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Synthesis and Biological Evaluation of Sphingosine Kinase Substrates as Sphingosine-1-Phosphate Receptor Prodrugs

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Abstract

In the search for bioactive sphingosine 1-phosphate (S1P) receptor ligands, a series of 2-amino-2-heterocyclic-propanols were synthesized. These molecules were discovered to be substrates of human-sphingosine kinases 1 and 2 (SPHK1 and SPHK2). When phosphorylated, the resultant phosphates showed varied activities at the five sphingosine-1-phosphate (S1P) receptors ($S1P_{1–5}$). Agonism at $S1P_1$ was displayed *in vivo* by induction of lymphopenia. A stereochemical preference of the quaternary carbon was crucial for phosphorylation by the kinases and alters binding affinities at the S1P receptors. Oxazole and oxadiazole compounds are superior kinase substrates to FTY720, the prototypical prodrug immunomodulator, fingolimod (FTY720). The oxazole-derived structure was the most active for human SPHK2. Imidazole analogues were less active substrates for SPHKs, but more potent and selective agonists of the $S1P_1$ receptor; additionally, the imidazole class of compounds rendered mice lymphopenic.

Introduction

Five membrane-bound sphingosine 1-phosphate (S1P, Figure 1) receptors control physiological processes, including heart rate, tissue permeability,[1] wound healing,[2] immune cell trafficking[3] and oligodendrocyte function.[4] Receptor expression and metabolism of sphingolipid signaling molecules enable endogenous S1P to control these diverse functions with specificity while being present at concentrations of 200 to 450 nM in plasma.[5,6] Our laboratories have attempted to describe these signaling pathways by investigating the structure-activity-relationship of individual S1P receptors through the synthesis and biological characterization of non-natural S1P receptor ligands. Previously, we reported diverse classes of S1P analogues with various receptor affinities; including $S1P_4$ and $S1P_{1,5}$ selective agonists,[7,8] as well as some of the first $S1P_{1,3}$ antagonists.[9,10]

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Important in the successful development of S1P ligands is their incorporation into sphingosine metabolism. In view of natural S1P biosynthesis and degradation, one pathway for ligand inactivation involves lysophospholipid phosphatases.[11,12] These enzymes dephosphorylate S1P and related molecules to primary alcohols that are physiologically inactive at the five receptors. We and others illustrated the synthesis of phosphonate mimetics that are more chemically resistant to phosphatase activity (bioactive **VPC44152**, Figure 1).[10,13] This report describes the synthesis and biological characterization of S1P ligands that are prone to phosphorylation by one or both of the known sphingosine kinases (SPHKs).[14] Substrates for SPHKs may obtain therapeutically useful equilibriums between their alcohol and phosphate states *in vivo*, as investigated by $S1P_1$ induced lymphopenia.

A series of 2-amino-2-heterocyclic-propanols were investigated, based on our previous discovery of $S1P_1$ selective agonists that contained *N*-aryl amide moieties within their linker region (Figure 1). This series was tested for activity at the known mouse (mSPHK) and human (hSPHK) sphingosine kinases. These potential kinase substrates were tested *in vitro*; and, following chemical phosphorylation, the compounds were evaluated at the five individual S1P receptors; and finally, the substrates were tested *in vivo*, for the induction of $S1P_1$ mediated lymphopenia.

Imidazole, oxazole, and oxadiazole containing compounds are phosphorylated by SPHKs, with hSPHK2 being the more active species. This activity was dependant on the chirality of the C-2 carbon. One of two oxadiazoles was a better kinase substrate for than FTY720, the prototypical S1P prodrug. The corresponding oxazole showed the highest activity at SPHK2. Imidazole based compounds were comparatively less active substrates at the SPHKs but their phosphorylated congeners were more potent and selective agonists at the $S1P_1$ receptor. Meta-substituted compounds in these series (found to be antagonists of $S1P_{1,3}$ receptors) were not substrates for SPHKs. This is consistent with our previous model, in which the stereochemical preference for antagonism is opposite to that favored by enzymatic phosphorylation.

Chemistry

The synthesis of 4(5)-phenylimidazoles (Scheme 1) was envisioned through a Davidson-like cyclodehydration.[15,16,17,18,19] Compound **1** was attained from the Freidel-Crafts acylation of commercially available 1-phenyloctane and 2-bromoacetyl bromide as previously described.[10] *N*-Boc-α-methylserines were converted to their cesium salts under sonication,[20] and alkylated with α-bromoketone **1** to form the desired -acyloxyketones, ***R*-** and ***S*-2**, in robust yields. α-Acyloxyketones were cyclized to optically active phenylimidazoles ***R*-**and ***S*-3** by careful heating with NH_4OAc in xylenes. The 2-amino-1-propanols, **3**, were deprotected under acidic conditions and neutralized to yield the optically active final compounds **VPC44211** and **VPC44217**. *N*-Boc protected compounds were also converted to the corresponding phosphates by standard phosphoramidite methodology. Subsequent deprotection provided the bis-ammonium trifluoroacetate salts **VPC44218** and **VPC44239**.

A procedure to create the 4-phenyloxazole ring system (Scheme 2) was readily available; however, the acidic conditions necessary for the cyclization meant that new protection schemes for the α-methyl-serine were necessary.[21] A *tert*-butyldiphenylsilyl (TBDPS) ether was used successfully[22] with a benzyloxycarbonyl (Cbz) protection for the amine. Literature procedures for the selective hydrolysis of the methyl ester protected acid (**6**) in the presence of the TBDPS ether further increased the utility of this protection scheme.[23] Formation of the corresponding α-acyloxyketone proceeded smoothly, but the cyclization step to form the desired intermediate **9** proved low yielding. The 4-phenyloxazole was converted by standard methods to amino alcohol **VPC92153** and amino phosphate **VPC92249**.

1,2,4-oxadiazoles are established peptide bond mimetics and comparable to the 4(5)-phenylimidazoles.[24] They are smaller in diameter, considerably less basic than their imidazole counterparts, and allow for hydrogen-bond acceptance, but not donation. Two isomers exist in which the nitrogen atom occupies a similar location compared with the imidazole ring. To approach the two isomers, a common pathway for construction of the oxadiazole ring was desired. Previously, 1,2,4-oxadiazoles were constructed by the condensation of activated carboxylic acids with amidoximes in the presence of strong base. [25,26,27,28,29,30] Several common condensation methods suggested by the literature for coupling carboxylic acids and amidoximes afforded little or no success (DCC,[31] EDC,[32] and DIC/HOBT[33]). Following the literature through extensions to mild condensation strategies, a general method for the conjoining of various carboxylic acids and amidoximes remained elusive.[34] We found PyBOP, the common and mild condensation reagent, worked well for the coupling of both oxadiazole isomers.

With this strategy in hand, the synthesis of the 1,2,4-oxadiazole isomer commenced with the conversion of commercially available 4-iodobenzonitrile to the *para*-alkynylaniline **12** through a Verkade-modified Sonogashira reaction (Scheme 3).[35] Selective reduction of the arylalkyne was accomplished by hydrogenation over Lindlar's catalyst to afford para-octylbenzonitrile **13**. Using methods pioneered by Tiemann and Kruger,[36] and optimized by Eitner and Weitz, [37] hydroxylamine heated in ethanol gave reliable yields of the amidoxime **14**.[38,39]

Commercially available 2-methyl-(*D*,*L*)-serine was converted to acid **15** in two convenient steps (Scheme 4). Carboxylic acid **15** was coupled with amidoxime **14** to form acylamidoxime **16** following our newly established PyBOP coupling strategy. The resulting intermediate was cyclized, providing near quantitative yields of **17**. Global deprotection was successful upon the addition of TFA to attain 3-phenyl-1,2,4-oxadiazole **VPC45064** following basic workup. Protected amino alcohol **17** was also converted to the ammonium phosphate **VPC45070** under standard conditions.

Inversion of the oxadiazole substitution pattern relied on the proper conversion of α-methyl serine to the amidoxime derivative **20** (Scheme 5). The desired 5-phenyl-1,2,4-oxadiazole more closely approximates the position of the nitrogens in the 4(5)-phenylimidazole compounds. Carboxylic acid **15** was converted to the primary amide **18** through formation of the mixed anhydride followed by addition of either $NH_{3(g)}$ or $NH_4OH_{(aq)}$. Convenient and effective dehydration conditions40 were used to convert the amide to the nitrile-serinoid **19**. Treatment of this nitrile with hydroxylamine yielded the desired amidoxime analogue of serine **20**.

The methyl benzoyl ester **21**, previously synthesized by esterification, (Scheme 6) provided the methyl *para*-octynylbenzoyl ester **22** by a modified Sonogashira coupling. Hydrogenation over Pd/C was achieved to yield the alkylbenzoyl ester **23**. Saponification of **23** provided the *para*-octylbenzoic acid **24** efficiently, which was condensed with the sterically congested amidoxime **20** to yield **25** under PyBOP coupling conditions. The purified intermediate was cyclized to the 5-phenyl-1,2,4-oxadiazole, **26**, as previously described. The *N*-Boc and *N*,*O*-isopropylidene were deprotected with TFA and treated with basic conditions, providing the desired 2-amino-1-propanol **VPC45129.**

Once obtained, **VPC45129** was subjected to anhydrous *N*-Boc protection and subsequent phosphorylation and deprotection to yield the corresponding phosphate (**VPC46023**) as a white solid (Scheme 7).

Synthesis of a 4-phenylthiazole derivative began with the serine-derived amide **18**, which was next converted to the thioamide **27** with the use of Lawesson's reagent (Scheme 8). The α-iminothioketone formed by the base-initiated *S*-alkylation of compound **27** was dehydrated *in situ* to give a separable mixture of the desired thiazole **28** and the incomplete dihydrothiazole

29.[41,42] This one pot reaction was not optimized, but on re-treatment of the dihydrothiazole intermediate **29** with dry lutidine and TFAA, the dehydration was completed in excellent yields. Thiazole **28** was deprotected with TFA and neutralized to provide the desired aminoalcohol **VPC45214**.

Biology

The final 2-heterocyclic-2-amino-1-propanols were analyzed as substrates of four SPHKs (h-SPHK1,2 and m-SPHK1,2, as previously described[43]). Phosphorylation was compared to that of the natural substrate of the kinases, D-*erythro*-sphingosine.

Most of our compounds (Figure 2) exhibited activity at SPHK2 with the exception of the 4-phenylthiazole (**VPC45124**). The (*S*)-stereoisomer of the imidazole (**VPC44217**) was virtually inactive at the kinases while the *R*-stereoisomer (**VPC44211**), having the natural configuration about the quaternary carbon, had approximately 20% the activity of sphingosine at hSPHK2. This stereoselective preference was upheld when comparing the racemic mixture of the 3-phenyl-1,2,4-oxadiazole (**VPC45064**) and its *R*- stereoisomer (**VPC45080**), and recapitulates the observed stereoselectivity of SPHK2 for the methylated FTY720 analogs, AAL149 and AAL151.[44] The 5-phenyl-1,2,4-oxadiazole (**VPC45129**) and the 4-phenyloxazole (**VPC92153**) performed exceptionally well in the phosphorylation assay. **VPC92153** displayed the best activity at SPHK2. While **VPC45129** displayed moderate activity at SPHK2, it was the only alcohol in the series to have significant activity at SPHK1. It should be noted that very few synthetic analogs display activity at SPHK1, making this particular oxadiazole-containing compound unusual.

Due to extensive work by our laboratories and others, it is now well understood that lymphopenia induced by S1P receptor agonists, such as FTY720, is the direct result of potency at the $S1P_1$ receptor after *in vivo* phosphorylation by SPHK2.[43] It has also been demonstrated that the bradycardia evoked by FTY720 is linked to agonism at the $S1P_3$ receptor, at least in rodents.[45] Thus it is of interest to determine receptor activity in assessing aminoalcohols as S1P receptor prodrug agonists. Each phosphate was subjected to our standard GTP-[γ-^{35}S] assay as previously described.[6–10]

On initial examination of the data (Table 1), the selectivity between the $S1P_1$ and $S1P_3$ receptor has been greatly improved relative to FTY720. In most cases a difference of two log orders of selectivity was observed. The only exception was the 3-phenyl-1,2,4-oxadiazole phosphate, (VPC45070) which was considerably less potent at $S1P_1$ and was equipotent at $S1P_3$. These phosphates also appear to be good agonists for the $S1P_4$ receptor, providing some insight into $S1P_4$ agonist SAR. However, while these analogs are approximately equipotent to the natural ligand, S1P itself is a surprisingly poor agonist. Experimental potencies of S1P at $S1P_4$ are in the high nanomolar range according to our assays. The 4-phenylthiazole phosphate was not included in the receptor screening because it was such a poor substrate for the SPHKs. The ligand was, therefore an unlikely candidate as a $S1P_1$ receptor prodrug. Because of the detrimental side effects of FTY720's potency at the $S1P_3$ receptor, and the ability of this class of heterocycles to discriminate between $S1P_1$ and $S1P_3$, the therapeutic potential of these compounds becomes immediately apparent.

While receptor data provides some insight as to how these compounds should work as a potential therapy, the ultimate test of these heterocyclic sphingosine analogs lies in their ability to induce lymphopenia *in vivo*. Disappointingly, most of these aminoalcohols were not effective at lowering lymphocyte counts despite their unprecedented activity at the SPHKs and receptor potencies (data not shown). However, the 4-phenylimidazole analogs performed exceptionally well; lowering lymphocyte counts approximately 75% in most cases (Figure 3).

The imidazole containing compounds that exhibited low nanomolar binding constants effectively induced lymphopenia in mice. S1P analogues containing the natural configuration (**VPC44211**) induced lymphopenia for more than 20 hours while the unnatural aminoalcohol (**VPC44217**) did not cause this effect. This finding is consistent with our initial kinase studies, where the analog with the unnatural stereochemistry was a poor substrate for either sphingosine kinase. Although this study produced only a single heterocyclic analog of desirable activity *in vivo*, we have demonstrated there is a clear structure-activity-relationship for this class of SPHK substrates. Elucidating the elements that make these amino alcohols substrates for SPHK1 and SPHK2 while dialing out $S1P_3$ potency, it becomes possible to deliver immunosuppressants with significantly less detrimental $S1P_3$ related side effects.

Conclusion

At the outset of this study, we sought to not only further our understanding of the elements of SPHK1 and SPHK2 substrate SAR, but also design more metabolically stable S1P1 receptor agonist prodrugs. Additionally, we hoped to improve S1P1/S1P3 receptor selectivity, which would make these compounds more attractive as potential therapeutic agents. While in vitro data was initially quite promising, our newly synthesized series of heterocyclic S1P receptor prodrugs did not prove viable therapeutic candidates after in vivo analysis of lymphocyte levels. A likely cause for this result is the rate of dephosphorylation of these analogs by any number of lysophospholipid phosphatases, which would be revealed by a low agonist (phosphate) : parent (alcohol) drug ratio in plasma. Another possibility is rapid clearance of these compounds in mice. We are currently evaluating these possibilities.

Due to their implication in a number of disease states, such as cancer and tumor growth, inhibitors of the sphingosine kinases are quite desirable. Here, we have presented a body of research that displays some of the most remarkable substrates of SPHK1 and SPHK2 yet reported in the literature; compounds whose rates of phosphorylation are beginning to approach that of the natural ligand. We hope to take this data forward in an effort to design a novel class of SPHK inhibitors that could be used as tools to answer questions about the role S1P in various disease states. Such tools have the potential to validate sphingosine kinases as drug targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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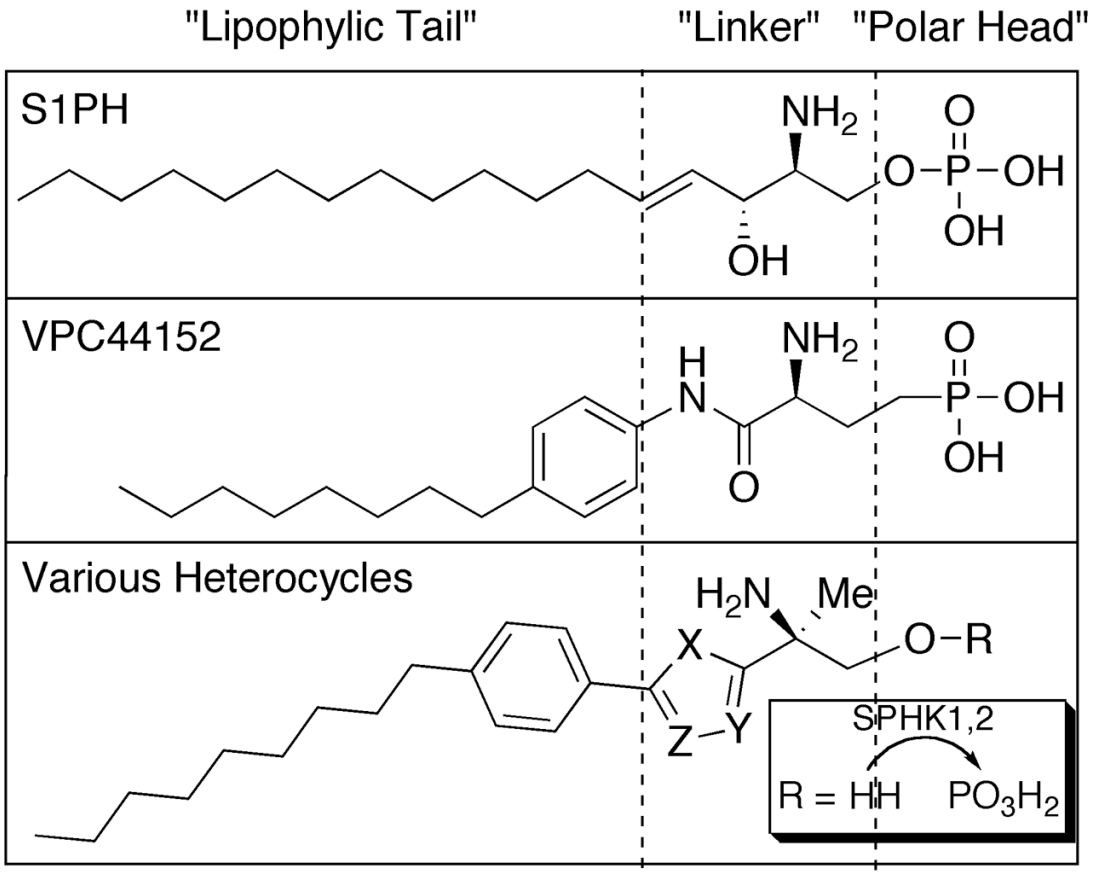


Figure 1.

S1P is active at five S1P receptors ($S1P_{1–5}$). **VPC44152** is a non-hydrolysable phosphonate agonist at $S1P_{1,4,5}$. Various 2-amino-2-heterocyclic propanols were designed as prodrugs (converted by sphingosine kinases to active phosphates) for targeting S1P receptors.

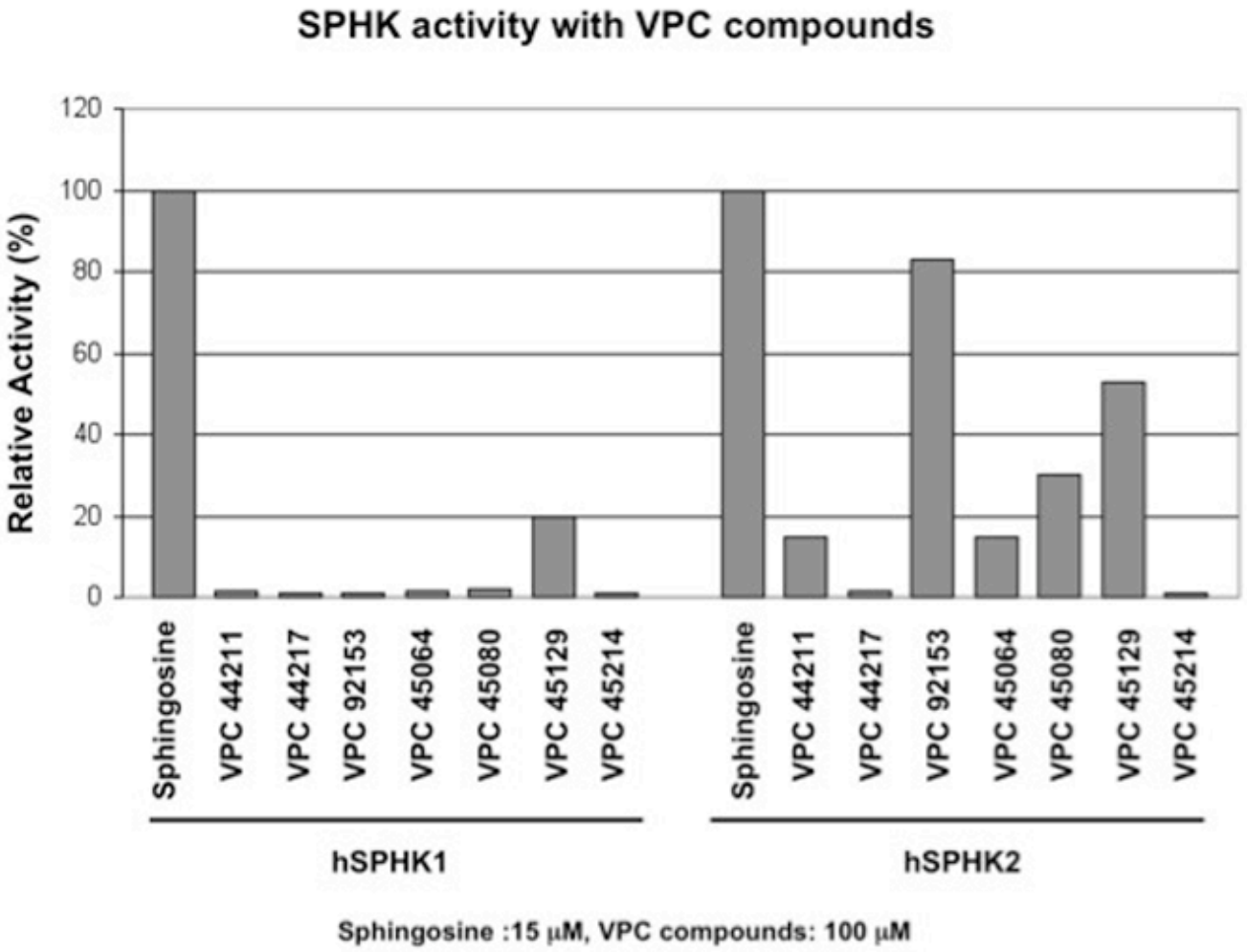
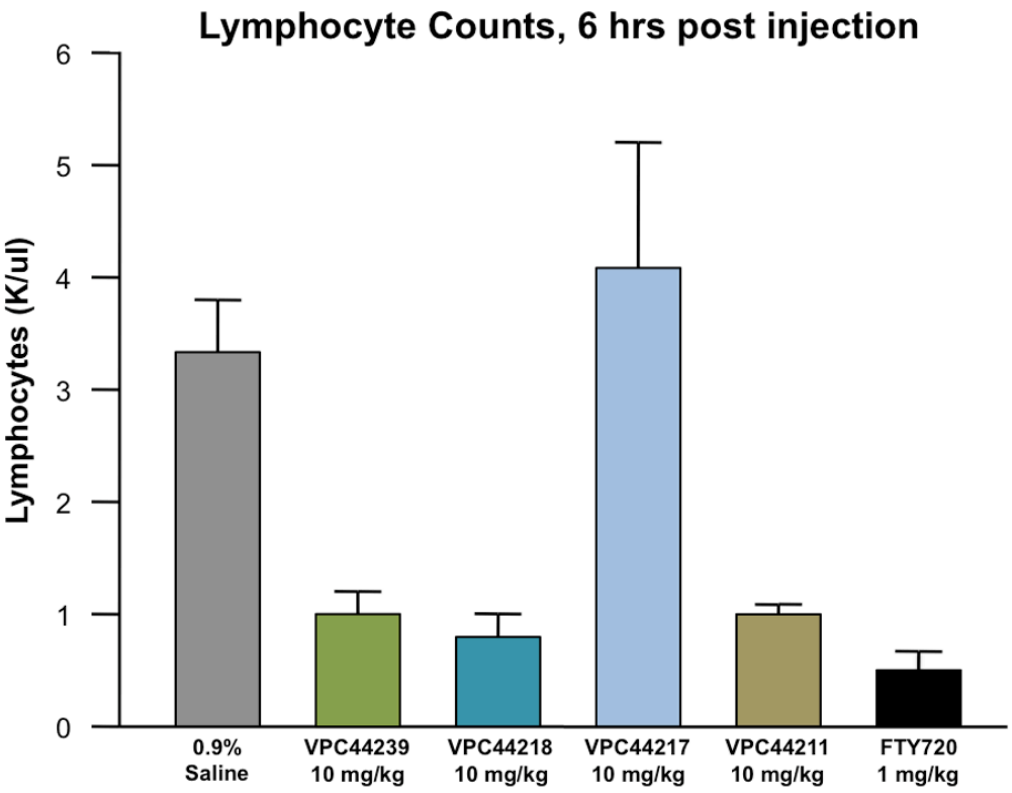


Figure 2.
Comparison of imidazole, thiazole, oxadiazole and oxazole 2-amino-alcohols. Compounds were analyzed as possible substrates for the kinases hSPHK1 (hSK1) and hSPHK2 (hSK2).



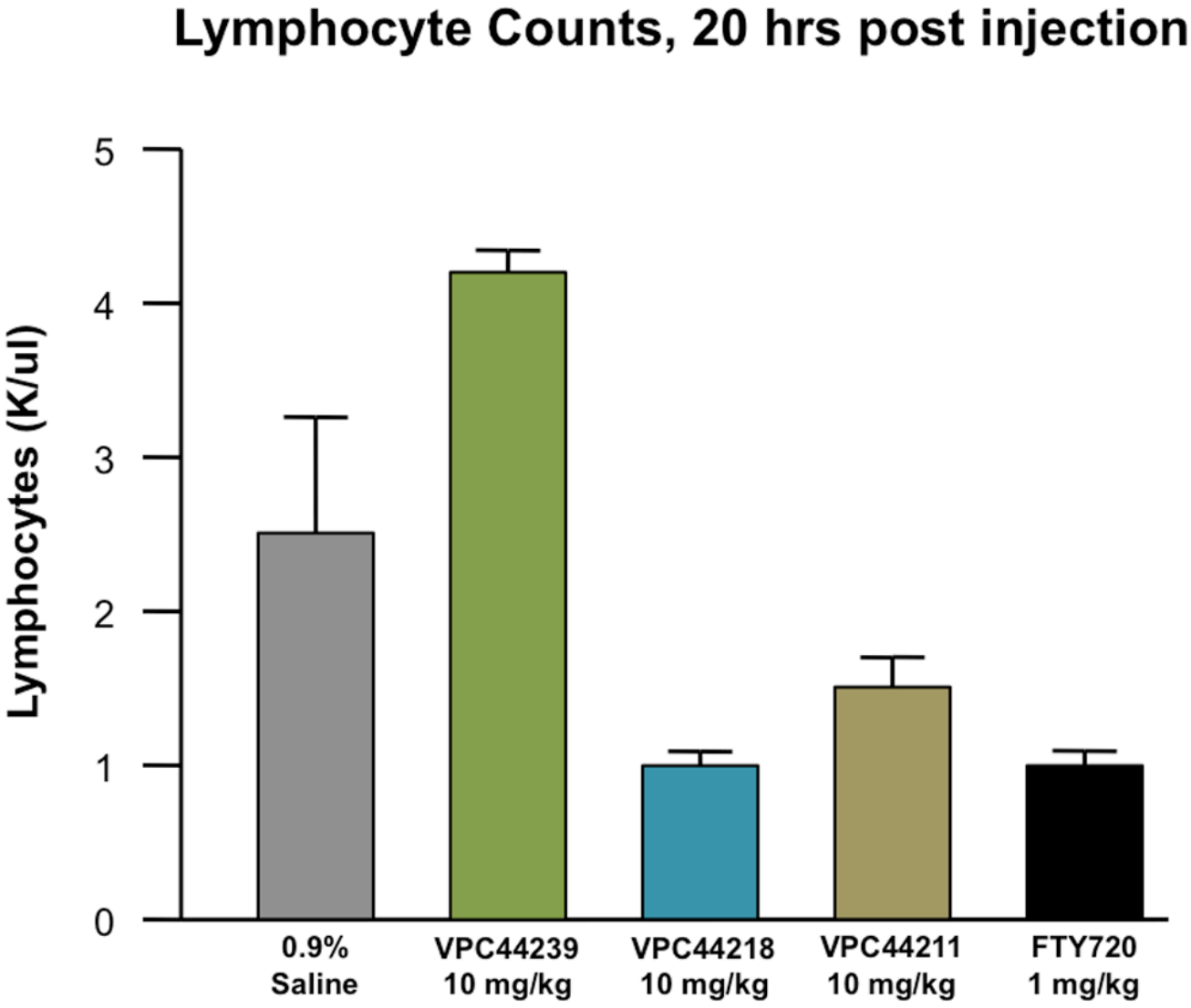
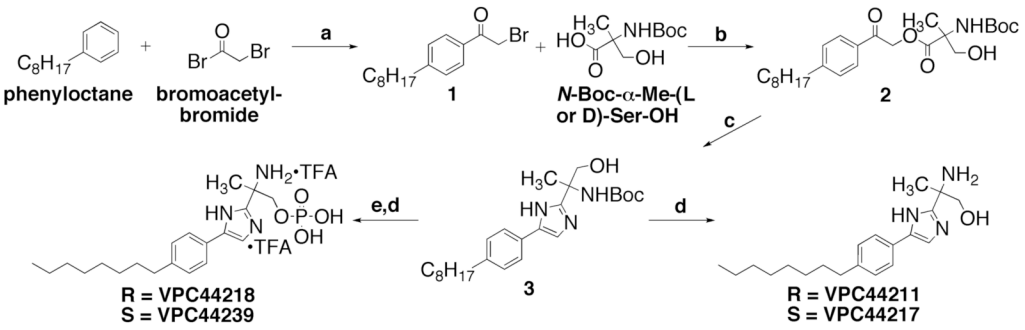


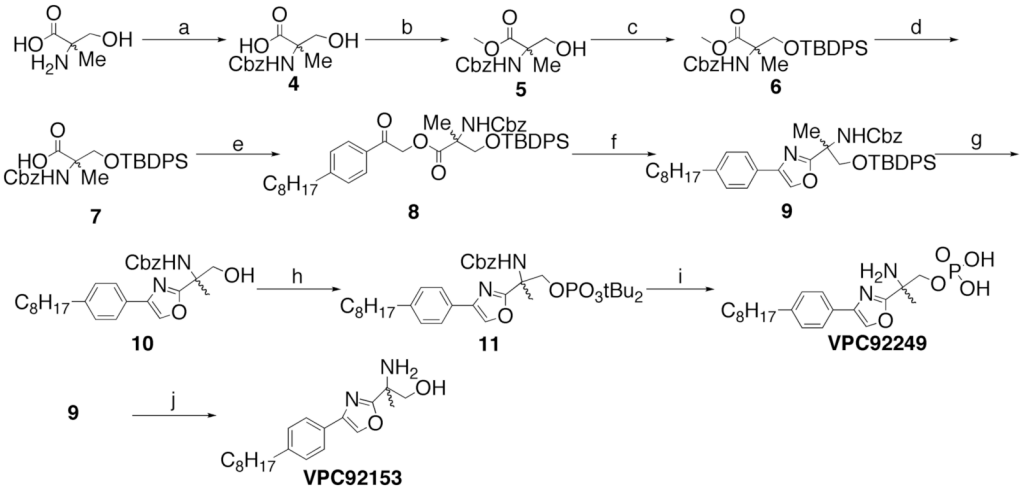
Figure 3.

A) Four chiral phenylimidazole compounds **VPC44239**, **VPC44218**, **VPC44217** and **VPC44211** were compared for their stimulation of lymphopenia using our standard *in vivo* assay.[6] B.) Twenty hours post ip injection, the alcohol **VPC44211** and phosphate **VPC44218** treated mice experienced nearly equivalent levels of lymphocyte depletion from the periphery.[46]



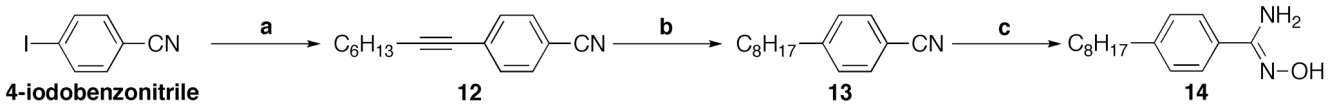
Scheme 1.

Synthesis of chiral 4(5)-phenylimidazoles. Reagents and conditions: a.) $AlCl_3$, neat, 0 °C to rt, 4h. (84%); b.) Cs_2CO_3, EtOH, sonication, 5 – 10 min.; then α-bromoketone in DMF, rt, overnight (86–94%); c.) NH_4OAc, Xylenes, Dean-Stark, 110–120 °C, 1–3h. (50–60%); d.) TFA, CH_2Cl_2, 0 °C to rt, 4h. (80–84%); e.) *N*,*N*-di-*iso*-propyl-di-*tert*-butyl-phosphoramidite, 3% tetrazole in acetonitrile, CH_2Cl_2, 4–8h.; then 30% $H_2O_{2(aq)}$ rt, 4h. (33–37%).



Scheme 2.

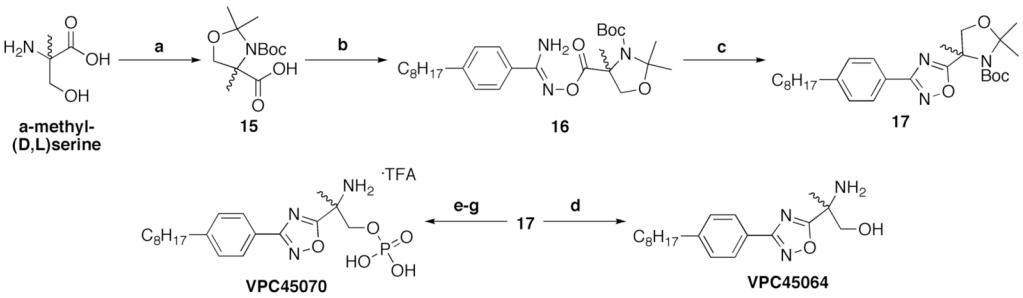
Synthesis of 4-phenyloxazoles. Reagents and Conditions: a.) 10% sat. aq. Na_2CO_3, Dioxanes, *N*-(Benzyloxycarbonyloxy)succinimide; b.) $TMSCHN_2$, 6:1 benzene/MeOH (79% over 2 steps); c.) TBDPSCl, imidazole, DMAP, CH_2Cl_2 (82%); d.) NaOH, H_2O/i-PrOH (96%); e.) Cs_2CO_3, EtOH, sonication, 5 – 10 min.; then α-bromoketone in DMF, rt, overnight (94%); f.) NH_4OAc, AcOH, 90 °C, 10h (33%); g.) *t*-Bu_4NF, THF (77%); h.) *N*,*N*-di-*iso*-propyl-di-*tert*-butyl-phosphoramidite, 3% tetrazole in acetonitrile, CH_2Cl_2, overnight.; then 30% $H_2O_{2(aq)}$ rt, 4h. (59%); i.) Pd/C, H_2, EtOH, rt, overnight (97%); then TFA, CH_2Cl_2, 4h (quantitative); j.) *t*-Bu_4NF, THF (77%); then Pd/C, H_2, EtOH (quantitative).



4-iodobenzonitrile **12** **13** **14**

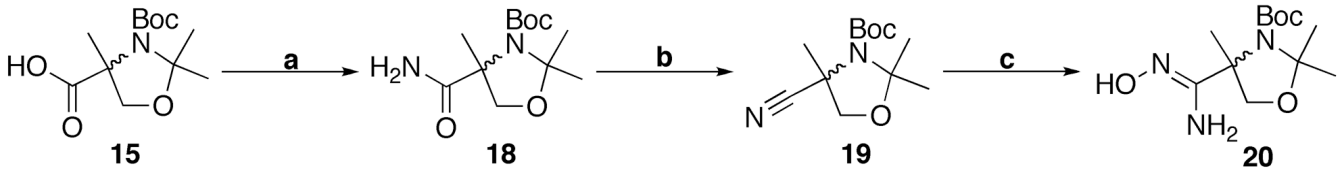
Scheme 3.

Synthesis of benzylamidoxime **14**. Reagents and conditions: a.) $Pd(OAc)_2$, Bu_4NOAc, 1-octyne, DMF, rt, overnight (85–99%); b.) H_2, Pd on $BaSO_4$, EtOH, 45 psi, rt, 1h. (>95%); c.) $NH_2OH{\cdot}HCl$, Et_3N, 95% $EtOH_{(aq)}$, 75 °C, 3h (74%).



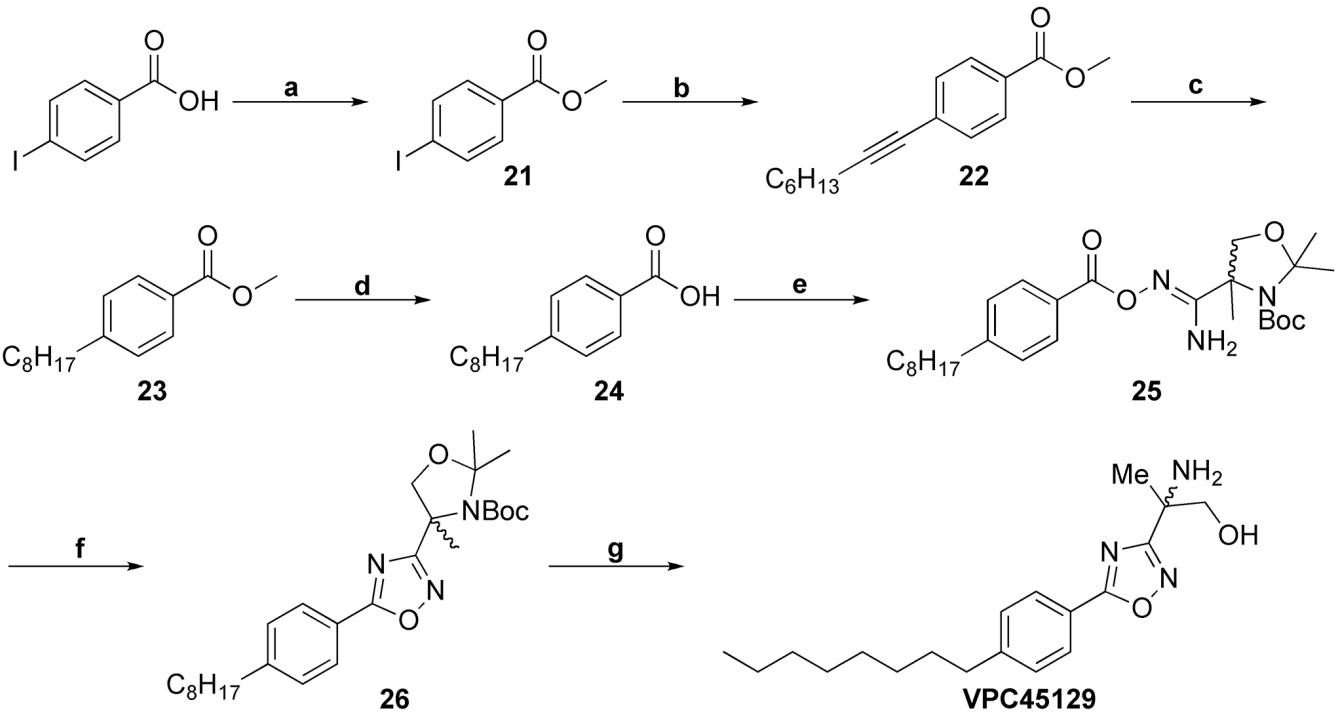
Scheme 4.

Reagents and Conditions. a.) i. 10% Na_2CO_3 in H_2O, rt, 5–10 min.; then Boc_2O in Dioxanes, rt, 0.5 – 2 days; ii. 2,2-dimethoxypropane, BF_3 OEt_2, Acetone, rt, 1–3 h. (>95%, two steps); b.) PyBOP, *i*-Pr_2NEt, CH_2Cl_2, rt, 4h. (88%); c.) DMF, 110 °C, 3–4 h (71%); d.) TFA, CH_2Cl_2, rt, 3h.; then $NaHCO_3$, rt, 15 min. (90%). e.) i. TFA, CH_2Cl_2, rt, 3h.; then $NaHCO_3$, rt, 15 min. (90%); ii. 10% Na_2CO_3 in H_2O, rt, 5–10 min.; then Boc_2O in Dioxanes, rt, 4 – 6 h. (67%, two steps); f.) *N*,*N*-di-*iso*-propyl-di-*tert*-butyl-phosphoramidite, 3% tetrazole in acetonitrile, CH_2Cl_2, overnight; then 30% $H_2O_{2(aq)}$ rt, 4h. (57%); g.) TFA, CH_2Cl_2, rt, 3h. (>80%).



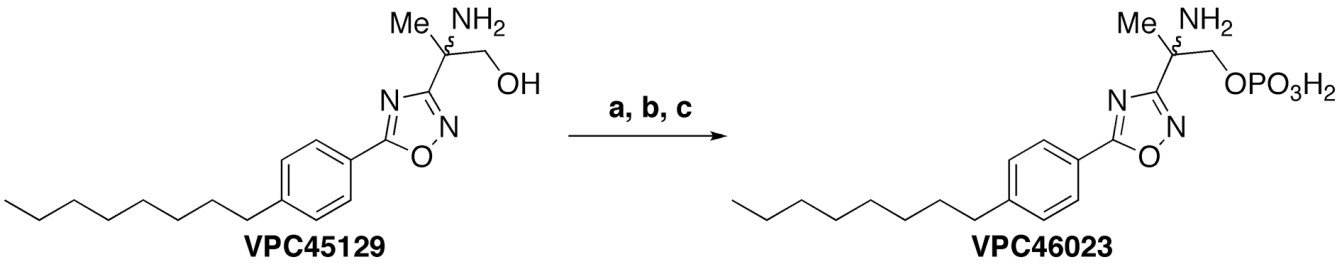
Scheme 5.

Reagents and Conditions: a.) i. *iso*-butyl chloroformate, Et_3N, THF, −10 °C, 30 min.; ii. NH_4OH, 0 °C to rt. 1h. (60–80%); b.) Et_3N, trifluoroacetic anhydride (TFAA), 0.1M THF 0 °C to rt, 30 min. (85–95%); c.) $NH_2OH{\cdot}HCl$, Et_3N, 95% EtOH(aq), 75 °C, 3–5h. (85%).



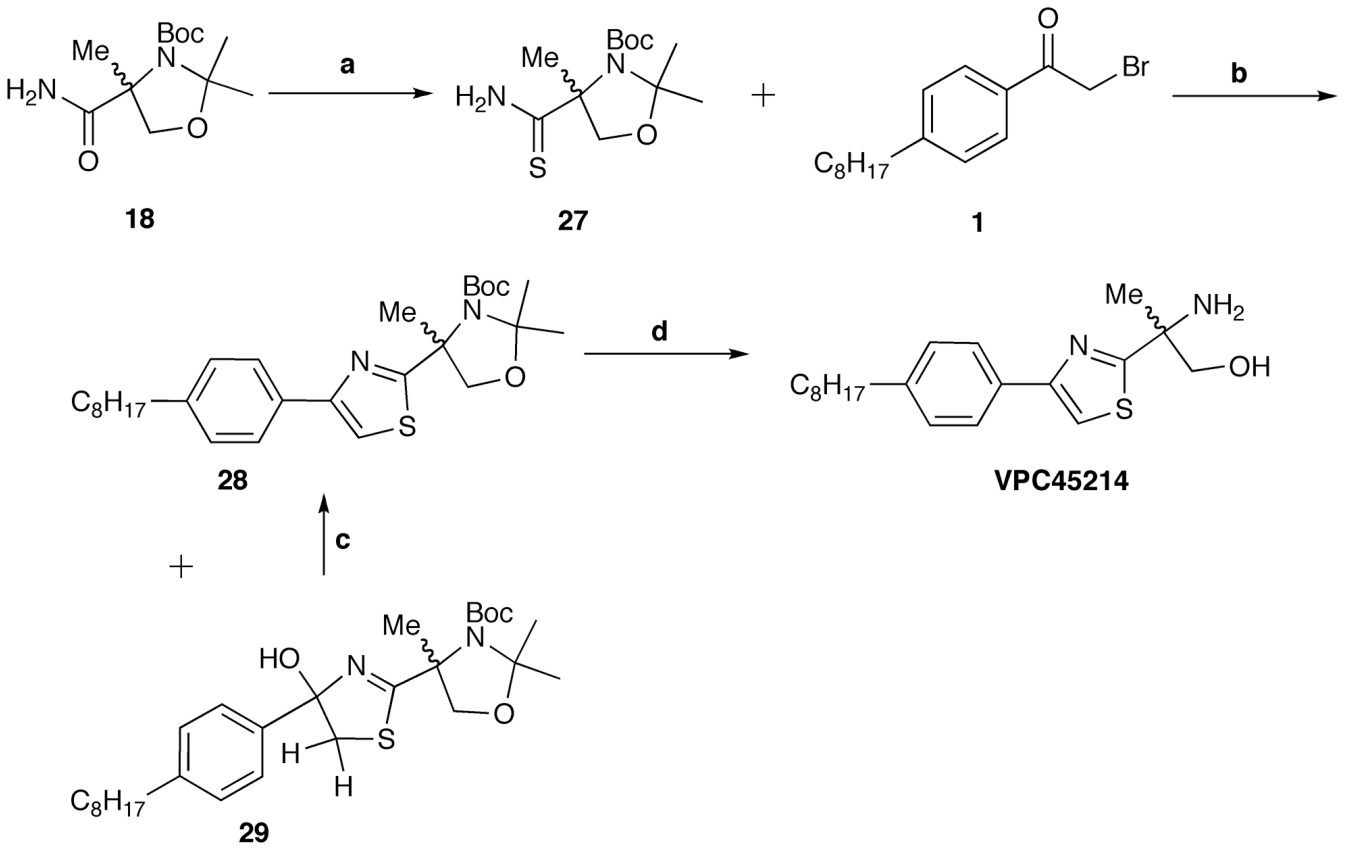
Scheme 6.

Reactants and Conditions. a.) MeOH, $SOCl_2$, 0 °C to rt, 24h. (81%); b.) 1-octyne, $Pd(OAc)_2$, Bu_4NOAc, DMF, rt, overnight (83%); c.) H_2, Pd/C, EtOH, rt, 4–6 h. (99%); d.) 20% $NaOH_{(aq)}$, 95% EtOH(aq), rt, 2h., then 1N H_2SO_4 rt, 15 min. (99%); e.) **20**, PyBOP, *i*-Pr_2NEt, CH_2Cl_2, rt, 4h (43%); f.) DMF, 110 °C, 16h. (60%); g.) TFA, CH_2Cl_2, rt, 3h.; then $NaHCO_3$, rt, 15 min. (>90%).



Scheme 7.

Synthesis of 5-phenyl-1,2,4-oxadiazole phosphate **VPC46032**. Reagents and conditions: a.) Boc_2O, TEA, CH_2Cl_2; b.) Tetrazole, MeCN, *N*,*N*-diisopropylditertbutylphosphoramidite; 30% H_2O_2; c.) TMSBr, DCM (32% over 3 steps).



Scheme 8.

Synthesis of thiazole **VPC45214**. a.) Lawesson's Reagent, THF, rt, 4h. (46%); b.) i. $KHCO_3$, DME, −15 °C, 15 min; ii. α-bromoketone, −15 °C, 30 min., then rt, 30 min.; iii. TFAA, Lutidine, DME, −15 °C to rt, 12 h. (39%); c.) TFAA, lutidine, DME, −15 °C to rt, overnight, (>95%); d.) TFA, CH_2Cl_2, rt, 6h.; then $NaHCO_3$, 15 min. (62%)

Table 1

GTP-[γ-^{35}S] ASSAYS

	S1P Receptors									
	$S1P_1$		$S1P_2$		$S1P_3$		$S1P_4$		$S1P_5$	
	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}
S1P	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
FTY720P	0.28	1.00	NAA	0.00	0.23	0.50	0.15	0.78	4.32	0.56
VPC44218	1.09	0.87	NAA	0.00	50.77	0.65	0.59	0.93	3.62	0.83
VPC44239	8.09	0.92	NAA	0.00	1,000	0.61	4.13	1.19	16.15	0.83
VPC45070	24.40	0.89	NAA	0.00	22.99	0.51	0.52	0.81	4.35	0.73
VPC46023	2.50	1.00	NA	0.00	NA	NA	NA	NA	21.60	1.00
VPC92249	4.28	0.80	NA	0.00	52.52	1.00	NA	NA	24.07	0.60

NAA = No Agonist Activity detected. NA = Not Assessed. [a]·EC_{50} and [b]·E_{MAX} values were normalized to those of S1P. In a typical assay, the EC_{50} value of S1P was 10 nanoM (100 nanoM at $S1P_4$) and the membrane bound GTP[γ-^{35}S] varied from 1,000 to 3,000 cpm in response to increasing S1P concentrations.