequent purification, the tetra-*n*-butylammonium ions were substituted by NH_4^+ prior to extraction, as carried out in analogous approaches.[8] However, we rather chose to perform the final chromatography step using an anion exchange material instead of cellulose. After lyophilization of the product, the purity of the end product was established on a Dionex HPLC anion exchange system.

In order to compare the Vγ9/Vδ2 T cell stimulating properties of HMB-PCP with that of synthetic HMB-PP, a procedure described previously was employed,[9] and subjected to the above purification protocol.

Biological Results and Discussion

In contrast to a number of recent protocols on its chemical synthesis,[8,10] our approach allows an absolute quantitation of the salt-free HMB-PP recovery (by measuring the phosphorus content of the pure product, in addition to the gross weight of the lyophilized material), and thus a reliable determination of its bioactivity. In the presence of HMB-PP, proliferation of human PBMC was restricted to Vγ9/Vδ2 T cells, leading to a significant expansion of this subpopulation.[11] Importantly, synthetic HMB-PP and the natural compound isolated from *Escherichia coli* Δ*lytB* mutants displayed identical activities in stimulating Vγ9/Vδ2 T cells, at concentrations in the nM range and below (Table 1). Compared with the bioactivities of the direct precursor and successor of HMB-PP within its biosynthesis pathway, 2-*C*-methyl-D-erythritol 2,4-cyclopyrophosphate (MEcPP) and isopentenyl pyrophosphate (IPP), respectively,[3] HMB-PP was 3–4 magnitudes more potent in stimulating Vγ9/Vδ2 T cells than IPP, and 6 magnitudes more potent than MEcPP. With an EC_{50} value of approx. 0.1 nM, HMB-PP is considerably more active than the strongest compounds described so far,[2] and is the only molecule known possessing a bioactivity of significantly less than 1 nM.

Strikingly, replacing the pyrophosphate by a P–CH_2–P function abrogated the bioactivity of HMB-PP by 4–5 magnitudes (yet leaving a residual activity still compar-

Table 1. Biological activities of selected low molecular weight antigens

Compd	EC_{50} range (μM) other studies (ref 2)	EC_{50} range (μM) this study	Mean EC_{50} (μM) this study (n = 5–13)	Activity against IPP (-fold) this study
Phosphoribosyl pyrophosphate	—	> 10,000	> 10,000	< 0.00005
D-ribose-1-phosphate	1000–10,000	—	—	—
MEcPP	—	10–200	114	0.0004–0.01
HMB-PCP	—	1–10	5.30	0.04–0.2
Pamidronate	4–8	0.07–10	2.21	0.6–1.0
IPP	1–10	0.06–4.0	1.02	1
Alendronate	0.9	0.1–1.0	0.75	0.7–1.5
Bromohydrin pyrophosphate	0.005–0.02	—	—	—
Iodohydrin pyrophosphate	0.0005–0.003	—	—	—
HMB-PP (*E. coli* Δ*lytB*)	—	0.000004–0.001	0.000181	1200–21,500
HMB-PP (synthetic)	—	0.00001–0.0003	0.000084	3600–19,700

able to that of the commonly used ligands, IPP and pamidronate), suggesting that the extensive bioactivity of HMB-PP resides mainly in the pyrophosphate moiety. This is in agreement with comparable data on methylenediphosphonate analogues of a synthetic γδ T cell activator, bromohydrin pyrophosphate ($EC_{50} = 10$ nM), with the PCP derivative having a bioactivity of $>> 12$ μM.[5] Even more, in the same publication dephosphorylation-resistant analogues of synthetic activators were described as competitive antagonists, with IC_{50} values ranging from 10 μM to 1 mM. However, the IC_{50} values of these compounds were 3–4 magnitudes higher than the EC_{50} values of the corresponding agonists, thus raising doubts about the inhibitory specificity of the antagonism observed. Indeed, in our hands the putative antagonist HMB-PCP showed no inhibitory activity at all, whether titrated in the presence of a given concentration of the agonist HMB-PP, or in the reciprocal experiment, when added at a fixed concentration to a serial dilution of HMB-PP (not shown).

Taken together, while for a number of synthetic compounds the γδ T cell stimulatory capacity was attributed to their strong chemical reactivity,[5] this seems not the case for the comparably inert natural activator, HMB-PP. Consistently, quantitative structure–activity relationship analyses rather identified the presence of two negative ionizable groups, an H-bond donor, and a hydrophobic group as the most important features to make a good γδ T cell activator.[2b] Yet, this model still does not consider the striking difference in the bioactivity between bromohydrin pyrophosphate and its imidodiphosphate analogue ($EC_{50} = 12$ μM),[5] or between HMB-PP and HMB-PCP (Table 1). While the proposed need for a hydrolysable pyrophosphate moiety[5] may explain the relatively low bioactivities of the aminobisphosphonates, alendronate and pamidronate, our own data indicate that the bridging oxygen atom between the two negative ionizable groups is clearly important for maximum activity.

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6. NMR-spectra were recorded on Bruker DRX 500 and AM 400 spectrometers. ^{1}H- and ^{13}C NMR spectra in D_2O were referenced to internal 3-trimethylsilyl propionic acid sodium salt (0.0 ppm and 1.7 ppm, respectively), or tetramethylsilane in $CDCl_3$, proton-decoupled ^{31}P NMR to external phosphoric acid (0.0 ppm). **(*E*)-4-(*tert*-Butyldimethylsilyl)oxy-3-methyl-but-2-enoic acid ethylester, (*E*)-2**. To a suspension of 2.40 g (60 mmol) NaH in 40 mL of dry THF under argon was dropped a solution of 8.97 g (40 mmol) ethyl P,P-diethylphosphonoacetate in 15 mL of dry THF at 0 °C. After 1 h at 0 °C, 7.53 g (40 mmol) of *tert*-butyldimethylsilyloxy-propanone **1** (Choo, H. Y.; Peak, K. H.; Park, J.; Kim, D. H.; Chung, H. S. *Eur. J. Med. Chem.* **2000**, *35*, 643) in 15 mL of dry THF were added, and stirring was continued for 15 min at 0 °C and 2 h at rt. Workup was carried out by slow addition of 50 mL of saturated NH_4Cl solution, followed by 10 mL of water and 30 mL of ether. The aqueous layer was separated and extracted with a further 30 mL of ether. The combined organic layers were washed with two 30 mL portions of saturated NaCl solution, dried with Na_2SO_4, and released from the solvent by rotary evaporation under reduced pressure to yield a crude *E*/*Z*-mixture [(*E*)-**2**:(*Z*)-**2** = 80:20; GC analysis], which was separated by flash chromatography on silica gel with *n*-hexane/*tert*-butylmethylether (90:10) as eluent to yield 7.03 g (61%) of the pure desired *E*-isomer (*E*)-**2**: ^{1}H NMR ($CDCl_3$/TMS), δ: 0.09 (s, 6H, $Si(CH_3)_2$); 0.93 (s, 9H, $SiC(CH_3)_3$); 1.29 (t, 3H, $J = 7.1$ Hz, $CH_2C\underline{H}_3$); 2.05 (s (broad), 3H, $C{=}CCH_3$); 4.11 (s (broad), 2H, CH_2OSi); 4.17 (q, 2H, $J = 7.1$ Hz, $\underline{C}H_2CH_3$); 5.99 (s (broad), 1H, C=CH). ^{13}C NMR ($CDCl_3$/TMS), δ: −5.4 ($Si(CH_3)_2$); 14.4 ($CH_2\underline{C}H_3$); 15.4 ($C{=}C\underline{C}H_3$); 18.4 ($Si\underline{C}(CH_3)_3$); 25.9 ($SiC(\underline{C}H_3)_3$); 59.6 ($\underline{C}H_2CH_3$) 67.1 (CH_2OSi); 113.4 (C=CH) 157.1 ($\underline{C}$=CH); 167.1 (C=O). (*Z*)-**2** isomer: ^{1}H NMR ($CDCl_3$/TMS), δ: 0.08 (s, 6H, $Si(CH_3)_2$); 0.91 (s, 9H, $SiC(CH_3)_3$); 1.27 (t, 3H, $J = 7.1$ Hz, $CH_2C\underline{H}_3$); 1.97 (s (broad), 3H, $C{=}CCH_3$); 4.13 (q, 2H, $J = 7.1$ Hz, $C\underline{H}_2CH_3$); 4.79 (s (broad), 2H, CH_2OSi); 5.67 (s (broad), 1H, C=CH). ^{13}C NMR ($CDCl_3$/TMS), δ: −5.4 ($Si(CH_3)_2$); 14.3 ($CH_2\underline{C}H_3$); 18.3 ($Si\underline{C}(CH_3)_3$); 21.4 ($C{=}C\underline{C}H_3$); 25.9 ($SiC(\underline{C}H_3)_3$); 59.7 ($\underline{C}H_2CH_3$) 62.7 (CH_2OSi); 115.0 (C=CH) 160.8 ($\underline{C}$=CH); 166.1 (C=O). **(*E*)-4-(*tert*-Butyldimethylsilyl)oxy-3-methyl-but-2-en-1-ol**, **3**. In a 50 mL flask were placed 0.891 g (3.45 mmol) of (*E*)-**2** in 10 mL of dry toluene under argon and cooled to −78 °C. After dropping 8.6 mL (8.6 mmol, 1 M in hexane) of DIBAH into this solution, the temperature was kept at −78 °C for 2 h, then raised to 0 °C during 1 h, and held at 0 °C for 30 min. Addition of 1 mL of methanol and 10 mL of saturated sodium potassium tartrate solution formed a white gelatinous precipitate, which was stirred for 1 h at rt prior to adding 10 mL of water and 30 mL of ether. The aqueous layer was separated and extracted twice more, each with 30 mL of ether. The combined organic layers were dried with $MgSO_4$, filtered and the solvent was removed under reduced pressure to yield the crude product quantitatively, which was used in the next step without further purification. ^{1}H NMR ($CDCl_3$/TMS), δ: 0.07 (s, 6H, $Si(CH_3)_2$); 0.92 (s, 9H, $SiC(CH_3)_3$); 1.64 (s (broad), 3H, $C{=}CCH_3$), 4.03 (s (broad), 2H, CH_2OSi); 4.18 (d, 2H, $J = 6.9$ Hz, CH_2OH); 5.66 (dq, 1H, $J_1 = 6.9$ Hz, $J_2 = 1.4$ Hz, C=CH). ^{13}C NMR ($CDCl_3$/TMS), δ: −5.6 ($Si(CH_3)_2$); 13.5 ($C{=}CCH_3$); 18.4 ($Si\underline{C}(CH_3)_3$); 25.7 ($SiC(\underline{C}H_3)_3$); 58.9 (CH_2OH); 67.8 (CH_2OSi); 123.0 ($C{=}\underline{C}H$); 137.9 ($\underline{C}$=CH). **(*E*)-4-Hydroxy-3-methyl-but-2-enyl methylenediphosphonate, HMB-PCP.** To a solution of 0.430 g (1.987 mmol) **3** in 10 mL of dry CH_2Cl_2 at

0 °C under argon were dropped in succession 0.625 g (2.384 mmol) PPh_3 and 0.791 g (2.384 mmol) CBr_4, each in 3 mL of dry CH_2Cl_2. After 1 h at 0 °C and 4 h at rt, the reaction mixture was filtered through a short column of silica gel. The solvent was removed by rotary evaporation under reduced pressure and the residue treated for 1 h in vacuo. The crude product was triturated 3 times with *n*-pentane and filtered. The combined filtrates were evaporated to leave 0.265 g of the bromide **4** as a yellow oil, which was dissolved immediately in 0.5 mL of dry acetonitril and dropped to (1.282 g, 1.424 mmol, 1.5 equiv) freshly prepared tris(tetra-*n*-butylammonium) hydrogen methylenediphosphonate suspended in 3 mL of dry acetonitril under argon. After stirring the product **5** overnight at RT, 0.449 g (1.424 mmol, 1.5 equiv) of TBAF·$3H_2O$ dissolved in 2 mL of acetonitril was added dropwise, and stirring was continued for a further 3 h. Subsequent filtering of the reaction mixture and evaporation of the solvent left a slightly brownish oil, which was dissolved in 1 mL of ion-exchange buffer (25 mM NH_4HCO_3/2% isopropanol). This solution was subjected to chromatography on a 2.6×40 cm column of Dowex 50Wx8 (100–200 mesh, NH_4^+-form, elution at 2.5 mL/min). Fractions containing the desired product, analyzed on a Dionex HPLC system using high capacity anion-exchange columns, conductivity detection, and KOH gradient elution, were combined and lyophilized. For removal of diphosphonate, the residue was dissolved in 1 mL of buffer (50 mM NH_4HCO_3), 2 mL of isopropanol were added, and the mixture was shaken vigorously on a vortex mixer. After centrifugation (2000 *g*, 5 min, rt), the supernatant was removed and the residue extracted a further two times with 3 mL of a 1:2 mixture of buffer/isopropanol, with each extraction step being monitored on the Dionex HPLC system. The combined supernatants were loaded on a 2.6×40 cm Source 15Q column (Pharmacia) and gradient-eluted (A, 25 mM NH_4HCO_3/acetonitril 10:1; B, 1 M NH_4HCO_3/acetonitril 10:1; equilibration, 1 h 100% A; gradient, 10 min 100% A, 120 min A→B, 10 min 100% B; flow rate 4 mL/min, each fraction 8 mL). Product containing fractions were lyophilized to yield 126.1 mg (corresponding to an overall yield for the last three steps of 24%) of HMB-PCP as a white solid material, using the method for phosphorus determination in organic compounds described before (ref. 3a), and calculated as free acid. ^{1}H NMR (D_2O), δ: 1.72 (s, 3H, CH_3); 2.09 (dd, $J_1 = J_2 = 19.6$ Hz, 2H, PCH_2P); 4.03 (s, 2H, CH_2OH); 4.49 (dd, $J_1 = J_2 = 7.2$ Hz, 2H, CH_2OP); 5.64 (t, $J = 6.7$ Hz, 1H, C=CH). ^{13}C NMR (D_2O), δ: 17.7 (CH_3); 32.8 (dd, $J_1 = 121.0$ Hz, $J_2 = 124.8$ Hz, PCH_2P); 65.3 (d, $J = 5.0$ Hz; CH_2OP); 71.1 (CH_2OH); 125.8 (d, $J = 6.5$ Hz, C=CH); 143.9 (C=CH). ^{31}P NMR (D_2O), δ: 13.76 (d, $J = 8.9$ Hz); 20.95 (d, $J = 8.9$ Hz). ESI–MS(m−H)$^-$, calcd 259.0137, found 259.0147.

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9. **(*E*)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate, HMB-PP.** (*E*)-4-hydroxy-3-methyl-but-2-enyl bromide (127 mg) was obtained according to ref 8, dissolved in 0.5 mL dry acetonitril, and dropped into (1.042 g, 1.155 mmol) freshly prepared Tris(tetra-*n*-butylammonium) hydrogen pyrophosphate, suspended in 3 mL dry acetonitril under argon. After 16 h at rt, the solvent was evaporated to leave a slightly brownish oil, which was dissolved in 1 mL of ion-exchange buffer. This solution was subjected to the same extraction protocol as described for HMB-PCP, yielding 36.2 mg of HMB-PP as a white solid material, calculated as free acid.

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11. Flow cytometrical analysis of human Vγ9/Vδ2 T cells was performed according to ref 3b, and Eberl, M.; Engel, R.; Beck, E.; Jomaa, H. *Cell. Immunol.* **2003**, *218*, 1. Samples were tested at appropriate dilutions and calibrated using serial dilutions of IPP (Sigma). Natural HMB-PP was purified from *E. coli* Δ*lytB* (ref 3a); MEcPP was isolated from benzylviologen treated *Corynebacterium ammoniagenes* (Ostrovsky, D.; Shashkov, A.; Sviridov, A. *Biochem. J.* **1993**, *295*, 901). Alendronate was purchased from Merck, pamidronate from Novartis. Phosphoribosyl pyrophosphate (Sigma) served as negative control.