Thiopyrophosphoantigens: Solid-phase Synthesis and in Vitro Characterization of a New Class of Vγ9 Vδ2 T Cells Activators

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The Vγ9 Vδ2 T cells mediate rapid, innate-like immune responses to pathogens and are important in several key immunoregulatory pathways, including those involved in infections and tumor development. Vγ9 Vδ2 T cells respond to low molecular weight isoprenoid phosphoantigens; the prototypic stimulatory compound is isopentenylpyrophosphate (IPP), an alkylphosphate intermediate of mevalonate metabolism that elicits proliferative, cytotoxic, and cytokine secretion responses. We studied the replacement of the pyrophosphate moiety with the thiopyrophosphate bioisostere, synthesizing thioanalogues of IPP and 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP, the most potent natural antigen known to date). Once their in vitro efficacy and stability had been demonstrated, we synthesized a small library of compounds through the development of an innovative solid-phase strategy. Biological results confirmed thioHMBPP to be the best compound of this first series. Future aims are (i) the exploitation of the parallel solid-phase strategy to further explore structure−activity relationships of this new class of synthetic antigens and (ii) the determination of the PK/PD profile of thioHMBPP.

Introduction

Basic research contributions toward the molecular and cellular understanding of immune cells have created opportunities to manipulate the immune system for new therapies against pathogens and tumors. Immunomodulation is one of the major aims of modern pharmacological research for the large number of diseases compromising the homeostasis of immune defenses.

Human T cells carrying the γδ T cell receptor account for 2−5% of CD3+ T cells in the peripheral blood. γδ T cells constitute an system of functionally specialized subsets that is implicated in the innate responses against tumors and pathogens, in the regulation of immune responses, including cell recruitment and activation, and in the antigen presentation.[1,2]

In most healthy individuals, T cells expressing Vδ2 gene paired with one particular Vγ chain (Vγ9) account for 50−90% of the γδ T cell pool. Indeed, Vγ9 Vδ2 T cells are the major population of γδ T cells in humans constituting several percents of CD3+ cells in adults, probably due to the consequence of their postnatal peripheral expansion. The highly restricted T cell receptor V region repertoire of γδ T cells is certainly one of the most salient features distinguishing these lymphocytes from conventional MHC restricted αβ T cells. Vγ9 Vδ2 T cells display broad reactivity against tumors and infectious agents as they are able to recognize both microbial and endogenous metabolites whose production is up-regulated during cell stress.[3,4]

Vγ9 Vδ2 T cell antigens (i.e., phosphoantigens) are ubiquitous in plants, bacteria, and eukaryotes, and they are recognized in a TCR[a] (T cell receptor)-dependent manner.[5,6] Several natural and synthetic phosphoantigens have been described in literature (Figure 1).[7] The most potent antigen appears to be the 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), an intermediate of 1-deoxy-D-xylulose-5-phosphate pathway, restricted to plant cells and some microrganisms.[8]

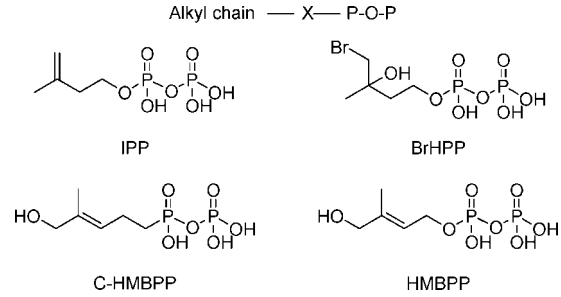

Figure 1. Structures of natural and synthetic phosphoantigens.

Metabolic intermediates such as isopentenyl pyrophosphate (IPP) can activate Vγ9 Vδ2 T cells at concentrations 100000-fold higher than those of microbial agonists.[9] IPP derives from the mevalonate pathway (MVA) used by mammalian cells and some bacteria and is essential for sterol synthesis, cell growth, and membrane integrity. Because IPP production has been found to be up-regulated in most tumor cells, its recognition by Vγ9 Vδ2 T cells allows the discrimination not only between infected and uninfected cells but also between normal and transformed cells.[10]

The first clinical evidence for in vivo manipulation of human Vγ9 Vδ2 T lymphocytes came from aminobisphosphonates

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[a] Abbreviations: TCR, T cell receptor; HMBPP, 4-hydroxy-3-methyl-but-2-enyl pyrophosphate; IPP, isopentenyl pyrophosphate; MVA, mevalonate pathway; ABP, aminobisphosphonates; IL-2, interleukin-2; BrHPP, bromohydrin pyrophosphate; SAR, structure-activity relationship PBMCs, peripheral blood mononuclear cells.

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(ABPs): in vivo γδ T cells stimulation by pamidronate and low dose IL-2 induced antitumor response in lymphoid malignancies.[11] ABPs have been shown to activate Vγ9 Vδ2 T cells through their ability to inhibit farnesyl pyrophosphate synthase, an enzyme acting downstream of IPP synthesis on the MVA pathway, promoting intracellular accumulation of IPP.[12] However, ABPs work at the micromolar range, rapidly binding to bone tissue with a very long half-life and blocking the mevalonate pathway with undesired effects.[13] These observations guided the development of novel synthetic phosphoantigens able to selectively stimulate Vγ9 Vδ2 T cells. Encouraging results have been achieved through the synthesis of BrHPP (bromohydrin pyrophosphate, *Phosphostim*[6]), which shows a systematic Vγ9 Vδ2 T cell expansion in vivo in combination with a low dose of IL-2.[14] Adoptive transfer of BrHPP-expanded Vγ9 Vδ2 T cells is currently in phase II for clinical evaluation in renal cell carcinoma, non-Hodgkin lymphoma, and myeloid chronic leukemia.[15,16] Nevertheless, the usage of this class of compounds shows serious drawbacks from a therapeutic perspective because of its rapid dephosphorylation under physiological conditions.[14]

Since the stability of these compounds is crucial for their clinical application in immunotherapy, the identification of synthetic phosphoantigens endowed with a suitable pharmacological profile and high stimulating potency is one of the most attractive goals of Vγ9 Vδ2-targeted immunotherapy.

Rationale. Structure−activity relationships (SARs) of different synthetic phosphoantigens suggest the critical role of two main moieties: the alkenyl chain and the pyrophosphoric group. The alkenyl chain associated with the pyrophosphate group may have from 3 to 5 carbon atoms and a double bond in position 2 or 3.[7,17] An increase in Vγ9 Vδ2 activating potency has been shown with the addition of a halohydrin in C3 position or with the replacement of a C3−C4 double bond with a carbonyl group in the C4 position.[18]

Investigations on the pyrophosphates moiety highlighted (i) the need for a hydrolyzable phosphodiester bond and (ii) the importance of the C−O−P sequence. Substitution of the P−O−P sequence with P−CH_2−P drastically reduces the activity,[19] and C−O−P replacement with the C−C−P sequence leads to synthetic compounds with an increased mean lifetime.[20,21]

We initially decided to focus on the C−O−P sequence to try to overcome the phosphoantigens limited stability.

Thiopyrophosphate analogues of isopentenyl pyrophosphate and its derivatives (geranyl, farnesyl pyrophosphates) are well-known inhibitor/substrates for the enzyme farnesyl/geranyl/undecaprenyl pyrophosphate synthetase.[22−24] For example, the conversion of geranyl pyrophosphate into the corresponding thiopyrophosphate transforms the natural substrate into an inhibitor of farnesyl pyrophosphate synthetase; the improved bond stability determines this behavior.[23−25] Thus we planned to incorporate the C−S−P−O−P sequence as bioisosteric replacement for the pyrophosphate (C−O−P−O−P) with the aim of improving the stability.

In particular, we designed and synthesized a new class of thioanalogues (patent WO2007/099117A1) to investigate (i) the efficacy and stability of the new isosteric group C−S−P−O−P and (ii) the effect of IPP alkyl chain replacement with alternatives.

Thioanalogues of the reference compound IPP and of the best natural phosphoantigen HMBPP were synthesized. Once we demonstrated the efficacy and stability of these thiopyrophosphate derivatives, an innovative synthetic strategy was developed to study the key features of the alkyl chain with the aim of improving Vγ9 Vδ2 stimulating activity. Since the molecular mechanism of phosphoantigens recognition is still unknown[7] and we cannot predict how changes in the structure of natural phosphoantigens may influence the function of γδ T lymphocytes, our approach to the chemical modifications of the alkyl chain was diversity-oriented.

We adopted a solid-phase synthetic approach that would allow the synthesis to be exploited in a parallel fashion. Different alcohols to be linked to the thiopyrophosphate moiety were chosen from our proprietary 2D database (Molecular Database, MoDa) according to one or more of the following criteria: (a) chain length from 2 to 7 carbon atoms and/or (b) the presence of a double bond, a halogen, a heteroatom, or a reactive group.

Chemistry. A very efficient and innovative synthetic procedure via solid-phase parallel synthesis was adopted for the preparation of the initial library of thiodiphosphate **1a**−**m** (Table 1) as shown in Scheme 1.

The Tris(tetra-*N*-butylammonium) salt of inorganic thiodiphosphate was used to convert activated alcohols on solid support to the corresponding thiodiphosphates esters by nucleophilic displacement. The sulfur atom of inorganic thiodiphosphate is more nucleophilic than the oxygen atoms and is responsible for the (*S*)-alkyl thiodiphosphates regioselectivity.[22]

The alcohols were activated by loading onto polystyrene sulfonyl chloride (PS-TsCl) resin (1.45 mmol/g). The alcohols **a**−**m** (commercially available except for alcohol **m**, which was obtained as previously described[26]) were dissolved in a solution of dichloromethane-pyridine (1:1), and the mixture was added to the resin. The loading was monitored qualitatively by the bromophenol blue test.[27] When the reaction was completed, the excess of reactants was removed by filtration and sequential washings with DCM, DMF, THF/H_2O, and THF. A solution of thiodiphosphate (0.8−1 equiv)[22] in acetonitrile (CH_3CN) was then added to the solid support to which the alcohols **a**−**m** had been bound. After about 24 h, the reaction was filtered and washed with CH_3CN and the filtrate was collected and concentrated to dryness. The residue was dissolved in water and passed through an ion exchange column (NH_4^+ form) to replace the tetra-*N*-butylammonium cations with ammonium cations. This step was carried out to facilitate a final purification step by chromatography on cellulose (tetrabutylammonium salts streaked when chromatographed on cellulose).[28]

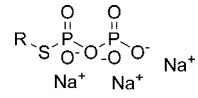
The product **3m** had to be deprotected before exchanging the tetrabutylammonium cation. To avoid partial hydrolysis of the pyrophosphate moiety, we decided to use non-hydrolytic conditions (1 equiv of $BF_3 \cdot OEt_2$ and 1.5 equiv of $NaCNBH_3$ in THF as described in Scheme 1, step e).

All products obtained as NH_4^+ salts were then purified by chromatography on cellulose by eluting with a mixture of 2% of 25 mM NH_4HCO_3 in isopropyl alcohol and passed through an ion exchange column (Na^+ form) to afford the desired products **1a**−**m** with yields from 30% to 96%.

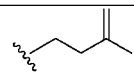
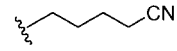
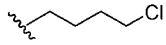
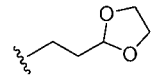
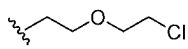
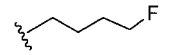
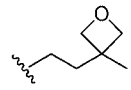
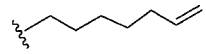
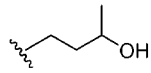
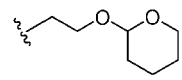
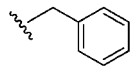
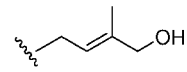
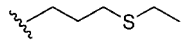
Thiodiphosphate structures were confirmed by ^{1}H NMR. The spectra were consistent with the bridging sulfur attached to the C(1) of the R moiety. The C(1) protons in **1m** (ThioHMBPP) appear as double doublet at 3.5 ppm with coupling constants to the proton at C(2) of 8.1 Hz, and at P_α of 10.7 Hz. In the corresponding HMBPP spectrum,[29] the C(1) methylene protons appear as double doublet at 4.5 ppm. We found the same results for the other compounds that present the protons C(1) at about 2.9−3.5 ppm.

In summary, we have demonstrated a valid strategy to perform the synthesis of a library of thio-diphosphate derivatives using an efficient and quick solid-phase parallel approach.

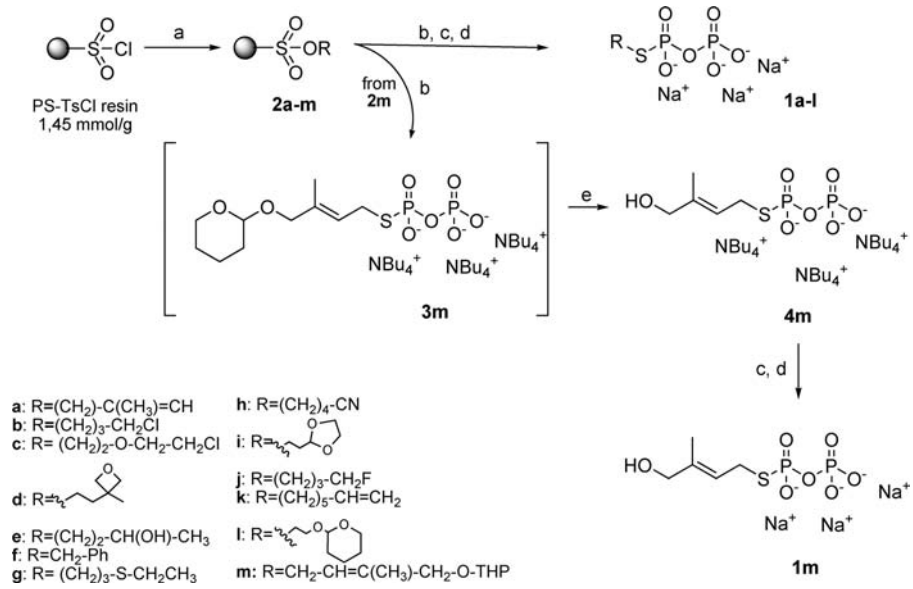
Table 1. EC_{50} Values Determined by Vγ9 Vδ2 T Cell Line Assay



1a-m

Cpd	R	EC_{50}(μM)	Cpd	R	EC_{50}(μM)
1a		3.75±0.50	1h		1.0±0.3
1b		100±15	1i		0.056±0.007
1c		0.39±0.10	1j		10.8±1.0
1d		14±3	1k		6.3±1.0
1e		75±6	1l		12.0±0.8
1f		55±4	1m		0.0048±0.0010
1g		0.055±0.010	IPP	-	5.9±1.0

Scheme 1[a]



[a] Reagents and conditions: (a) R-OH (**a**−**m**), DCM/Py 1/1, rt, 24 h; (b) tris(tetra-*N*-butylammonium)thiodiphosphate, CH_3CN, rt, 24 h; (c) Dowex(NH_4^+), chromatography on cellulose; (d) Dowex(Na^+); (e) $BF_3 \cdot OEt_2$, $NaCNBH_3$, THF, rt, 1 h.

Biological Evaluation. The immunostimulating activity of compounds was determined in vitro on lymphocytes obtained from healthy donors. Preliminary assay on total peripheral blood mononuclear cells (PBMCs) were carried out.

We screened a selection of compounds for their ability to stimulate Vγ9 Vδ2 T cells in terms of expansion and production of TNF-α and IFN-γ. After purification, PBMCs were cultured with 10 μM of the compounds in presence of IL-2. IPP was used as reference drug at the same concentration of the tested compounds. As shown in Figure 2, we observed that three out of eight compounds were able to stimulate an expansion of Vγ9 Vδ2 T cells comparable to IPP reference stimulation.

To perform structure−activity relationship (SAR) studies, Vγ9 Vδ2 T cell lines were used to estimate half-maximal effective concentration (EC_{50}) data for each compound. EC_{50} obtained by measuring TNF-α production through the ELISA test are shown in Table 1.

Table 2. IPP and ThioIPP (**1a**) in Vitro Stability towards Alkaline Phosphatase[a]

	percentage of parent compound			
compd	$T = 0$ min	$T = 20$ min	$T = 30$ min	$T = 60$ min
IPP	94	48	54	27
1a	100	90	90	90

[a] Percentage of parent compound was determined by HPLC-MS analysis.

To better characterize the newly synthesized class of phosphoantigens, we decided to evaluate (i) the in vitro stability toward alkaline phosphatase and (ii) the in vitro efficacy and stability of the most potent compound of the series.

Alkaline phosphatase is the main enzyme responsible for pyrophosphate hydrolysis to phosphate.[30] To compare the resistance of IPP and its thioanalogue **1a** to alkaline phosphatase, we decided to use a resin-bound enzyme. A simple filtration of the enzyme at different incubation intervals provided samples, which were analyzed by HPLC-MS to detect the percentage of remaining parent compound (Table 2).

To evaluate the in vitro stability of **1m** as near as possible to "physiological" conditions, we incubated **1m** with human serum and evaluated its residual Vγ9 Vδ2 activating capacity after different incubation intervals (Figure 4).

An additional objective of this work was to better characterize in vitro the most potent compound of the series (**1m**). We analyzed Vγ9 Vδ2 response variability to **1m** stimulation by testing PBMCs from seven healthy donors (Figure 3); these experiments could demonstrate its efficacy on a wider spectrum of phenotypes.

Discussion

We designed and synthesized a new class of thiopyrophosphoantigens and evaluated their Vγ9 Vδ2 stimulating capacity in comparison with the natural metabolite IPP. First we carried out the synthesis of IPP and HMBPP thioanalogues in order to test their efficacy and stability.

ThioIPP (**1a**) is equipotent with IPP (Table 1) but shows better resistance to alkaline phosphatase in our in vitro assay with resin-bound enzyme. HPLC-MS supports our claims: after incubation for 60 min, the percentage of IPP is drastically reduced (27%) while **1a** is stable (Table 2).

ThioHMBPP (**1m**), which is about 1000-fold more potent than IPP (Table 1) and 50-fold less potent than HMBPP according to literature data,[31] shows a higher in vitro stability. Residual Vγ9 Vδ2 activating capacity was outlined in a biological assay in which 20 μM IPP/**1m** were incubated with human serum, and samples at different incubation intervals were used to stimulate PBMCs. Again **1m** expansion indices are higher than IPP and they decrease more slowly for the thiophosphoantigen ranging from 63 ($T = 0$) to 47 ($T = 1$ h) for thioHMBPP and from 19.6 ($T = 0$) to 1 ($T = 1$ h) for IPP (Figure 4).

To better characterize **1m** efficacy, PBMCs from seven healthy donors were used to test Vγ9 Vδ2 response variability. **1m** and IPP expansion indices were calculated at 20 μM concentration; **1m** indices range from 1.5- to 33-fold greater than IPP (Figure 3A). These data confirm thioHMBPP efficacy in vitro; further experiments will be carried out to test its in vivo potency.

Once we demonstrated the efficacy and stability of the new thiopyrophosphate bioisosteric group through IPP and HMBPP thioanalogues, our aim was to develop a parallel strategy to better explore the SAR of this new class of synthetic antigens. We worked on a parallel solid-phase synthetic methodology to obtain a small library of new thioderivatives with different alkyl chains (Table 1).

First, 4C-length saturated alkyl chains with halogen (Cl, F), hydroxyl, or cyano groups were used. A decrease of activity was observed for compounds **1b**, **1e**, and **1j** as well as for **1k** characterized by the introduction of a C6−C7 unsaturated chain. The cyano derivative **1h** is about 5-fold more potent than IPP. These data confirm the importance of key features such as the length of the chain (up to 6 C) and the presence of a double or triple bond.[7,17]

We also investigated the effect of a heteroatom insertion into the chain: **1c** and **1g** are respectively 12- and 100-fold more potent than IPP. The simultaneous presence of a halogen (Cl) and O in compound **1c** and the introduction of S in a six carbon chain in **1g** improved the bioactivity to low micromolar or nanomolar range.

Finally, bulky (benzyl) and reactive (3-methyl-oxoetane-3-ethyl; 1,3-dioxolane-2-ethyl; tetrahydro-2-pyranyloxyethyl) moieties were introduced. **1d** shows an EC_{50} in low micromolar range, while the benzyl derivative **1f** is 10-fold less potent than IPP.

Reactive groups as tetrahydropyranyloxy and 1,3-dioxolanyl are usually used to protect hydroxyl and aldehydic functions during chemical reactions. Since certain phosphoantigens with chemically reactive groups have been reported to be relatively potent Vγ9 Vδ2 stimulators,[32] we decided to test these protected compounds that may undergo chemical in vitro/in vivo transformation. While compound **1l** was equipotent with IPP, **1i** derivative shows an EC_{50} in the nanomolar range. The chemical reactivity of the molecule may explain this result.

Conclusion

Improving phosphoantigen stability is one of the major goals in pharmaceutical research on Vγ9 Vδ2 activators and different approaches have been reported.[20,21] We worked to the bioisosteric replacement of phosphate with a thiophosphate, which led to the successful identification of a new class of more stable phosphoantigens.

ThioIPP and ThioHMBPP were synthesized and tested showing a drug-like profile in terms of in vitro potency and stability. Further experiments will be carried out to better define ThioHMBPP pharmacokinetic/pharmacodynamic profile.

One of the main goals of our work was the development of an optimized parallel solid-phase strategy to synthesize small libraries of thiophosphoantigens. It can be considered an innovative tool to perform SAR studies and to search for new potent Vγ9 Vδ2 activators.

Experimental Section

The reactions on solid-phase were performed in a 24-position Bohdan Miniblock parallel synthesizer (Mettler Toledo).

The analytical characterizations of the compounds were carried out by means of a HPLC-MS Nebula system (Gilson-Thermofinnegan), a Synergy Polar RP column (150 × 4.6; Phenomenex) or ODS3 column (150 × 4.6; GL Science) and nuclear magnetic resonance spectroscopy (^{1}H NMR) recorded on a Bruker Avance 300 MHz instrument. Elemental microanalyses of C, H, N were performed on a Carlo Erba model 1106 analyzer and were within 0.4% of the calculated values.

General Procedure for the Loading of the Alcohol onto the Solid Support. The alcohol (0.850 mmol) was dissolved in 0.6 mL of DCM-pyridine (1:1). The mixture was added to PS-TsCl resin (1.45 mmol/g) (0.170 mmol) swelled in THF.

The reaction mixture was shaken for 24 h, the excess of reactant was filtered off, and the resin washed sequentially with DCM (3 ×

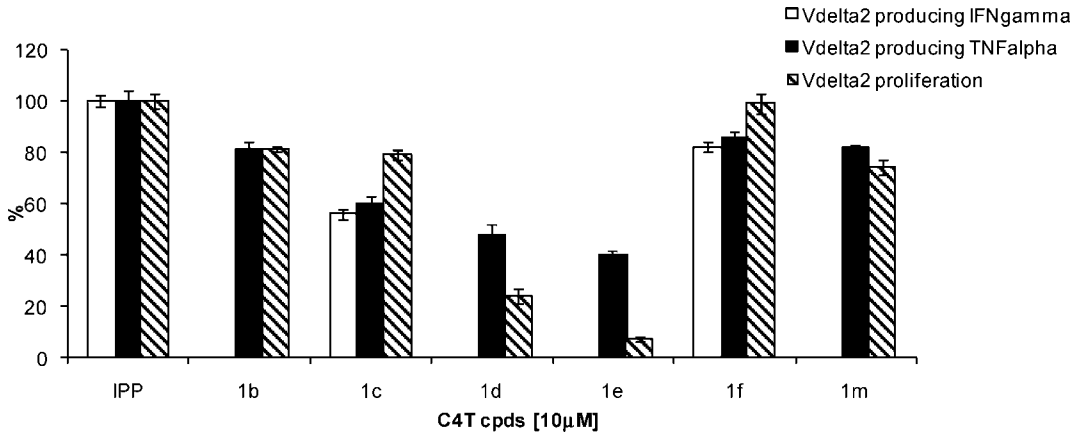


Figure 2. Activation of Vγ9 Vδ2 T cells by stimulating PBMCs from healthy donors. Vγ9 Vδ2 proliferation and cytokines (IFN-γ and TNF-α) production were evaluated after stimulation with tested compounds (10 μM). Results have been expressed as percentage increases after being normalized to IPP index at 10 μM.

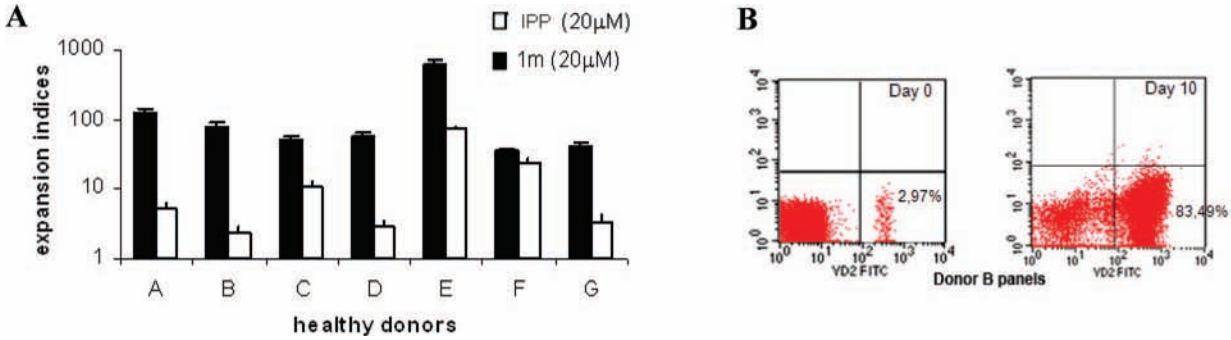


Figure 3. (A) Comparison between expansion indices of IPP (20 μM) and **1m** (20 μM) by testing PBMCs from seven healthy donors. (B) Example of analysis panels.

1 mL), DMF (3 × 1 mL), 3:1 THF/H_2O (3 × 1 mL), and THF (3 × 1 mL). The loading was monitored with the bromophenol blue test.

General Procedure for the Synthesis of Pyrophosphates on Solid Support. The resin (**2a**−**o**) was reswelled in THF and tetrabutylammonium thiopyrophosphate (0.17 mmol) dissolved in acetonitrile (0.6 mL) was added. The reaction mixture was allowed to stir for 24 h; after filtration, the filtrate, containing the product, was collected and passed through a Dowex (NH_4^+) resin to convert the tetrabutylammonium salt into ammonium salt by eluting with a mixture of 2% of 25 mM NH_4HCO_3 in isopropyl alcohol. The product was purified by chromatography on cellulose using a mixture (4.5:2.5:3) *i*PrOH:CH_3CN:(0.1 M) NH_4HCO_3 as eluent. The fractions were collected and lyophilized. The product was finally converted into the sodium salt using an ion-exchange column (Dowex (Na^+) resin, eluting with deionized water).

Trisodium *S*-(3-Methyl-3-butenyl)thiodiphosphonate (1a). Yield 49%. ^{1}H NMR (300 MHz, D_2O) δ (ppm) 4.85 (2H, m), 3.04−2.97 (2H, m), 2.44 (2H, m), 1.77 (3H, s). LC-MS (ESI^-) *m*/*z* 261.0 (M − 3Na + 3H − 1, 100%). Anal. ($C_5H_9Na_3O_6P_2S$) C, H, Na, S.

Trisodium *S*-(4-Chlorobutyl)thiodiphosphonate (1b). Yield 61%. ^{1}H NMR (300 MHz, D_2O) δ (ppm) 3.70 (2H, m), 2.92−2.85 (2H, m), 2.01−1.80 (4H, m). LC-MS (ESI^-) *m*/*z* 283.2 (M − 3Na + 3H − 1, 9%). Anal. ($C_4H_8ClNa_3O_6P_2S$) C, H, Cl, Na, S.

Trisodium *S*-[2-(2-Chloroethoxy)ethyl]thiodiphosphonate (1c). Yield 70%. ^{1}H NMR (300 MHz, D_2O) δ (ppm) 4.05−3.95 (4H, m), 3.92−3.84 (2H, m), 3.24−3.12 (2H, m). LC-MS (ESI^-) *m*/*z* 299.1 (M − 3Na + 3H − 1, 93%). Anal. ($C_4H_8ClNa_3O_7P_2S$) C, H, Cl, Na, S.

Trisodium *S*-[(3-Methyloxyethane-3-yl)methyl]thiodiphosphonate (1d). Yield 92%. ^{1}H NMR (300 MHz, D_2O) δ (ppm) 4.80 (2H, d, *J* = 6.0), 4.55 (2H, d, *J* = 6.6), 3.27 (2H, d, *J* = 9.9), 1.51 (3H, s). LC-MS(ESI^-) *m*/*z* 277.3 (M − 3Na + 3H − 1, 68%). Anal. ($C_6H_{11}Na_3O_7P_2S$) C, H, Na, S.

Trisodium *S*-(3-Hydroxybutyl)thiodiphosphonate (1e). Yield 86%. ^{1}H NMR (300 MHz, D_2O) δ (ppm) 4.12−4.07 (1H, m), 3.07−2.97 (1H, m), 2.90−2.84 (1H, m), 1.98−1.85 (2H, m), 1.30 (3H, d, *J* = 6.0). LC-MS (ESI^-) *m*/*z* 265.3 (M − 3Na + 3H − 1, 58%). Anal. ($C_4H_9Na_3O_7P_2S$) C, H, Na, S.

Trisodium *S*-(Benzyl)thiodiphosphonate (1f). Yield 81%. ^{1}H NMR (300 MHz, D_2O) δ (ppm) 7.51 (2H, d, *J* = 7.7), 7.45−7.38 (2H, m), 7.37−7.32 (1H, m), 4.13 (2H, d, *J* = 9.3). LC-MS (ESI^-) *m*/*z* 283.2 (M − 3Na + 3H − 1, 85%). Anal. ($C_7H_7Na_3O_6P_2S$) C, H, Na, S.

Trisodium *S*-[2-(Ethylthio)propyl]thiodiphosphonate (1g). Yield 96%. ^{1}H NMR (300 MHz, D_2O) δ (ppm) 2.92 (2H, dt, *J* = 13.2, 7.1), 2.70 (2H, t, *J* = 7.4), 2.60 (2H, q, *J* = 7.5), 2.03−1.94 (2H, m), 1.24 (3H, t, *J* = 7.5). LC-MS (ESI^-) *m*/*z* 295.2 (M − 3Na + 3H − 1, 75%). Anal. ($C_5H_{11}Na_3O_6P_2S_2$) C, H, Na, S.

Trisodium *S*-(4-Cyanobutyl)thiodiphosphonate (1h). Yield 62%. ^{1}H NMR (300 MHz, D_2O) δ (ppm) 2.96−2.80 (2H, m), 2.57−2.48 (2H, m), 1.89−1.71 (4H, m). LC-MS (ESI^+) *m*/*z* 276.1 (M − 3Na + 3H − 1, 100%). Anal. ($C_5H_8NNa_3O_6P_2S$) C, H, N, Na, S.

Trisodium *S*-(2-Ethyl-1,3-dioxolanyl)thiodiphosphonate (1i). Yield 72%. ^{1}H NMR (300 MHz, D_2O) δ (ppm) 5.07 (1H, t, *J* = 4.6), 4.09−3.89 (4H, m), 2.92 (2H, dt, *J* = 13.2, 7.1), 2.10 (2H, td, *J* = 7.4, 4.6). LC-MS (ESI^+) *m*/*z* 295.1 (M − 3Na + 3H + 1, 100%). Anal. ($C_5H_9Na_3O_8P_2S$) C, H, Na, S.

Trisodium *S*-(4-Fluorobutyl)thiodiphosphonate (1j). Yield 93%. ^{1}H NMR (300 MHz, D_2O) δ (ppm) 4.58 (2H, dt, *J* = 47.2, 6.0), 2.99−2.79 (2H, m), 1.97−1.72 (4H, m). LC-MS (ESI^+) *m*/*z* 269.1 (M − 3Na + 3H + 1, 100%). Anal. ($C_4H_8FNa_3O_6P_2S$) C, H, F, Na, S.

Trisodium *S*-(6-Heptenyl)thiodiphosphonate (1k). Yield 85%. ^{1}H NMR (300 MHz, D_2O) δ (ppm) 6.02−5.79 (1H, m), 5.06 (1H,

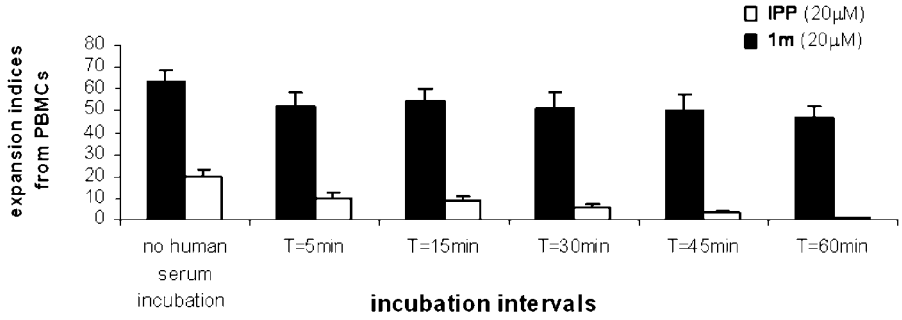


Figure 4. Comparison between expansion indices of IPP and **1m** after incubation with human serum. The residual Vγ9 Vδ2 activating capacity was evaluated by PBMCs stimulation with samples sorted at different incubation intervals.

d, $J = 16.2$), 4.97 (1H, d, $J = 9.2$), 2.83 (1H, dt, $J = 12.0, 7.4$). LC-MS (ESI^+) *m*/*z* 291.1 (M − 3Na + 3H + 1, 95%). Anal. ($C_7H_{13}Na_3O_6P_2S$) C, H, Na, S.

Trisodium *S*-(Ethyl-2-tetrahydro-2*H*-2-pyranyloxy)thiodiphosphonate (1l). Yield 57%. ^1H NMR (300 MHz, D_2O) δ (ppm) 4.81−4.75 (1H, m), 4.06−3.77 (3H, m), 3.68−3.55 (1H, m), 3.07 (2H, dt, $J = 12.9, 6.0$), 1.88−1.73 (2H,m), 1.66−1.47 (4H, m). LC-MS (ESI^+) *m*/*z* 239.1 (M − 3Na + 3H + 1, 100%). Anal. ($C_7H_{13}Na_3O_8P_2S$) C, H, Na, S.

Synthesis of Pyrophosphate 1m. Synthesis of Trisodium *S*-[(*E*)-4-Hydroxy-3-methyl-2-butenyl] thiodiphosphonate. The solution obtained after resin **2m** treatment with thiodiphosphate was dried under reduced pressure. The residue containing the intermediate **3m** (24.9 mg, 0.023 mmol) was dissolved in 5 mL of THF; 1 equiv of $BF3 \cdot OEt_2$, and 1.5 equiv of $NaCNBH_3$ were added. The reaction mixture was allowed to stirr for 1 h and then was quenched at 0 °C with 0.1 M NH_4HCO_3 and stirred again for 10 min. THF was evaporated, and the resulting mixture was passed through a Dowex (NH_4^+) resin to convert the tetrabutylammonium salt into ammonium salt by eluting with a mixture of 2% of 25 mM NH_4HCO_3 in isopropyl alcohol. The product was purified by chromatography on cellulose using *i*PrOH:CH_3CN:NH_4HCO_3 (0.1 M) mixture (4.5:2.5:3) as eluent. The fractions were collected and lyophilized. The product was redissolved in water and passed through a Dowex(Na^+) resin to exchange the ammonium ion. The final product was obtained, after lyophilization, as a white solid (30% yield). ^1H NMR (300 MHz, D_2O) δ (ppm) 5.57 (1H, t), 3.90 (2H, s), 3.48−3.40 (2H, m), 1.62 (3H, s). LC-MS (ESI^-) *m*/*z* 277.2 (M − 1). Anal. ($C_5H_9Na_3O_7P_2S$) C, H, Na, S.

Biological Procedures. PBMCs Assay. PBMCs were isolated from buffy coats of healthy donors by density gradient centrifugation using Lympholeyte-H (Cederlane Laboratories). Mononuclear cells were cultured at 1×10^6 cells/mL into 24-well flat-bottom tissue culture plates (Falcon) in RPMI 1640 (Euroclone, UK), supplemented with 10% FCS (HyClone, Invitrogen Life Technologies), L-glutamine (2 mM), HEPES buffer (10 mM), and gentamicin (10 g/mL) (Sigma-Aldrich). Phosphoantigen-specific stimulation of Vγ9 Vδ2 T cells was performed using 10 μM of the compounds to be tested, while IPP at the same concentration was used as reference drug. After 1 day incubation at 37 °C in 5% CO_2, a single-cell analysis of cytokine synthesis was performed. Monensin (10 μM; Sigma-Aldrich) was added during the last 4 h of culture to block intracellular transport processes and allow cytokine accumulation. Intracellular TNF-α and IFN-γ production was detected by intracellular staining and flow cytometry as previously described.[33]

The expansion of Vγ9 Vδ2 T cells was followed by cytometric analysis after one week of culture monitoring the percentage of Vγ9 Vδ2 T cells vs total CD3+ cells by using a double staining with anti-CD3 (PE) and Vδ2 (FITC) mAbs (BD Biosciences). All results are expressed as percentage increases normalized to IPP stimulation index at 10 μM.

Vγ9 Vδ2 T Cell Lines Assay. Vγ9 Vδ2 T cell lines were obtained by stimulating PBMCs of healthy donors with isopentenyl pyrophosphate (IPP) (160 ng/mL) and IL-2 (100 U/mL) for 10 days. The purity of cell fraction was ≥90% in all experiments as measured by flow cytometric analysis. Increased concentrations of the compounds to be tested were used to stimulate Vγ9 Vδ2 (2.5×10^5 cells/mL) for another 12 h. Dose-ranging concentrations of IPP represented the positive control. Supernatants were collected and dosed for TNF-α concentration with hTNF-α screening set kit (Endogen). Each compound was tested in duplicate and EC_{50} values were calculated through GraphPad Prism analysis (GraphPad Software, version 4.00 for Windows, San Diego, CA).

1m Expansion Indices from Seven Healthy Donors PBMCs. Stimulation and flow cytometric analysis of Vγ9 Vδ2 T cells from seven healthy donors PBMCs were basically performed as previously described in PBMCs assay section. Compound **1m** and the reference drug IPP were tested in duplicate at a 20 μM fixed concentration. After incubation, the absolute number of Vδ2 T cell in each well was calculated as follows: (percentage of Vδ2 T cells among total cells) × (total cells count)/100. The Vδ2 expansion index was then calculated by dividing the absolute number of Vδ2 T cells in specifically stimulated cultures by the absolute number of Vδ2 T cells before culture.[34]

In Vitro Stability of Thiopyrophosphoantigens. Alkaline Phosphatase Assay. First, 20 μL of agarose-bound alkaline phosphatase (5 U/mL) in Tris-HCl/$MgCl_2$ buffer (SIGMA phosphatase, alkaline-Agarose from calf intestine) was added to 0.5 mL of 2 mM IPP or **1a** solution. Then after stirring at room temperature for settled time intervals up to 1 h, the mixture was filtered to remove the enzyme and HPLC-MS analysis was carried out to determine the percentage of parent compound.

Incubation with Human Serum. First, 130 μL of 2 mM IPP or **1m** solution were added to 1.17 mL of human serum. Then after incubation at 37 °C in 5% CO_2 atmosphere for settled time intervals up to 1 h, 100 μL from each solution were collected. The samples sorted at different times were used to stimulate Vγ9 Vδ2 T cells from PBMCs according to the same procedure previously described in PBMCs assay section. Non human serum incubated samples were used as controls.

After 24 h and 1 week of incubation, TNF-α producing Vδ2 percentages and Vδ2 expansion indices were calculated.

Acknowledgment. We dedicate this work to the memory of Fabrizio Poccia to honour his enthusiasm and his great scientific skill.

Supporting Information Available: Elemental analysis data for compounds **1a**−**1m**. This material is available free of charge via the Internet at http://pubs.acs.org.

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