Aryloxy Triester Phosphonamidates of Phosphoantigens Exhibit Favorable Stability and Potent Activation of Vγ9/Vδ2 T-Cells

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ABSTRACT
We previously reported the application of the aryloxy triester phosphoramidate prodrug technology to the phosphoantigen (E)-4-hydroxybut-2-enyl phosphate (HMBP). Although these prodrugs exhibited potent activation of Vy9/Vδ2 T-cell immune responses (EC50 = 0.45-10.62 nM), their stability was low due to the rapid cleavage of the -O-P- bond. To address this, we herein report the application of the same prodrug strategy to two HMBP phosphonates, which have stable -CH2-P- or -CF2-P- bonds. These HMBP phosphonate prodrugs, phosphonamidates, exhibited excellent serum stability and potent activation of Vy9/Vδ2 T-cells making them attractive compounds for further development as potential immunotherapeutics.
INTRODUCTION

Vγ9/Vδ2 T-cells are a key subset of γδ T-cells that is involved in immune responses in many disease contexts, such as tuberculosis, leprosy, typhoid, malaria, and toxoplasmosis.\(^1\) Studies in primate models have also implicated Vγ9/Vδ2 T-cells in immunity to *Mycobacterium tuberculosis*.\(^2\) These cells have been shown to target and lyse a diverse range of cancer cells *in vitro*.\(^1\) Together, these observations have made the Vγ9/Vδ2 subset a major focus of investigation for the therapeutic exploitation of γδ T-cells.\(^3\)

To date, a number of small molecule activators of Vγ9/Vδ2 T-cells have been reported. Among these are the microbially derived phosphoantigen (PAg) (E)-4-hydroxy-3-methylbut-2-enyl pyrophosphate (HMBPP), and the host-derived P-Ag isopentenyl pyrophosphate (IPP) (Figure 1).\(^4\) Additionally, two synthetic molecules, risedronate and zoledronate (Figure 1), which are currently used in the clinic to treat osteoporosis and some types of cancer,\(^5\) are also known to activate Vγ9/Vδ2 T-cells.\(^6\) This activatory effect is due to their ability to inhibit IPP catabolism via farnesyl diphosphate (FPP) synthase, leading to its intracellular accumulation.\(^6\)

![Figure 1. Chemical structure of reported small molecule Vγ9/Vδ2 T-cells activators.](image)

The most potent Vγ9/Vδ2 T-cells activator reported to date is the small molecule PAg HMBPP, EC\(_{50}\) = 0.00051 μM,\(^7\) which activates these T-cells by binding the type-1 transmembrane protein butyrophilin 3A1.\(^8\) Although the binding site for these PAg remained unclear amid conflicting reports as to whether they bind the extracellular or intracellular domains of this transmembrane protein, there is now compelling evidence that supports the notion that HMBPP binds the intracellular B30.2 domain of butyrophilin 3A1.\(^8\)-\(^9\)

Encouraged by the potency of the PAg HMBPP in activating Vγ9/Vδ2 T-cells and given our interest in developing phosphate-containing drugs,\(^10\) we recently reported the application of the aryloxy triester phosphoramidate prodrug technology\(^11\) to the monophosphate derivative of HMBPP, i.e. HMBP, as a means of improving its drug-like properties.\(^12\) In this prodrug approach, the monophosphate or monophosphonate groups are masked by an aryl group or an amino acid ester (Figure 2), which are both enzymatically cleaved off inside cells to release the monophosphate or monophosphonate species.\(^11a\) As these are prodrugs of phosphoantigens, we previously\(^12\) termed them ProPAgens to distinguish them from ProTides\(^11a\), prodrugs of nucleotides. Although the HMBP ProPAgens that we reported previously\(^12\) exhibited potent activation of Vγ9/Vδ2 T-cells (EC\(_{50}\) = 0.45-11 nM), their stability was rather low.\(^12\) The observed instability was shown to be due to the cleavage of the -P-O-
bond of these compounds.\textsuperscript{[12]} To circumvent this problem, we herein report our application of the aryloxy phosphonamidate prodrug technology to two HMBP phosphonates (Figure 2).

![Figure 2. Chemical structures of HMBP ProPAgens (previous work) and the ProPAgens of their phosphonate derivatives (this work) as well as a general indication of their stability and Vγ9/Vδ2 T-cells activation.](image)

**RESULTS AND DISCUSSION**

In the design of HMBP phosphonates, we switched the liable -O-P- to a more stable -CH$_2$-P- or -CF$_2$-P- bond. The introduction of a phosphonate bond (-CH$_2$-P-) instead of a phosphate one (-O-P-) is quite common in drug discovery and has been used with success in addressing stability issues observed with phosphate-containing compounds.\textsuperscript{[13]} A prime example of this are the antiviral drugs cidofovir and adefovir.\textsuperscript{[13]} Although the change from a -O-P- bond to a -CH$_2$-P- bond achieves better stability, the pKa value of the second deprotonation of the phosphonate group (pKa = 7.49) is significantly different from that of the phosphate group (pKa = 6.31) (Figure 3).\textsuperscript{[14]} This as a result affects their full ionization under physiological pH (< 7.4) and hence their binding to the target protein, which requires the full ionization of the phosphate group to bind a positively charged pocket (arginine-rich) on the butyrophilin 3A1 intracellular domain.\textsuperscript{[9d]} To address this, switching the phosphate (-O-P-) bond to a difluoromethyl phosphonate (-CF$_2$-P-) has emerged as a better substitution since these have excellent stability in physiological environments coupled with a pKa value for the second deprotonation (pKa = 6.7) very close to that of the second deprotonation of the native phosphate (Figure 3).\textsuperscript{[14]}
Figure 3. pKa values of phosphate and different phosphonate groups.

With this in mind, we synthesised the aryloxy phosphoramidate prodrugs of both HMBP methylphosphonate and difluoromethyl phosphonate (4a-d and 9a-d, Scheme 1). For the synthesis of the HMBP phosphonate ProPAgens, we used a new synthetic approach, which employs Grubbs olefin metathesis,\textsuperscript{[15]} to access these PAgs prodrugs. The synthesis of ProPAgens 4a-d started by treating the commercially available diethyl 3-butenylphosphonate (1) with bromotrimethylsilane (TMSBr) at room temperature to remove the ethoxy groups.\textsuperscript{[16]} This was followed by chlorination reaction using oxalyl chloride in the presence of a catalytic amount of DMF. The product of this reaction, 2, was subsequently treated with phenol in the presence of triethylamine and then with the appropriate amino acid ester to yield phosphonamidates 3a-d in moderate yields (38-61%). Subsequently, these compounds underwent Grubbs olefin metathesis with 2-methyl-2-propenol employing Hoveyda-Grubbs second generation catalyst in the presence of 1,4-benzoquinone.\textsuperscript{[17]} This gave ProPAgens 4a-d in good yields (57-64%). In terms of the synthesis of ProPAgens 9a-d, the commercially available diethyl (bromodifluoromethyl)phosphonate (5) was reacted with allyl bromide in the presence of zinc and copper bromide in DMF as reported.\textsuperscript{[18]} The generated compound, 6, was subsequently chlorinated and then treated with phenol and the appropriate amino acid ester to yield phosphoramidates 8a-d in moderate yields (24-46%) as described for the synthesis of compounds 3a-d. Subsequently, these phosphonamidates were treated with 2-methyl-2-propenol in the presence of Hoveyda-Grubbs second generation catalyst and catalytic amounts of 1,4-benzoquinone to prevent alkene isomerization.\textsuperscript{[17]} The final ProPAgens 9a-d were generated in good yield (58-69%). For the ProPAgen prodrugs, L-alanine was used as the amino acid of choice in the synthesis of these pro-drugs since it has historically shown the optimum biological activity,\textsuperscript{[11]} while the phenol motif was chosen as it has been used successfully in the discovery of two FDA-approved drugs; sofosbuvir and tenofovir alafenamide.\textsuperscript{[11]} Four different ester motifs have been chosen in the synthesis of ProPAgens 4a-d and 9a-d, methyl (Me), isopropyl (iPr), tert-butyl (tBu) and benzyl (Bn), because these show varying biological activities that vary from low activity (tBu) to high activity (Bn).\textsuperscript{[10, 11, 12]}
Scheme 1. Reagents and conditions: (i) TMSBr, DCM, rt, 2 h then (COCl)$_2$, DMF cat, DCM, rt, 18 h; ii) a. Phenol, Et$_3$N, DCM, -78 °C for 30 mins then rt, 3 h; b. Substituted L-alanine ester hydrochloride, Et$_3$N, DCM, rt, 12 h, yields: 38-61%; (iii) 2-methyl-2-propenol, 1,4-benzoquinone, Hoveyda-Grubbs Catalyst 2nd generation, DCM, rt, yields: 57-64%; (iv) Diethyl (bromodifluoromethyl)phosphonate, DMF, zinc powder, rt, N$_2$, 3 h then CuBr, allyl bromide, rt, 40 h; (v) (i) TMSBr, DCM, rt, 2 h then (COCl)$_2$, DMF cat, DCM, rt, 18 h; ii) a. Phenol, Et$_3$N, DCM, -78 °C for 30 mins then rt, 3 h; b. Substituted L-alanine ester hydrochloride, Et$_3$N, DCM, rt, 12 h, yields: 24-46%; (iii) 2-methyl-2-propenol, 1,4-benzoquinone, Hoveyda-Grubbs Catalyst 2nd generation, DCM, rt, yields: 58-69%.

Since the motive for synthesizing the HMBP phosphonate ProPAgens was to address the poor stability observed with their parent HMBP phosphate ProPAgens,$^{[12]}$ upon the completion of the synthesis of these compounds, we first studied their stability in human serum. As an example, we incubated ProPAgen 4d with human serum at 37 °C for 12 h and monitored the
sample by $^{31}$P-NMR as we reported previously.\textsuperscript{[10a]} As shown in Figure 4, ProPagen 4d had two singlets at $\delta_P = 33.60$ and 34.05 ppm, on the $^{31}$P-NMR corresponding to the two diastereoisomers that arise from the chiral phosphorous center, which is typical of these prodrugs. Following the addition of human serum and monitoring of the sample by $^{31}$P-NMR, there was no degradation observed since no new phosphorous peaks were detected for the period studied (12 h). A similar stability profile was observed for the ProPagen 9a, the difluoromethyl phosphonate (Figure 5). Together, the data indicate the superior stability of these compounds relative to that previously observed with the HMBP phosphate ProPAgens.\textsuperscript{[12]} The observed -CH$_2$-P- and -CF$_2$-P- groups stability profile is in line with what had been observed for difluoromethyl and methyl phosphonates.\textsuperscript{[13]} Beyond the favorable stability of these ProPAgens, they exhibited potent activation (sub-nanomolar EC$_{50}$) of Vy9/V62 T-cells (data not shown).

Figure 4. Stability of HMBP phosphonate ProPagen 4d in human serum at 37 °C for 12 h as monitored by $^{31}$P NMR. Prodrug 4d (5.0 mg) was dissolved in DMSO-d$_6$ (0.10 mL) and D$_2$O (0.15 mL). All $^{31}$P NMR spectra were recorded at 37 °C. Initially, a $^{31}$P NMR scan of prodrug 4d (5.0 mg) in DMSO-d$_6$ (0.10 mL) and D$_2$O (0.15 mL) was recorded (shown as compound 4d alone in the figure). Following this, a previously defrosted human serum (0.30 mL) was added to the NMR tube and spectra were recorded at 30 min after the addition and then at even time intervals over 12 h.
Figure 5. Stability of HMBP phosphonate ProPAgen 9a in human serum at 37 °C for 12 h as monitored by $^{31}$P NMR. Same as for compound 4d above (Figure 4). The $^{31}$P NMR of prodrug 9a shows six phosphorous peaks because of the fluorine-phosphorous coupling. In fact, these are eight $^{31}$P NMR peaks (two doublet of doublets corresponding to each diastereoisomer that arise from the chiral phosphorous center) as predicted and two extra peaks are seen when zoomed into these peaks to make the predicted eight phosphorous peaks of such compounds.

CONCLUSION
A series of HMBP methyl and difluoromethyl phosphonate ProPAgens were synthesised using Olefin Grubbs metathesis, the first report of this approach in synthesizing the HMBP phosphonate core. These ProPAgens exhibited superior serum stability compared to their phosphate ProPAgens derivatives, which we reported previously. Critically, these prodrugs were potent activators Vy9/Vδ2 T-cells (sub-nanomolar EC$_{50}$). The stability and potency profiles of these new HMBP phosphonate ProPAgens make them ideal candidates for further in vitro and in vivo safety and efficacy studies and future development as new immunotherapeutics for treating challenging cancers and infections that can be targeted by Vy9/Vδ2 T cell responses.
EXPERIMENTAL SECTION

General Information. All reagents and solvents were of general purpose or analytical grade and were purchased from Sigma-Aldrich Ltd., Fisher Scientific, Fluorochem or Acros. $^{31}$P, $^1$H and $^{13}$C NMR data were recorded on a Bruker Avance DPX500 spectrometer operating at 202, 500 and 125 MHz. Chemical shifts (δ) are quoted in ppm, and J values are quoted in Hz. In reporting spectral data, the following abbreviations were used: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), td (triplet of doublets), and m (multiplet). All of the reactions were carried out under nitrogen atmosphere and were monitored with analytical thin layer chromatography (TLC) on pre-coated silica plates (kiesel gel 60 F254, BDH). Compounds were visualized by illumination under UV light (254 nm) or by the use of KMnO$_4$ stain followed by heating. Flash column chromatography was performed with silica gel 60 (230–400 mesh) (Merck).

But-3-en-1-ylphosphonic dichloride (2). Trimethylsilyl bromide (13.72 mL, 104.06 mmol, 10 eq.) was slowly added over 30 min to diethylbut-3-en-1-ylphosphonate 1 (2 g, 10.40 mmol, 1 eq.) in CH$_2$Cl$_2$ (50 mL) under nitrogen at room temperature. The mixture was stirred for 2 h followed by the removal of volatiles under reduced pressure to obtain a yellow liquid δ$_p$ NMR (202 MHz, CDCl$_3$): 24.70. This was then dissolved in 50 mL CH$_2$Cl$_2$ and two drops of dry DMF were added and the mixture was cooled to 0 °C. Oxalyl chloride (2.68 mL, 31.20 mmol, 3 eq.) was then added dropwise and the reaction mixture was allowed to warm to room temperature and stirred for 18 h. The volatiles were evaporated and additional CH$_2$Cl$_2$ (10mL) was evaporated three more times to give the crude product (1.79 g, 100 %) as a brown liquid which was used in the next step without further purification. δ$_p$ NMR (202 MHz, CDCl$_3$): 49.66.

General procedure 1. Synthesis of allylphosphonamidates 3a–d. The crude product but-3-en-1-ylphosphonic dichloride (2) was dissolved in 5 mL CH$_2$Cl$_2$ and added dropwise to a solution of phenol (1 eq.), dry Et$_3$N (2 eq.) and CH$_2$Cl$_2$ (10 mL) at -78 °C. After stirring at -78 °C for 30 min, the reaction mixture was allowed to warm to room temperature and stirring was continued for another 3 h. Once the reaction is complete as indicated by $^{31}$P NMR [δ$_p$ NMR (202 MHz, CDCl$_3$): ~ 39.93], the mixture was filtered, and the volatiles were removed under reduced pressure, washed twice with Et$_2$O, which was subsequently removed under reduced pressure to give a crude oil. This product was then dissolved in CH$_2$Cl$_2$ (10 mL) and was added dropwise over 15 min to a stirring mixture of L-alanine ester hydrogen chloride (1 eq.) and dry Et$_3$N (2 eq.) in dry CH$_2$Cl$_2$ (10 mL) under nitrogen at -78 °C. After stirring at -78 °C for 30 mins, the reaction was allowed to warm to room temperature and was left stirring overnight. The solvents were removed under reduced pressure, and the mixture was filtered and washed with Et$_2$O, which was then removed under reduced pressure to give a crude oil. The final products were then purified by column chromatography (6:4 Hex/ EtOAc) as colorless oils.

Methyl (but-3-en-1-yl(phenoxy)phosphoryl)-L-alaninate (3a). Synthesised following general procedure 1 using phenol (0.210 g, 2.23 mmol, 1 eq.) and L-alanine methyl ester hydrogen chloride (0.373 g, 2.23 mmol, 1 eq.) to give product 3a (0.248 g, 38%) as a colorless oil. δ$_p$ NMR (202 MHz, CDCl$_3$): 30.88, 31.22. δ$_H$ NMR (500 MHz, CDCl$_3$): 7.30 (m, 2H, Ar), 7.10 (m, 3H, Ar), 5.82 (m, 1H, CH$_2$=CH), 5.00 (m, 2H, CH$_2$=CH), 4.11-3.86 (m, 1H, CH-NH), 3.60 (d, J = 6.6 Hz,
3H, OCH₃), 3.18 (m, 1H, NH), 2.48-2.27 (m, 2H, =CH-CH₂), 1.92 (m, 2H, CH₂-P), 1.21 (2 d, J = 7.1 Hz, 3H, CH-CH₃). δc NMR (126 MHz, CDCl₃): 174.68 (d, J = 6.3 Hz, C=O), 174.38 (d, J = 5.1 Hz, C=O), 150.72 (d, J = 9.1 Hz), 150.51 (d, J = 9.4 Hz), 129.78, 124.78 (d, J = 5.5 Hz, CH=CH₂), 120.86 (d, J = 4.6 Hz, C-AR), 120.71 (d, J = 4.7 Hz), 115.56, 52.52 (d, J = 3.1 Hz, CH₃-O), 49.58 (d, J = 14.7 Hz, CH-NH), 27.88 (d, J = 130.9 Hz, CH₂-P) 27.60 (d, J = 131.6 Hz, CH₂-P), 26.59 (d, J = 4.1 Hz, CH₂-CH₂-P), 21.68 (2 d, J = 4.3 Hz, CHCH₃).

**Isopropyl (but-3-en-1-yl)(phenoxy)phosphoryl-L-alaninate (3b).** Synthesised following general procedure 1 using phenol (0.210 g, 2.23 mmol, 1 eq.) and L-alanine isopropyl ester hydrogen chloride (0.373 g, 2.23 mmol, 1 eq.) to give product 3b (0.348g, 48%) as a colorless oil. δp NMR (202 MHz, CDCl₃): 30.93, 31.21. δh NMR (500 MHz, CDCl₃): 7.30 (m, 2H, Ar), 7.21 (m, 3H, Ar), 5.81 (m, 1H, CH₂=CH), 5.12 (m, 2H, CH₂=CH), 4.96 (m, 1H, CH=CH₂), 4.04–3.94 (m, 1H, CH-NH), 3.21 (m, 1H, NH), 2.45 (m, 2H, =CH-CH₂), 2.02-1.88 (m, 2H, CH₂-P), 1.29-1.18 (m, 9H, CH₃-CH-NH-CH=CH₂). δc NMR (126 MHz, CDCl₃): 173.67 (d, J = 6.3 Hz, C=O), 150.74 (d, J = 9.1 Hz), 129.76 (d, J = 6.7 Hz), 124.73 (d, J = 5.0 Hz), 120.86 (d, J = 4.6 Hz), 120.69 (d, J = 4.6 Hz), 115.50, 69.23 (d, J = 5.6 Hz, CH=CH₂), 49.75 (d, J = 9.5 Hz, CH-NH), 27.89 (d, J = 130.9 Hz, CH₂-P), 27.54 (d, J = 131.4 Hz, CH₂-P), 26.74 (d, J = 4.3 Hz, CH₂-CH₂-P), 26.57 (d, J = 4.0 Hz, CH₂-CH₂-P), 21.58 (2 d, J = 4.4 Hz, CHCH₃).

**tert-Butyl (but-3-en-1-yl)(phenoxy)phosphoryl-L-alaninate (3c).** Synthesised following general procedure 1 using phenol (0.210 g, 2.23 mmol, 1 eq.) and L-alanine tert-butyl ester hydrogen chloride (0.405 g, 2.23 mmol, 1 eq.) to give product 3c (0.461g, 61%) as a colorless oil. δp NMR (202 MHz, CDCl₃): 30.95, 31.21. δh NMR (500 MHz, CDCl₃): 7.32 (m, 2H, Ar), 7.20 (m, 3H, Ar), 5.87 (m, 1H, CH₂=CH), 5.12(m, 2H, CH₂=CH), 4.00-3.86 (m, 1H, CH-NH), 3.26 (m, 1H, NH), 2.45 (m, 2H, =CH-CH₂), 2.04-1.85 (m, 2H, P-CH₂), 1.42 (s, 9H, tBu-H), 1.22 (2 d, J = 7.2 Hz, 3H, CH-CH₃). δc NMR (126 MHz, CDCl₃): 173.13 (d, J = 5.5 Hz, C=O), 150.77 (d, J = 9.2 Hz), 137.43 (d, J = 7.1 Hz), 137.32 (d, J = 7.6 Hz), 129.76 (d, J = 6.2 Hz), 124.71 (d, J = 4.0 Hz), 120.88 (d, J = 4.6 Hz), 120.72 (d, J = 4.7 Hz), 115.47, 81.99 (d, J = 8.2 Hz), 60.54, 50.18 (d, J = 4.0 Hz, CH-NH), 28.05, 27.91 (d, J = 131.6 Hz, CH₂-P), 27.20 (d, J = 130.2 Hz, CH₂-P), 21.82 (2 d, J = 4.2 Hz, CHCH₃).

**Benzyl (but-3-en-1-yl)(phenoxy)phosphoryl-L-alaninate (3d).** Synthesised following general procedure 1 using phenol (0.210 g, 2.23 mmol, 1 eq.) and L-alanine benzyl ester hydrogen chloride (0.405 g, 2.23 mmol, 1 eq.) to give product 3d (0.461 g, 55 %) as a colorless oil. δp NMR (202 MHz, CDCl₃): 30.85, 31.21. δh NMR (500 MHz, CDCl₃): 7.38-7.27 (m, 7H, Ar), 7.21-7.18 (m, 2H, Ar), 7.16-7.09 (m, 1H, Ar), 5.85 (m, 1H, CH₂=CH), 5.04 (m, 4H, CH₂=CH, -OCH₂), 4.28-4.03 (m, 1H, CH-NH), 3.24 (m, 1H, NH), 2.56-2.34 (m, 2H, =CH-CH₂), 2.08-1.82 (m, 2H, P-CH₂), 1.24 (2 d, J = 7.1 Hz, 3H, CH-CH₃). δc NMR (126 MHz, CDCl₃): 173.74 (d, J = 5.2 Hz, C=O), 150.70 (d, J = 9.1 Hz), 137.35, 135.40 (d, J = 6.7 Hz), 129.79 (d, J = 6.1 Hz), 128.79 (d, J = 2.8 Hz), 128.64 (d, J = 6.4 Hz), 128.33, 120.86 (d, J = 4.6 Hz), 120.68 (d, J = 4.7 Hz), 115.55, 67.31 (d, J = 3.0 Hz, CH₂-O), 49.72 (d, J = 11.1 Hz, CH-NH), 27.88 (d, J = 130.7 Hz, CH₂-P), 27.57(d, J = 131.3 Hz, CH₂-P), 26.74 (d, J = 4.3 Hz, CH₂-CH₂-P), 26.57 (d, J = 4.1 Hz, CH₂-CH₂-P), 21.70 (2 d, J = 4.4 Hz, CHCH₃).
General procedure 2. Synthesis of phosphonoamidates 4a-d through Hoveyda-Grubbs cross metathesis. To a solution of allylphosphonoamidates 3a-d (1 eq.) and 2-methyl-2-propen-1-ol (85 µL, 1 mmol, 2 eq.), 1,4-benzoquinone (5.40 mg, 10mol%) in dry DCM (10 mL) was added Hoveyda-Grubbs catalyst 2nd generation (23.5 mg, 0.038 mmol, 7.5 mol%). The catalyst was added in three equal portions of 2.5 mol% (7.8 mg, 0.013 mmol) at t = 0, 2 and 4 h over the course of the reaction. The solution was then heated to reflux at 45 °C under nitrogen atmosphere for 18 h. After cooling to room temperature, a scoop of activated carbon was added, and the mixture stirred for another 2 hr then filtered through a Celite pad. Volatiles were evaporated and the residue was purified by extensive silica gel column chromatography (Hexane/EtOAc, 7:3 to 0:1) to give 4a-d as colorless oils.

**Methyl ((E)-5-hydroxy-4-methylpent-3-en-1-yl)(phenoxy)phosphoryl)-L-alaninate (4a).**

Synthesised following general procedure 2 using 3a (150 mg, 0.5 mmol, 1 eq.) to give 4a (91 mg, 57 %) as a colorless oil. $\delta^p$ NMR (202 MHz, CDCl$_3$): 30.89, 31.31. $\delta^h$ NMR (500 MHz, CDCl$_3$): 7.31 (m, 2H, Ar), 7.18 (m, 3H, Ar), 5.48 (m, 1H, =CH), 4.24 – 3.98 (m, 3H, CH$_2$OH, CH-NH), 3.68 (d, J = 7.9 Hz, 3H, OCH$_3$), 3.39-3.18 (m, 1H, NH), 2.55-2.41 (m, 2H, =CH-CH$_2$), 2.12-1.87 (m, 2H, CH$_2$-P), 1.71 (d, J = 6.6 Hz, 3H, CH$_3$(CH$_2$)=), 1.27 (2 x d, J = 7.1 Hz, 3H, CH-CH$_2$). $\delta^c$ NMR (126 MHz, CDCl$_3$): 176.71 (d, J = 5.9 Hz, C=O), 150.72 , 129.81 (d, J = 6.9 Hz), 124.81 (d, J = 8.6 Hz, CH=CH$_2$), 124.17, 124.04, 120.94, 120.69 (d, J = 4.6 Hz), 68.56 (d, J = 9.1 Hz, CH$_2$-OH), 52.64 (d, J = 3.5 Hz, CH$_3$-O), 49.51 (d, J = 4.1 Hz, CH-NH), 28.33 (d, J = 129.6 Hz CH$_2$-P), 28.06 (d, J = 130.2 Hz, CH$_2$-P ), 21.86 (2 d, J = 4.9 Hz, CHCH$_3$), 21.00 (d, J = 4.8 Hz, CH$_2$-CH$_2$-P), 20.89 (d, J = 4.4 Hz, CH$_2$-CH$_2$-P). HRMS (ES+, m/z) calcd. for (M+Na)+ C$_{15}$H$_{24}$NaO$_5$P: 364.1290; found: 364.1293.

**Isopropyl ((E)-5-hydroxy-4-methylpent-3-en-1-yl)(phenoxy)phosphoryl)-L-alaninate (4b).**

Synthesised following general procedure 2 using 3b (0.150 g, 0.46 mmol, 1 eq.) to give product 4b (97 mg, 59%) as a colorless oil. $\delta^p$ NMR (202 MHz, CDCl$_3$): 31.11, 31.49. $\delta^h$ NMR (500 MHz, CDCl$_3$): 7.28 (m, 2H, Ar), 7.20 (m, 3H, Ar), 5.46 (m, 1H, =CH), 4.95 (m, 1H, CH-iPr), 4.08 – 3.79 (m, 3H, CH$_2$OH, CH-NH), 3.47-3.25 (m, 1H, NH), 2.51-2.35 (m, 2H, =CH-CH$_2$), 2.02-1.85 (m, 2H, CH$_2$-P), 1.73-1.56 (d, J = 6.0 Hz, 3H, CH$_3$(CH$_2$)=), 1.35-1.08 (m, 9H, CH$_3$-CH-NH, CH-iPr). $\delta^c$ NMR (126 MHz, CDCl$_3$): 173.62 (d, J = 5.8 Hz, C=O), 150.74 (d, J = 9.0 Hz), 136.76, 124.75, 123.95 (d, J = 5.4 Hz), 123.84 (d, J = 6.9 Hz), 120.90 (d, J = 4.5 Hz), 120.67 (d, J = 4.6 Hz), 119.83, 115.58, 69.41 (d, J = 4.7 Hz, CH-iPr) 68.39 (d, J = 7.0 Hz, CH$_2$-OH), 49.65, 28.36 (d, J = 129.7 Hz, CH$_2$-P), 27.92 (d, J = 131.1 Hz, CH$_2$-P), 21.83 (2 d, J = 6.2 Hz, CHCH$_3$), 20.90 (d, J = 4.4 Hz, CH$_2$-CH$_2$-P), 20.84 (d, J = 4.4 Hz, CH$_2$-CH$_2$-P). HRMS (ES+, m/z) calcd. for (M+Na)+ C$_{18}$H$_{26}$NO$_5$NaP: 392.1603; found: 392.1613.

**t-Butyl ((E)-5-hydroxy-4-methylpent-3-en-1-yl)(phenoxy)phosphoryl)-L-alaninate (4c).**

Synthesised following general procedure 2 using 3c (0.150 g, 0.44 mmol, 1 eq.) to give product 4c (108 mg, 64 %) as a colorless oil. $\delta^p$ NMR (202 MHz, CDCl$_3$): 31.05, 31.42. $\delta^h$ NMR (500 MHz, CDCl$_3$): 7.30 (m, 2H, Ar), 7.17 (m, 3H,Ar), 5.46 (m, 1H, =CH), 4.17-3.78 (m, 3H, CH$_2$OH, CH-NH), 3.41-3.18 (m, 1H, NH), 2.60-2.30 (m, 2H, =CH-CH$_2$), 2.01-1.87 (m, 2H, CH$_2$-P), 1.69 (d, J = 6.8 Hz, 3H, CH$_3$(CH$_2$)=), 1.42 (s, 9H, tBu), 1.27 (2 d, J = 7.9 Hz, 3H, CH-CH$_3$). $\delta^c$ NMR (126 MHz, CDCl$_3$): 173.34 (d, J = 6.0 Hz, C=O), 150.79 (d, J = 9.0 Hz), 136.81 (d, J = 9.6 Hz), 115.59,
82.22 (d, J = 5.6 Hz), 68.45 (d, J = 10.4 Hz, CH₂-OH), 50.07 (d, J = 4.5 Hz, CH-NH), 28.01 (d, J = 129.7 Hz, CH₂-P), 27.26 (d, J = 130.4 Hz, CH₂-P), 21.98 (d, J = 3.8 Hz, CH₂CH₃), 20.97 (d, J = 4.7 Hz, CH₂-CH₂-P), 20.88 (d, J = 4.4 Hz, CH₂-CH₂-P), 13.86 (CH₃). HRMS (ES+, m/z) calcd. for (M+Na)+ C₁₉H₃₉NO₅NaP: 406.1759; found: 406.1762.

**Benzyl ((E)-5-hydroxy-4-methylpent-3-en-1-yl)(phenoxo)phosphoryl)-L-alaninate (4d).**

Synthesised following general procedure 2 using 3d (0.150 g, 0.4 mmol, 1 eq.) to give product 4d (100 mg, 59 %) as a colorless oil. δₓ NMR (202 MHz, CDCl₃): 30.90, 31.32, δₓ NMR (500 MHz, CDCl₃): 7.39-7.28 (m, 7H, Ar), 7.22-7.17 (m, 2H, Ar), 7.12 (m, 1H, Ar), 5.44 (m, 1H, =CH), 5.09 (m, 2H, OCH₃), 4.22-3.86 (m, 3H, m, 3H, CH₂OH, CH-NH), 3.49-3.17 (m, 1H, NH), 2.64 - 2.36 (m, 2H, =CH-CH₂), 2.04-1.80 (m, 2H, CH₂-P), 1.69 (d, J = 6.8 Hz, 3H, CH₃(CH₂)C=), 1.33 (2 d, J = 7.0 Hz, 3H, CH₃-CH₂), δc NMR (126 MHz, CDCl₃): 176.76, 129.81 (d, J = 7.4 Hz), 128.75 (d, J = 14.9 Hz), 128.39, 124.77, 124.15 (d, J = 15.0 Hz), 120.93-120.84, 120.67 (d, J = 4.7 Hz), 68.57 (d, J = 9.6 Hz), 67.42 (d, J = 2.9 Hz), 49.65, 28.05 (d, J = 129.9 Hz), 21.77 (d, J = 4.1 Hz), 20.87 (d, J = 4.4 Hz), 13.87. HRMS (ES+, m/z) calcd. for (M+Na)+ C₂₂H₂₈N₂O₅NaP: 440.1603; found: 440.1609.

**Diethyl (1,1-difluorobut-3-en-1-yl)phosphonate (6).**[18] Anhydrous DMF (20 mL) was added to a 250 mL round bottom flask containing activated zinc powder (2.50 g, 38.23 mmol, 1 eq.) under nitrogen. This was followed by slow dropwise addition of diethyl (bromodifluoromethyl)phosphonate (6.80 mL, 38.23 mmol, 1 eq.) and the mixture was stirred for 3 h at room temperature. CuBr (5.48 g, 38.23 mmol, 1 eq.) was added followed by slow dropwise addition of allyl bromide (3.96 mL, 45.87 mmol, 1.2 eq.) to prevent exothermic reaction. After stirring for 40 h, the mixture was filtered and then partitioned between DCM and 10% aqueous NH₄Cl. The aqueous phase was extracted three times with DCM. The combined organic phases were dried over anhydrous MgSO₄ and concentrated under reduced pressure and the obtained residue was purified by column chromatography using 20% EtOAc in hexane to give 6 (5.41 g, 62 %) as a pale-yellow oil. δₓ NMR (202 MHz, CDCl₃): 6.93 (t, J = 107.4 Hz). δₓ NMR (500 MHz, CDCl₃): 5.84 (m, 1H, =CH), 5.26 (m, 2H, CH₂-en), 4.32-4.21 (m, 4H, 2 x OCH₂CH₃), 2.82 (m, 2H, =CH-CH₂), 1.37 (t, J = 7.1 Hz, 6H, 2 x OCH₂CH₃).

**1,1-Difluorobut-3-en-1-ylphosphonic dichloride (7).** Synthesised as described for 2 using 6 (2.5 g, 10.95 mmol, 1 eq.) to give the crude product 7 (2.28 g, 100 %) as a brown liquid which was used in the next step without further purification. δₓ NMR (202 MHz, CDCl₃): 31.56 (t, J = 138.8 Hz).

**Methyl ((1,1-difluorobut-3-en-1-yl)(phenoxo)phosphoryl)-L-alaninate (8a).** Synthesised following general procedure 1 using phenol (0.210 g, 2.23 mmol, 1 eq.) and L-alanine methyl ester hydrogen chloride (0.261 g, 1.87 mmol, 1 eq.) to give product 8a (0.150 g, 24 %) as a colorless oil. δₓ NMR (202 MHz, CDCl₃): 9.05 (dd, J = 101.1, 49.9), 8.41 (dd, J = 100.0, 51.1 Hz). δₓ NMR (500 MHz, CDCl₃): 7.35 (m, 2H, Ar), 7.21 (m, 3H, Ar), 5.89 (m, 1H, CH₂=CH), 5.29 (m, CH₂=CH), 4.14 (m, 1H, CH-NH), 3.69 (d, J = 6.6 Hz, 3H, OCH₃),3.64 (m, 1H, NH), 3.13-2.84 (m, 2H, =CH-CH₂), 1.38 (2 x d, 7.1 Hz, 3H, CH-CH ). δc NMR (126 MHz, CDCl₃): 173.76 (d, J = 4.1 Hz, C=O), 130.02 (d, J = 4.1 Hz), 127.40-127.07 (m), 125.64, 121.54 (d, J = 9.3 Hz), 120.54 (t, J = 4.8 Hz), 52.69, 50.09 (d, J = 6.6 Hz), 39.39-37.22 (m), 21.74(2d, J = 3.5 Hz, CH₃).
Isopropyl 1,1-difluorobut-3-en-1-yl)(phenoxy)phosphoryl-L-alaninate (8b). Synthesised following general procedure 1 using phenol (0.210 g, 2.23 mmol, 1 eq.) and L-alanine isopropyl ester hydrogen chloride (0.314 g, 1.87 mmol, 1 eq.) to give product 8b (0.165 g, 25 %) as a colorless oil. \( \delta \) NMR (202 MHz, CDCl3): 9.17 (dd, \( J = 101.1, 30.3 \) Hz), 8.46 (dd, \( J = 99.9, 38.0 \) Hz). \( \delta \)H NMR (500 MHz, CDCl3): 7.34 (m, 2H, Ar), 7.20 (m, 3H, Ar), 5.89 (m, 1H, CH2=CH), 5.29 (m, 2H, CH2=CH), 4.99 (m, 1H, CH-iPr), 4.14-3.99 (m, 1H, CH-NH), 3.68 (m, 1H, NH), 3.00-2.89 (m, 2H, =CH-CH2), 1.35-1.17 (m, 9H, CH3-CH-NH, CH-iPr). \( \delta \)C NMR (126 MHz, CDCl3): 172.68 (d, \( J = 5.9 \) Hz, C=O), 149.46, 129.88 (d, \( J = 4.2 \) Hz), 127.11 (d, \( J = 5.4 \) Hz), 125.46, 121.36 (d, \( J = 8.9 \) Hz), 120.40 (t, \( J = 4.9 \) Hz), 119.69 (3H, CH3), 50.15, 38.63-37.99 (m), 21.58 (d, \( J = 1.6 \) Hz), 21.44 (2 d, \( J = 3.2 \) Hz, CHCH3).

tert-Butyl 1,1-difluorobut-3-en-1-yl)(phenoxy)phosphoryl-L-alaninate (8c). Synthesised following general procedure 1 using phenol (0.210 g, 2.23 mmol, 1 eq.) and L-alanine tertbutyl ester hydrogen chloride (0.340 g, 1.87 mmol, 1 eq.) to give product 8c (0.320 g, 46%) as a colorless oil. \( \delta \)F NMR (202 MHz, CDCl3): 9.23 (dd, \( J = 101.0, 34.4 \) Hz), 8.52 (dd, \( J = 99.6, 36.0 \) Hz). \( \delta \)H NMR (500 MHz, CDCl3): 7.34 (m, 2H, Ar), 7.21 (m, 3H, Ar), 5.89 (m, 1H, CH2=CH), 5.28 (m, 2H, CH2=CH), 4.08-3.98 (m, 1H, CH-NH), 3.67 (m, 1H, NH), 3.01-2.84 (m, 2H, =CH-CH2), 1.42 (d, \( J = 5.2 \) Hz,9H, tBu), 1.30 (d, \( J = 7.2 \) Hz, 3H, CH3-CH3). \( \delta \)C NMR (126 MHz, CDCl3): 172.48 (d, \( J = 4.5 \) Hz, C=O), 149.72, 129.99 (d, \( J = 2.1 \) Hz), 128.59-126.95 (m), 125.54, 121.46 (d, \( J = 9.2 \) Hz), 120.54 (t, \( J = 4.8 \) Hz), 82.32 (d, \( J = 5.8 \) Hz), 50.69 (d, \( J = 2.8 \) Hz, CH-NH), 38.80-38.12 (m), 27.99, 21.85 (2 d, \( J = 3.1 \) Hz, CH3).

Benzy1 1,1-difluorobut-3-en-1-yl)(phenoxy)phosphoryl-L-alaninate (8d). Synthesised following general procedure 1 using phenol (0.210 g, 2.23 mmol, 1 eq.) and L-alanine benzy1 ester hydrogen chloride (0.403 g, 1.87 mmol, 1 eq.) to give product 8d (0.350 g, 45% yield) as a colorless oil. \( \delta \)F NMR (202 MHz, CDCl3): 9.08 (dd, \( J = 101.2, 45.4 \) Hz), 8.39 (dd, \( J = 100.1, 46.5 \) Hz). \( \delta \)H NMR (500 MHz, CDCl3): 7.41-7.29 (m, 7H, Ar), 7.25-7.17 (m, 3H, Ar), 5.87 (m, 1H, CH2=CH), 5.27 (m, 2H, CH2=CH), 5.11 (m, 2H, -OCH2), 4.33-4.09 (m, 1H, CH-NH), 3.68 (m, 1H, NH), 2.93 (m, 2H, -CH2), 1.36 (d, \( J = 7.2 \) Hz, 3H, CH3-CH3). \( \delta \)C NMR (126 MHz, CDCl3): 173.15 (d, \( J = 3.2 \) Hz, C=O), 149.68, 135.26 (d, \( J = 6.4 \) Hz), 130.01 (d, \( J = 4.1 \) Hz), 128.87-128.52 (m), 128.35 (d, \( J = 1.1 \) Hz), 127.38-127.03 (m), 125.62, 122.24-120.38 (m), 120.51, 67.49, 50.20, 38.74-38.07 (m), 21.96 (2 d, \( J = 3.5 \) Hz, CH3-CH3).

Methyl (((E)-1,1-difluoro-5-hydroxy-4-methylpent-3-en-1-yl)(phenoxy)phosphoryl)-L-alaninate (9a). Synthesised following general procedure 2 using 8a (0.150 g, 0.45 mmol, 1 eq.) to give product 9a (97 mg, 58 %) as a colorless oil. \( \delta \)F NMR (202 MHz, CDCl3) \( \delta \) 9.27 (dd, \( J = 112.1, 18.4 \) Hz), 8.44 (dd, \( J = 126.4, 20.2 \) Hz). \( \delta \)H NMR (500 MHz, CDCl3): 7.35 (m, 2H, Ar), 7.21 (m, 3H, Ar), 5.56 (m, 1H, =CH), 4.18 (m, 1H, CH-NH), 4.07 (m, 2H, =CH-CH2), 3.75-3.46 (m, 4H, NH, OCH3), 3.10-2.79 (m, 2H, =CH-CH2), 1.83 (m,1H, OH), 1.76 (s, 3H, CH3(CH2)2=CH), 1.37 (2 x d, \( J = 7.1 \) Hz, 3H, CH3-CH3). \( \delta \)C NMR (126 MHz, CDCl3): 176.76, 141.38 (d, \( J = 7.6 \) Hz), 130.04 (d, \( J = 3.2 \) Hz), 125.65 (d, \( J = 4.0 \) Hz), 120.56 (d, \( J = 4.6 \) Hz), 120.47 (d, \( J = 4.7 \) Hz), 68.25 (d, \( J = 1.9 \) Hz), 52.80 (CH2-O), 50.09 (d, \( J = 4.1 \) Hz, CH-NH), 32.82, 21.82 (2 d, \( J = 2.6 \) Hz, CHCH3), 14.15 (CH3). HRMS (ES+, m/z) calcd. for (M+Na)+ C16H22F2NO5NaP: 400.1101; found: 400.1109.
Isopropyl (([(E)-1,1-difluoro-5-hydroxy-4-methylpent-3-en-1-yl](phenoxy)phosphoryl)-L-alaninate (9b). Synthesised following general procedure 2 using 8b (0.150 g, 0.41 mmol, 1 eq.) to give product 9b (117 mg, 69 %) as a colorless oil. δº NMR (202 MHz, CDCl₃): 9.30 (dd, J = 109.1, 34.4 Hz), 8.42 (dd, J = 111.1, 38.4 Hz). δ₇ NMR (500 MHz, CDCl₃): 7.35 (m, 2H, Ar), 7.21 (m, 3H, Ar), 5.56 (m, 1H, =CH), 5.00 (m, 1H, CH-iPr), 4.17-3.91 (m, 3H, CH-NH, =CH-CH₂), 3.67 (m, 1H, NH), 3.08-2.75 (m, 2H, =CH-CH₂), 1.86 (m, 1H, OH), 1.70 (s, 3H, CH₃(CH₂)C=CH), 1.34-1.19 (m, 9H, CH₃-CH-NH, CH-iPr). δε NMR (126 MHz, CDCl₃): 173.09 (d, J = 6.8 Hz, C=O), 141.37 (d, J = 7.9 Hz), 130.03 (d, J = 2.6 Hz), 125.60 (d, J = 4.4 Hz), 120.50 (dd, J = 10.6, 4.7 Hz), 113.79 – 113.43 (m), 69.71 (d, J = 4.5 Hz), 68.25, 50.29 (d, J = 9.6 Hz), 30.88-28.72 (m), 21.73 (d, J = 8.2 Hz), 21.84 (2d, J = 4.1 Hz, CHCH₃), 14.18 (CH₃). HRMS (ES+, m/z) calcd. for (M+Na)+ C₁₉H₂₆F₂NO₅NaP: 428.1414; found: 428.1414.

tert-Butyl (([(E)-1,1-difluoro-5-hydroxy-4-methylpent-3-en-1-yl](phenoxy)phosphoryl)-L-alaninate (9c). Synthesised following general procedure 2 using 8c (0.150 g, 0.39 mmol, 1 eq.) to give product 9c (105 mg, 63 %) as a colorless oil. δº NMR (202 MHz, CDCl₃): 9.36 (dd, J = 109.1, 34.4 Hz), 8.48 (dd, J = 111.1, 36.4 Hz). δ₇ NMR (500 MHz, CDCl₃): 7.36 (m, 2H, Ar), 7.22 (m, 3H, Ar), 5.56 (m, 1H, =CH), 4.16-3.93 (m, 3H, CH-NH, =CH-CH₂), 3.63 (m, 1H, NH), 3.08-2.77 (m, 2H, =CH-CH₂), 1.90 (m, 1H, OH), 1.71 (s, 3H, CH₃(CH₂)C=CH), 1.43 (d, J = 5.2 Hz, 9H, t Bu), 1.30 (2 x d, J = 7.1 Hz, 3H, CH-CH₃). δε NMR (126 MHz, CDCl₃): 172.79 (d, J = 6.8 Hz, C=O), 130.02, 125.56 (d, J = 5.8 Hz), 120.55 (d, J = 4.6 Hz), 120.47 (d, J = 4.4 Hz), 82.59, 68.26, 50.72 (d, J = 7.4 Hz, CH-NH), 32.97-32.80 (m), 28.02 (d, J = 1.1 Hz, 3 x CH₃), 21.98 (2 d, J = 3.8 Hz, CHCH₃), 14.16. HRMS (ES+, m/z) calcd. for (M+Na)+ C₁₉H₂₆F₂NO₅NaP: 442.1571; found: 442.1578.

Benzyl (([(E)-1,1-difluoro-5-hydroxy-4-methylpent-3-en-1-yl](phenoxy)phosphoryl)-L-alaninate (9d). Synthesised following general procedure 2 using 8d (0.150 g, 0.36 mmol, 1 eq.) to give product 9d (101mg, 61 %) as a colorless oil. δº NMR (202 MHz, CDCl₃): 9.21 (dd, J = 113.2, 22.3 Hz), 8.37 (dd, J = 111.1, 24.3 Hz). δ₇ NMR (500 MHz, CDCl₃): 7.39-7.28 (m, 7H, Ar), 7.24-7.16 (m, 3H, Ar), 5.55 (m,1H, =CH), 5.13 (m, 2H, OCH₂), 4.27-4.14 (m, 1H, CH-NH), 4.06 (m, 2H, =CH-CH₂), 3.68 (m, 1H, NH), 3.05-2.73 (m, 2H, =CH-CH₂), 1.80 (m, 1H, OH), 1.69 (s, 3H, CH₃(CH₂)C=CH), 1.35 (2 x d, J = 7.1 Hz, 3H, CH-CH₃). δε (126 MHz, CDCl₃): 173.38 (d, J = 7.9 Hz, C=O), 149.71, 141.37 (d, J = 8.3 Hz), 130.04 (d, J = 3.1 Hz), 128.76 (d, J = 12.8 Hz), 128.35 (s), 125.63 (d, J = 5.5 Hz), 120.49 (dd, J = 13.7, 4.6 Hz), 113.75-113.34 (m), 68.25 (d, J = 2.4 Hz), 76.61, 50.22 (d, J = 8.0 Hz), 34.32-31.18 (m), 21.84 (d, J = 3.8 Hz, CHCH₃), 14.15 (CH₃). HRMS (ES+, m/z) calcd. for (M+Na)+ C₂₂H₂₆F₂NO₅NaP: 476.1414; found: 476.1421.

Author Contributions
H.K. and Q.X. synthesized all of the reported compounds. T.E.T., R.T.B., and B.E.W. designed and carried out the biological testing of the prodrugs. B.E.W. and Y.M. supervised the experiments. Y.M. wrote the manuscript, and all of the authors gave approval to the final version of the manuscript.
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**Notes**
The authors declare no competing financial interest.

**ABBREVIATIONS**
HMBP, (E)-4- hydroxy-3-methylbut-2-enyl monophosphate; HMBPP, (E)-4- hydroxy-3-methylbut-2-enyl pyrophosphate; IPP, isopentenyl pyrophosphate; iPr, isopropyl; Me, methyl; PAg, phosphoantigen; PBMC, peripheral blood mononuclear cell; ProPAgen, prodrug of a phosphoantigen; tBu, tert-butyl.

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