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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 14 (2004) 4471–4477

Synthesis of chiral phosphoantigens and their activity in γδ T cell stimulation☆

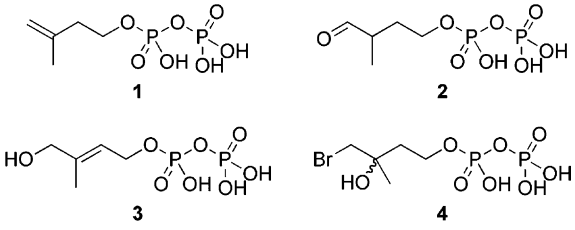
Yongcheng Song,[a] Yonghui Zhang,[a] Hong Wang,[b] Amy M. Raker,[b] John M. Sanders,[a] Erin Broderick,[a] Allen Clark,[a] Craig T. Morita[b] and Eric Oldfield[a,*]

[a]*Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, IL 61801, USA*
[b]*Department of Internal Medicine and the Interdisciplinary Group in Immunology, University of Iowa, EMRB 340F, Iowa City, IA 52242, USA*

Received 6 May 2004; accepted 16 June 2004
Available online 17 July 2004

Abstract—γδ T cells expressing Vγ2Vδ2 T cell receptors are activated by a broad range of phosphorus-containing small molecules, termed phosphoantigens, and are of interest in the context of the chemotherapy of B cell malignancies. Here, we report the synthesis of four pairs of chiral phosphoantigens: the bromohydrins of isopentenyl diphosphate (Phosphostim™), the epoxides of isopentenyl diphosphate (EIPP); and the corresponding bromohydrin and epoxide analogs of but-3-enyl diphosphate. The ability of each compound to stimulate human Vγ2Vδ2 T cells was determined by TNF-α release and cell proliferation. In these assays, the (*R*)-bromohydrin diphosphates were, on average, about twice as active as the (*S*)-bromohydrin diphosphates. In contrast, the (*S*)-form of EIPP was about twice as active as (*R*)-EIPP. The activities of the epoxy but-3-enyl diphosphates were both very low. These results suggest that chiral phosphoantigens, as opposed to racemic mixtures, may have utility in immunotherapy.

A wide variety of pathogenic bacteria and protozoa produce 'phosphoantigens', small molecule phosphorus-containing metabolites that stimulate human γδ T cells of the immune system.[1,2] The first natural bacterial phosphoantigen to be identified was the molecule isopentenyl diphosphate (IPP, **1**):



This was followed by the discovery of a much more active species, initially described as 3-formyl-butyl diphosphate (**2**),[3] but based on NMR and isotopic labeling experiments,[4,5] this compound is now thought to be (*E*)-4-hydroxyl-3-methyl-but-2-enyl diphosphate (HMBPP, **3**). Based on the structure of **2**, Fournié and co-workers developed the bromohydrin of **1**, termed Phosphostim™ (**4**), for use in the immunotherapy of myeloma, lymphoma, head, neck, and renal carcinoma.[6,7] Although Phosphostim™ is a potent activator of human γδ T cells in vitro,[3] in monkeys, rather large doses (~160 mg/kg) have been used for activitation.[6] Thus, it would be of interest to determine whether these doses might be reduced by use of enantiopure, chiral species. The synthesis of such chiral bromohydrins (**5**, **6**), as well as the epoxides of isopentenyl diphosphate (EIPP),[8] such as **7** and **8**, might, however, be expected to be somewhat complex, due to the presence of the diphosphate moiety (which may hydrolyze) and the bromohydrin or epoxide groups, both of which are quite reactive. Nevertheless, the commercial development of racemic Phosphostim™ and Innacell™ (Phosphostim™ for use in autologous cell

☆This work was supported by the US National Institutes of Health (grants GM-65307 to E.O. and AR-045504 to C.T.M.). J.M.S. is an American Heart Association, Midwest Affiliate, Predoctoral Fellow.
*Corresponding author. Tel.: +1-217-333-3374; fax: +1-217-244-0997; e-mail: eo@chad.scs.uiuc.edu

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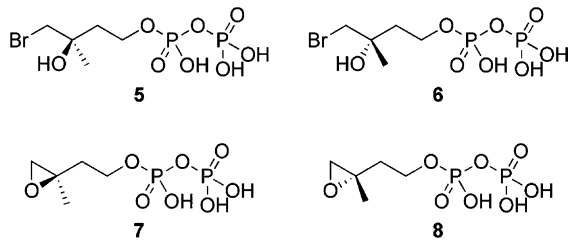
doi:10.1016/j.bmcl.2004.06.052

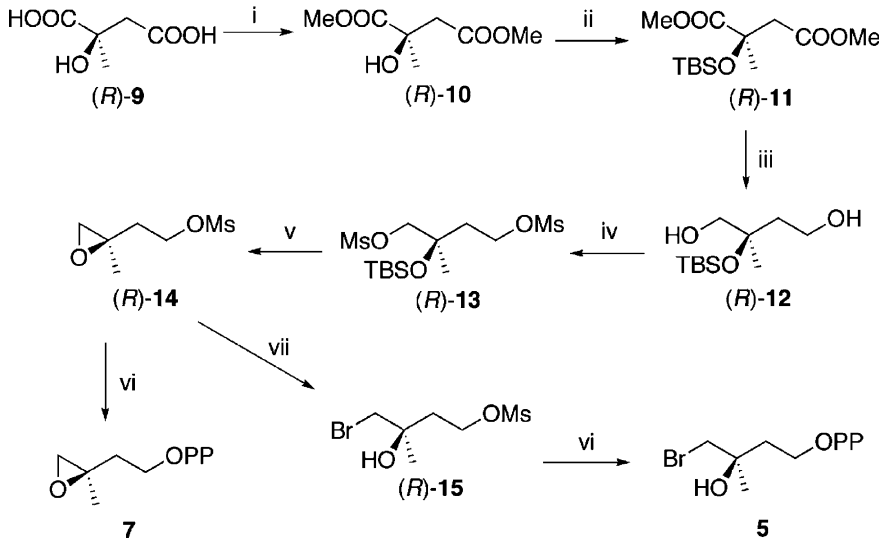
therapy[7]) makes the synthesis of chiral analogs of potential clinical importance, since the possibility exists that there might be differences in both the activity and the toxicity of the different enantiomers.



One route to the production of **5**–**8** would be chiral resolution using chromatographic techniques. However, the lack of large hydrophobic/aromatic groups in these molecules make this approach less attractive. Fortunately, the ready availability of the chiral synthon citramalic acid, **9**, enables the synthesis of **5**–**8**, using Scheme 1.

Our synthesis of optically active Phosphostim™ (**5**, **6**) and the two epoxides of isopentenyl diphosphate (**7**, **8**) started from commercially available (*R*)- and (*S*)-citramalic acid (**9**), as shown in Scheme 1. Treatment of (*R*)-**9** with $SOCl_2$ (5 equiv) in MeOH gave the corresponding dimethyl ester (*R*)-**10** in almost quantitative yield, which, without purification, was reacted with *t*-butyldimethylsilyl trifluoromethanesulfonate in the presence of NEt_3 in CH_2Cl_2 at 0 °C to afford (*R*)-**11**. The yield, after column chromatography, was 84%. Compound (*R*)-**11** was subsequently reduced to the corresponding diol (*R*)-**12** using DIBAL (5 equiv) in THF. Compound (*R*)-**12** was then mesylated with methanesulfonyl chloride, then treated with tetrabutylammonium fluoride (2 equiv) in THF to afford the key intermediate epoxide, (*R*)-**14**, in 50% yield, after column chromatography. Compound (*R*)-**14** was then reacted with 1.5 equiv of $(Bu_4N)_3HP_2O_7$ (0.5 M in CH_3CN) at room temperature for 8 h and the resulting product subjected to standard ion-exchange purification procedures[9] to give the optically active tris-ammonium salt of the epoxide of isopentenyl diphosphate, (*R*)-**7**, in high purity[10] and with an 80% yield ($[\alpha]_D^{25}$ −16.5; *c* 0.4, H_2O). The optically active (*R*)-**7** had identical ^1H and ^{31}P NMR spectra[11] to those of (±)-**7** prepared by using a published method[12] and are shown in Figure 1A (500 MHz ^1H NMR) and Figure 1C (202 MHz ^{31}P NMR). The only impurities clearly visible in the ^1H NMR spectrum occur at ~1 ppm and are attributed to incompletely removed solvent molecules (mainly EtOH). The large peaks in the 7–8 ppm range are due to imidazole, which was added as an internal spin-count quantitation standard, which enables very accurate (<1%) concentration determinations for bioassays (described below). The ^{31}P NMR spectrum shows no evidence of PPi (which based on the signal-to-noise ratio would place PPi levels at ⩽1–2 mole%).

When (*R*)-**14** was reacted with TMSBr[13] at room temperature, then, without purification, subsequently treated with 1.5 equiv of $(Bu_4N)_3HP_2O_7$,[9] optically active (*R*)-Phosphostim™, **5**, was obtained in a 60% yield as the triammonium salt ($[\alpha]_D^{25}$ −3.7; *c* 1.1, H_2O). The optically active (*R*)-Phosphostim™ (**5**) exhibited very similar ^1H and ^{31}P NMR spectra[14] to those of (±)-Phosphostim™, prepared by bromohydration of IPP.[15] The ^1H spectrum of **5** is shown in Figure 1B and, as with the epoxide **7**, the only visible impurities are trace amounts (~2 mole%) of solvent in the ~1 ppm region. The ^{31}P NMR spectrum of **5** is shown in Figure 1D. Here, there is a small PPi contaminant (indicated by the asterisk), which comprises ~5 mole%, a level which is not expected to have any measurable effects in bioassays (cell and plasma PPi levels are typically low micromolar since PPi is a by-product of numerous metabolic pathways). It should be noted that the TMSBr-mediated



Scheme 1. Reagents and conditions: (i) $SOCl_2$, MeOH; (ii) TBSOTf, NEt_3, 84% for two steps; (iii) DIBAL, 88%; (iv) MsCl, NEt_3, cat. DMAP; (v) TBAF, 50% for two steps; (vi) $(Bu_4N)_3HP_2O_7$, 80%; (vii) TMSBr (2 equiv), cat. PPh_3 (5%), CH_2Cl_2, 4 h, 95%.

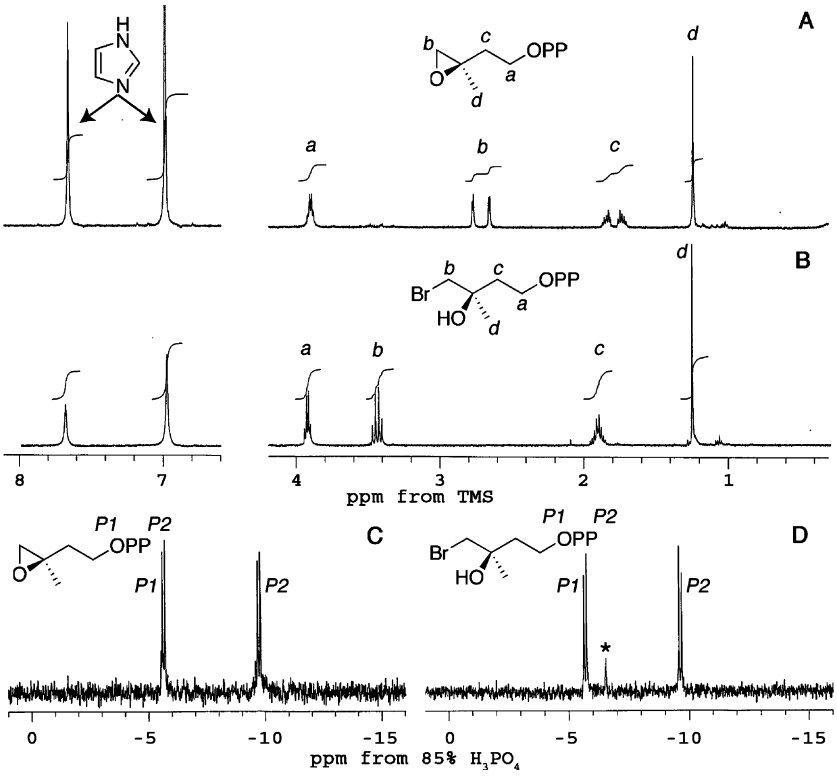
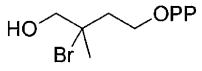
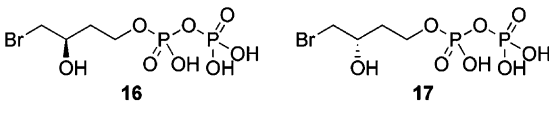


Figure 1. 500 MHz ^{1}H and 202 MHz ^{31}P solution NMR spectra of chiral phosphoantigens **7** and **5**. (A) ^{1}H NMR spectrum of **7**. The peak assignments (a–d) are shown on the spectrum and inset structure. The two big peaks in the 7–8 ppm region arise from internal imidazole spin-count quantitation standards. The main impurities (~2 mole%) occur at ~1 ppm and are attributed to solvent molecules used in the synthesis (mainly EtOH). (B) same as A, but ^{1}H NMR of **5**. (C) ^{31}P NMR spectrum of **7**. (D) ^{31}P NMR spectrum of **5**. There is a ~5 mole% impurity of PPi (shown asterisked).

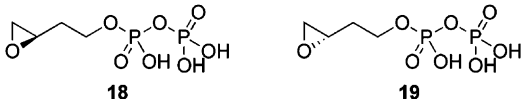


ring opening is highly regioselective, because the other possible regioisomer (shown above) was not present in the ^{1}H NMR spectrum. Synthesis of the enantiomeric (*S*)-epoxide of isopentenyl diphosphate (**8**, $[\alpha]_D^{25}$ +16.6; *c* 0.44, H_2O) and (*S*)-Phosphostim™ (**6**, $[\alpha]_D^{25}$ +3.8; *c* 0.9, H_2O) were then carried out using the same procedure, but starting this time with (*S*)-citramalic acid, resulting in similar yields to the (*R*)-enantiomers and with similar ^{1}H and ^{31}P NMR spectra.

After obtaining initial bioactivity data on **5**–**9** (see below), we were prompted to synthesize two additional pairs of chiral phosphoantigens, **16**–**19**:

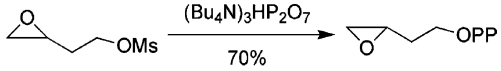


16 **17**

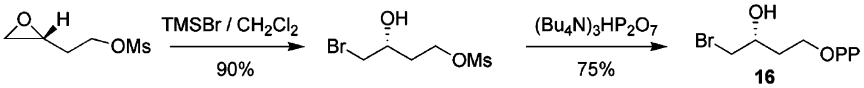


18 **19**

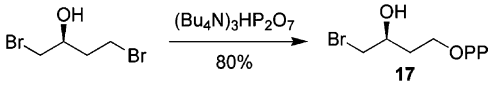
in which the 3-methyl group is absent. Mesylate epoxides were prepared from chiral malic acid, as reported,[16] and were readily converted to the corresponding epoxybutane diphosphates (EBPPs):



The (*R*)-bromohydrin diphosphate was synthesized from the (*R*)-epoxide:



while the (*S*) enantiomer was made in a single step from commercially available (*S*)-1,4-dibromo-2-butanol:



500 MHz ^{1}H NMR spectra indicated only trace levels (≤3%) of impurities, basically as described above for **5**–**8**. The $[\alpha]_D^{25}$ results for all four species are given in Table 1.

Table 1. Optical rotation results for chiral phosphoantigens

Compound	$[\alpha]_D^{25}$	*c*	Solvent
5, (*R*)-Phosphostim™	−3.7	1.1	H_2O
6, (*S*)-Phosphostim™	+3.8	0.9	H_2O
7, (*R*)-EIPP	−16.5	0.4	H_2O
8, (*S*)-EIPP	+16.6	0.44	H_2O
16, (*R*)-BHPP	+13.9	0.46	H_2O
17, (*S*)-BHPP	−13.3	0.51	H_2O
18, (*R*)-EBPP	+12.0	0.36	H_2O
19, (*S*)-EBPP	−11.7	0.34	H_2O

We first investigated the ability of **5**–**8** to stimulate Vγ2Vδ2 T cells to release TNF-α and to proliferate, using the JN.24 and 12G12 T cell clones, as described previously.[17,18] The 12G12 T cell clone expresses the CD8αα homodimer and NKG2D, whereas the JN.24 clone expresses CD4 and is NKG2D negative (data not shown). Both (*R*)- and (*S*)-bromohydrins stimulated TNF-α production and cell proliferation for each T cell clone (Figs. 2A and B and 3A and B), as did both the (*R*)- and (*S*)-epoxides (Figs. 2C and D and 3C and D). The dose responses of the individual enantiomers were very similar. For the bromohydrins (**5**, **6**), the EC_{50} (*S*)/EC_{50} (*R*) ratios were 2.6 (±0.8, $N=8$) for TNF-α release and 1.6 (±0.8, $N=7$) for T cell proliferation (Table 2). For the epoxides (**7**, **8**), the EC_{50} (*R*)/EC_{50} (*S*) ratios were 1.8 (±1.2, $N=5$) for TNF-α release and 1.9 (±0.7, $N=5$) for T cell proliferation (Table 2). Clearly, both (*R*)- and (*S*)-forms of the bromohydrins have very similar activity as do the (*R*)- and (*S*)-forms of the epoxide EIPP, with slightly stronger activity for the (*R*)-form of bromohydrin and the (*S*)-form of the epoxide (Table 2). Although the relative potencies of the enantiomers were similar, the $CD8\alpha\alpha^+$ $NKG2D^+$ Vγ2Vδ2 clone, 12G12, exhibited higher antigen sensitivity. The increased sensitivity of the 12G12 clone is likely due to costimulation through the NKG2D receptor by its interaction with the NKG2D ligands, MICA, ULBP2, and ULBP3, expressed by the Va2 antigen presenting cell line used here. We have previously shown that the NKG2D/MICA interaction significantly increases antigen sensitivity.[19]

In the case of the des-methyl species **16**–**19**, there is an overall decrease in activity, both in TNF-α production and γδ T cell proliferation (Table 2). However, as in the case of the more active bromohydrins (**5**, **6**), the (*R*)-form of des-methyl Phosphostim™ (**16**) is more active than the (*S*)-form (Table 2). Thus, the 3-methyl group does not account for the activity differential observed between **5** and **6**, although it does contribute

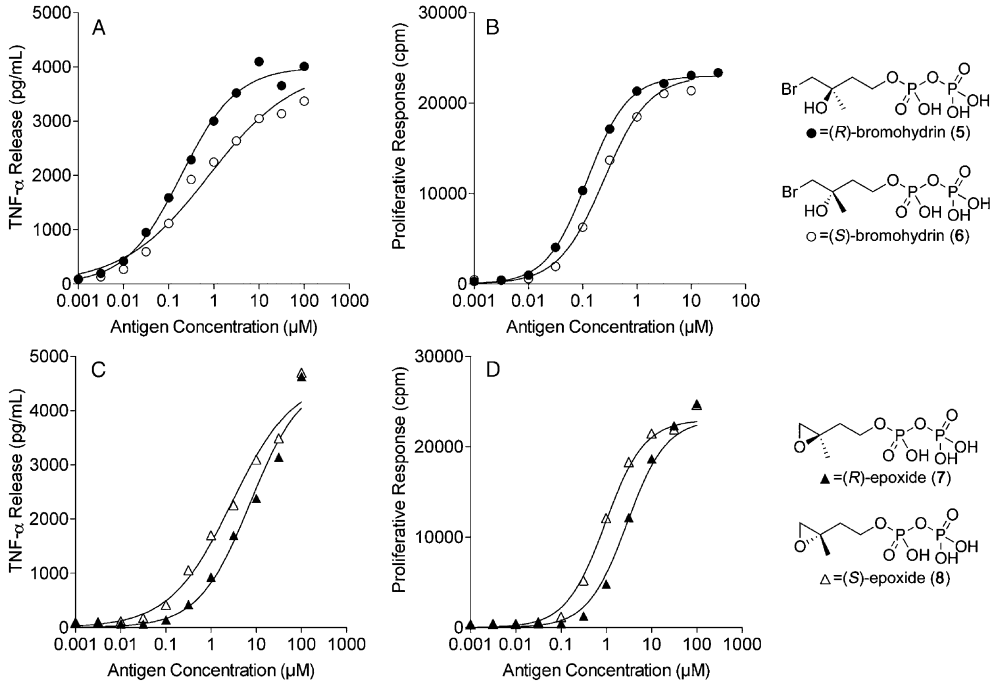


Figure 2. Vγ2Vδ2 T cell activation of the $CD4^+$, $NKG2D^-$ JN.24 clone as measured by TNF-α release and proliferation for chiral Phosphostim™ and chiral EIPP. Data were fitted by using a rectangular hyperbolic function (Prism Program[23]). A variable Hill slope was used to fit the TNF-α release data. (A) Phosphostim™, TNF-α release ●=**5** (*R*), ○=**6** (*S*). (B) same as (A) but proliferation assay. (C) ▲=**7** (*R*), △=**8** (*S*), TNF-α release. (D) as (C) but proliferation assay. For the bioassays, the JN.24 $CD4^+$ Vγ2Vδ2 T cell clone was stimulated by antigens presented by the CP.EBV B cells line (an EBV-transformed B cell line). For these experiments, the CP.EBV line was fixed with 0.05% glutaraldehyde (EM grade, Sigma, MO) (Ref. 18) making the CP.EBV cell line unable to proliferate or to produce TNF-α. Half-log dilutions of phosphoantigens were made in 96-well round-bottom plates before T cells were added in duplicate or triplicate at 1×10^5 cells per well with glutaraldehyde-fixed APCs at 1×10^5 cells per well (1:1 ratio T cell:APC). After 18 h, 100 μL of media was removed for TNF-α measurement and replaced with fresh media. The cultures were pulsed with 1 μCi of ^{3}H-thymidine (2 Ci/mmol) after supernatant removal and harvested 16–18 h later. Mean proliferation and SEM of duplicate or triplicate cultures is shown. To measure TNF-α release, supernatants were harvested after 18 h and assayed for TNF-α by sandwich ELISA (R&D Systems, Minneapolis, MN). A TNF-α standard curve was derived from serial dilution of a TNF-α standard and used to calculate the TNF-α concentration in picogram/milliliter by a Molecular Dynamics ELISA reader. Note that no TNF-α release was seen in control wells containing APC and antigen alone.

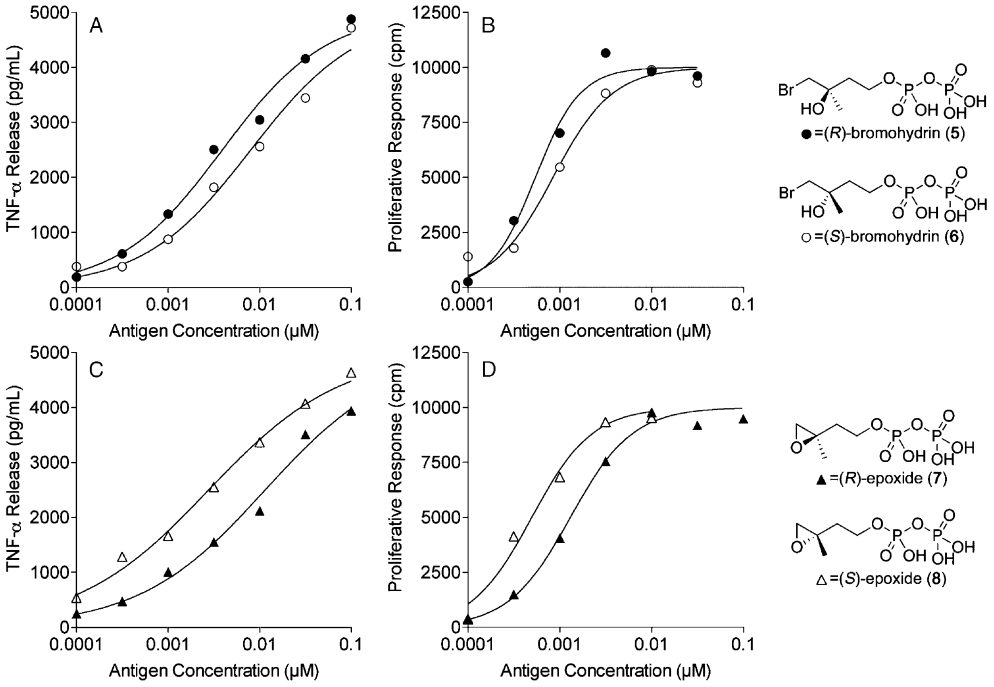
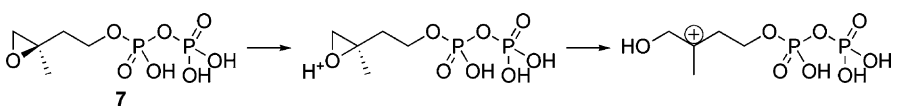


Figure 3. Vγ2Vδ2 T cell activation of the CD8αα$^+$, NKG2D$^+$ 12G12 clone as measured by TNF-α release and proliferation for chiral Phosphostim™ and chiral EIPP. Data were fitted by using a rectangular hyperbolic function (Prism Program[23]). A variable Hill slope was used to fit the TNF-α release data. (A) Phosphostim™, TNF-α release ●=**5** (*R*), ○=**6** (*S*). (B) same as (A) but proliferation assay. (C) ▲=**7** (*R*), △=**8** (*S*), TNF-α release. (D) as (C) but proliferation assay. TNF-α release and proliferation assays are as in Figure 2 except that 12G12 Vγ2Vδ2 T cells were used instead of JN.24 T cells and that fixed Va2 cells (transformed human fibrosarcoma cells) were used as APC, instead of CP.EBV B cells. Note that the use of Va2 cells as APC enhanced antigen presentation to non-CD4 Vγ2Vδ2 T cells by 10–30-fold as compared to using EBV-transformed B cells. This is similar to the effects we observed with nonpeptide antigen presentation to the NKG2D$^+$ DG.SF13 T cell clone, where we directly showed that MICA expression by APC enhances antigen presentation through a costimulation effect (Ref. 19). In contrast to 12G12 T cells, Va2 and CP.EBV cells showed no difference in their ability to present nonpeptide antigens to CD4$^+$ JN.24 T cells, consistent with the lack of NKG2D on JN.24 T cells.

to overall activity. With the epoxide samples **18** and **19**, the EC_{50} levels were $\geqslant$100 μM, and at these low response levels, a difference in activity could not be discerned.

The observation of only a small difference in activity between the most active enantiomers is surprising, given that in some bioassays, EC_{50} values in the low nanomolar range are seen (Table 2). Since these chiral epoxides and bromohydrins are not expected to racemize, these results suggest the intriguing possibility that binding to the γδ*T* cell target might involve formation of a planar carbonium ion, for example:



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in which case much of the anticipated difference in reactivity between (*R*)- and (*S*)-enantiomers could be lost. Species such as **7** might also be intermediates in the reactions of the bromohydrins with their T cell target, although further work will of course be necessary to test these hypotheses.

Overall, these results are of interest since they show that both the (*R*)- and (*S*)-forms of Phosphostim™ and EIPP, as well as the bromohydrin of but-3-enyl diphosphate, have activity in γδ T cell stimulation. The observation that both enantiomers of all three molecules have activity was unexpected, with there being only relatively small differences in the ED_{50}s observed, with each enantiomer. This is in contrast to the large differences in bioactivity noted with changes in alkyl chain length, double bond additions, phosphorylation, and halogenation.[20,21] The availability of chiral forms of such phosphoantigens should facilitate more detailed studies of the molecular basis for phosphoantigen recognition by Vγ2Vδ2 T cells and may also have utility in the development of such species for immunotherapy. In addition, the general synthetic strategies we have shown here can be readily extended to the synthesis of chiral immunosuppressive agents, such as the diphosphonate analogs of (**5**–**8**).[22]

Table 2. Results of γδ T cell stimulation by chiral phosphoantigens

Assay	Clone	Compound	EC_{50} (nM)	Compound	EC_{50} (nM)	Ratio[a]	
TNF-α	JN.24	**5**, (*R*)-Phosphostim™	40	**6**, (*S*)-Phosphostim™	140	3.5	2.9 ± 0.8
		5, (*R*)-Phosphostim™	21	**6**, (*S*)-Phosphostim™	71	3.4	
		5, (*R*)-Phosphostim™	440	**6**, (*S*)-Phosphostim™	860	2.0	
		5, (*R*)-Phosphostim™	190	**6**, (*S*)-Phosphostim™	480	2.5	
		5, (*R*)-Phosphostim™	200	**6**, (*S*)-Phosphostim™	790	4.0	
		5, (*R*)-Phosphostim™	330	**6**, (*S*)-Phosphostim™	590	1.8	
		8, (*S*)-EIPP	2900	**7**, (*R*)-EIPP	7400	2.6	1.3 ± 1.0
		8, (*S*)-EIPP	350	**7**, (*R*)-EIPP	420	1.2	
		8, (*S*)-EIPP	1100	**7**, (*R*)-EIPP	230	0.2	
		16, (*R*)-BHPP	500	**17**, (*S*)-BHPP	2,000	4	
		18, (*R*)-EBPP	>100,000	**19**, (*S*)-EBPP	>100,000	—	
	12G12	**5**, (*R*)-Phosphostim™	5	**6**, (*S*)-Phosphostim™	9	1.8	1.9 ± 0.1
		5, (*R*)-Phosphostim™	4	**6**, (*S*)-Phosphostim™	8	2.0	
		8, (*S*)-EIPP	8	**7**, (*R*)-EIPP	10	1.3	2.5 ± 1.2
		8, (*S*)-EIPP	3	**7**, (*R*)-EIPP	11	3.7	
Proliferation	JN.24	**5**, (*R*)-Phosphostim™	120	**6**, (*S*)-Phosphostim™	250	2.1	1.9 ± 0.8
		5, (*R*)-Phosphostim™	110	**6**, (*S*)-Phosphostim™	330	3.0	
		5, (*R*)-Phosphostim™	170	**6**, (*S*)-Phosphostim™	130	0.8	
		5, (*R*)-Phosphostim™	120	**6**, (*S*)-Phosphostim™	280	2.3	
		5, (*R*)-Phosphostim™	85	**6**, (*S*)-Phosphostim™	90	1.1	
		8, (*S*)-EIPP	160	**7**, (*R*)-EIPP	220	1.4	1.9 ± 0.8
		8, (*S*)-EIPP	950	**7**, (*R*)-EIPP	2900	3.1	
		8, (*S*)-EIPP	64	**7**, (*R*)-EIPP	83	1.3	
		16, (*R*)-BHPP	1200	**17**, (*S*)-BHPP	4000	3.3	
		18, (*R*)-EBPP	>100,000	**19**, (*S*)-EBPP	>100,000	—	
	12G12	**5**, (*R*)-Phosphostim™	1.4	**6**, (*S*)-Phosphostim™	0.9	0.6	1.1 ± 0.5
		5, (*R*)-Phosphostim™	0.5	**6**, (*S*)-Phosphostim™	0.8	1.6	
		8, (*S*)-EIPP	0.6	**7**, (*R*)-EIPP	1	1.7	1.9 ± 0.2
		8, (*S*)-EIPP	0.5	**7**, (*R*)-EIPP	1	2.0	

[a] Ratio of EC_{50}s. As discussed in the text, for the bromohydrins the ratio is the EC_{50} (*S*)/EC_{50} (*R*) and for EIPP the ratio is EC_{50} (*R*)/EC_{50} (*S*). Overall, there is about a factor of two difference between the activities of the three most active pairs of enantiomers.

Acknowledgements

We thank Professor Scott E. Denmark for use of his polarimeter and for helpful discussions.

References and notes

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11. **7/8**: ^{1}H NMR (500 MHz, D_2O): δ 1.25 (3H, s, CH_3), 1.70–1.90 (2H, m, CH_2CH_2O), 2.66 (1H, d, $J_{H,H}$ = 4 Hz, H at C4), 2.77 (1H, d, $J_{H,H}$ = 4 Hz, H at C4), 3.90 (2H, m, CH_2CH_2O); ^{31}P NMR (202 MHz, D_2O): δ −9.7 (1P, d, $J_{P,P}$ = 21.4 Hz) −5.6 (1P, d, $J_{P,P}$ = 21.4 Hz).
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