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WO 02/064616 A3

(54) Title: AGONISTS AND ANTAGONISTS OF SPIINGOSINE-1-PIOSPILATE RECEPTORS

(57) Abstract: The present invention relates to S1P analogs that have activity as S1P receptor modulating agents and the use of such compounds to treat diseases associated with inappropriate S1P receptor activity. The compounds have the general structure (I), wherein R₁ is C₃-C₂₂ alkyl, C₈-C₂₂ alkenyl or R₁₂ is O, or R₁ and R₁₂ taken together form an optionally substituted aryl or an optionally substituted heteroaryl; R₁₇ is H, alkyl or alkylaryl; R₁₈ is N or CH; R₂ and R₃ are independently selected from the group consisting of H, NH₂, and OH, with the proviso that at least one of R₂ and R₃ is NH₂; R₄ is selected from the group consisting of hydroxyl, phosphate, phosphonate methylene phosphonate, α-substituted methylene phosphonate, thiophosphate and thiophosphonate; and R₅ is C₈-C₂₂alkenyl.

Agonists and Antagonists of Sphingosine-1-phosphate Receptors

US Government Rights

This invention was made with United States Government support
5 under Grant No. NIH R01 GM52722 and NIH R01 CA88994 awarded by National
Institutes of Health. The United States Government has certain rights in the
invention.

Related Applications

10 This application claims priority under 35 U.S.C. §119(e) to provisional
patent application nos. 60/264,927 filed January 30, 2001; and 60/327,814 filed
October 9, 2001 the disclosures of which are incorporated herein.

Field of the Invention

15 The present invention is directed to compositions comprising
sphingosine-1-phosphate analogs and methods of using such analogs as agonist or
antagonists of sphingosine-1-phosphate receptor activity. Selective agonists and
antagonists at sphingosine-1-phosphate receptors will be useful therapeutically in a
wide variety of human disorders.

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Background of the Invention

Sphingosine-1-phosphate (S1P) has been demonstrated to induce many
cellular effects, including those that result in platelet aggregation, cell proliferation,
cell morphology, tumor-cell invasion, endothelial cell chemotaxis and endothelial cell
25 *in vitro* angiogenesis. For these reasons, S1P receptors are good targets for
therapeutic applications such as wound healing and tumor growth inhibition.
Sphingosine-1-phosphate signals cells in part via a set of G protein-coupled receptors
named S1P1, S1P2, S1P3, S1P4, and S1P5 (formerly Edg-1, Edg-5, Edg-3, Edg-6,
and Edg-8, respectively). These receptors share 50-55% identical amino acids and
30 cluster with three other receptors (LPA1, LPA2, and LPA3 (formerly Edg-2, Edg-4
and Edg-7)) for the structurally-related lysophosphatidic acid (LPA).

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A conformational shift is induced in the G-Protein Coupled Receptor (GPCR) when the ligand binds to that receptor, causing GDP to be replaced by GTP on the α - subunit of the associated G-proteins and subsequent release of the G-proteins into the cytoplasm. The α - subunit then dissociates from the $\beta\gamma$ - subunit and each subunit can then associate with effector proteins, which activate second messengers leading to a cellular response. Eventually the GTP on the G-proteins is hydrolyzed to GDP and the subunits of the G-proteins reassociate with each other and then with the receptor. Amplification plays a major role in the general GPCR pathway. The binding of one ligand to one receptor leads to the activation of many G-proteins, each capable of associating with many effector proteins leading to an amplified cellular response.

S1P receptors make good drug targets because individual receptors are both tissue and response specific. Tissue specificity of the S1P receptors is important because development of an agonist or antagonist selective for one receptor localizes the cellular response to tissues containing that receptor, limiting unwanted side effects. Response specificity of the S1P receptors is also of importance because it allows for the development of agonists or antagonists that initiate or suppress certain cellular responses without affecting other responses. For example, the response specificity of the S1P receptors could allow for an S1P mimetic that initiates platelet aggregation without affecting cell morphology.

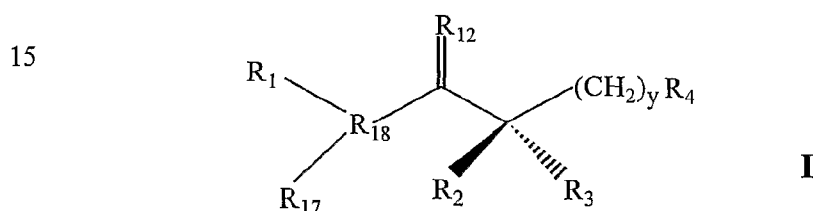
Sphingosine-1-phosphate is formed as a metabolite of sphingosine in its reaction with sphingosine kinase and is stored in abundance in the aggregates of platelets where high levels of sphingosine kinase exist and sphingosine lyase is lacking. S1P is released during platelet aggregation, accumulates in serum and is also found in malignant ascites. Biodegradation of S1P most likely proceeds via hydrolysis by ectophosphohydrolases, specifically the sphingosine 1- phosphate phosphohydrolases.

The physiologic implications of stimulating individual S1P receptors are largely unknown due in part to a lack of receptor type selective ligands. Therefore there is a need for compounds that have strong affinity and high selectivity for S1P receptor subtypes. Isolation and characterization of S1P analogs that have potent agonist or antagonist activity for S1P receptors has been limited due to the

complication of synthesis derived from the lack of solubility of S1P analogs. The present invention is directed to a series of substituted amides that vary in degrees of size, hydrophobicity, and stereochemistry and are active at S1P receptors.

5 Summary of the Invention

The present invention is directed to compositions comprising sphingosine-1-phosphate analogs and methods of using such analogs as agonist or antagonists of sphingosine-1-phosphate receptor activity to treat a wide variety of human disorders. S1P analogs of the present invention have a range of activities including agonism, with various degrees of selectivity at individual S1P receptor subtypes, as well as compounds with antagonist activity at the S1P receptors. More particularly, the S1P analogs of the present invention include compounds with the general structure:



wherein R_1 is selected from the group consisting of alkyl, alkenyl, alkynyl, alkyl(optionally substituted aryl), arylalkyl and arylalkyl(optionally substituted)aryl;

R_{12} is O or R_1 and R_{12} taken together form an optionally substituted aryl or an optionally substituted heteroaryl;

R_{17} is H, alkyl or alkylaryl;

25 R_{18} is N or CH;

R_2 and R_3 are independently selected from the group consisting of H, NH_2 , OH, C_1 - C_6 alkyl, $-(C_1-C_4 \text{ alkyl})OH$, and $-(C_1-C_4 \text{ alkyl})NH_2$; y is an integer from 1-10,

and R_4 is selected from the group consisting of hydroxyl, phosphate, methylene phosphonate, α -substituted methylene phosphonate, thiophosphate, thiophosphonate and other phosphate analogs and phosphonate analogs or a pharmaceutically acceptable salt thereof. Selective agonists and antagonists at S1P receptors will be useful therapeutically in a wide variety of human disorders.

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Brief Description of the Drawings

Fig. 1A-1F are graphic representations of [γ - 35 S]GTP binding to HEK293T cell membranes (containing different S1P receptors) in response to S1P, VPC23019 and VPC23031. Fig. 1A = S1P1 receptor, Fig. 1B = S1P3 receptor, Fig. 1C = S1P2 receptor, Fig. 1D = S1P4 receptor, Fig. 1E = S1P5 receptor, and Fig. 1F = S1P3 receptor. Each data point represents the mean of three determinations.

Fig. 2A-2E are graphic representations of [γ - 35 S]GTP binding to HEK293T cell membranes (containing different S1P receptors) in response to S1P, VPC23065 and VPC23069. Fig. 2A = S1P1 receptor, Fig. 2B = S1P3 receptor, Fig. 2C = S1P2 receptor, Fig. 2D = S1P4 receptor, and Fig. 2E = S1P5 receptor. Each data point represents the mean of three determinations.

Fig. 3A-3E are graphic representations of [γ - 35 S]GTP binding to HEK293T cell membranes (containing different S1P receptors) in response to S1P, VPC23075 and VPC23079. Fig. 3A = S1P1 receptor, Fig. 3B = S1P3 receptor, Fig. 3C = S1P2 receptor, Fig. 3D = S1P4 receptor, and Fig. 3E = S1P5 receptor. Each data point represents the mean of three determinations.

Fig. 4A-4E are graphic representations of [γ - 35 S]GTP binding to HEK293T cell membranes (containing different S1P receptors) in response to S1P, VPC23087 and VPC23089. Fig. 4A = S1P1 receptor, Fig. 4B = S1P3 receptor, Fig. 4C = S1P2 receptor, Fig. 4D = S1P4 receptor, and Fig. 4E = S1P5 receptor. Each data point represents the mean of three determinations.

Fig. 5A and 5B. Fig. 5A is a graphic representation of [γ - 35 S]GTP binding to HEK293T cell membranes containing the S1P1 receptor, in response to S1P, VPC23087 and VPC23087 + S1P. Fig. 5B is a graphic representation of [γ - 35 S]GTP binding to HEK293T cell membranes containing the S1P3 receptor, in response to S1P, VPC23089 and VPC23089 + S1P. Each data point represents the mean of three determinations.

Detailed Description of the Invention

Definitions

In describing and claiming the invention, the following terminology will be used in accordance with the definitions set forth below.

5 As used herein, the term "purified" and like terms relate to the isolation of a molecule or compound in a form that is substantially free of contaminants normally associated with the molecule or compound in a native or natural environment.

As used herein, the term "treating" includes prophylaxis of the specific
10 disorder or condition, or alleviation of the symptoms associated with a specific disorder or condition and/or preventing or eliminating said symptoms.

As used herein, an "effective amount" means an amount sufficient to produce a selected effect. For example, an effective amount of an S1P receptor antagonist is an amount that decreases the cell signaling activity of the S1P receptor.

15 As used herein, the term "halogen" means Cl, Br, F, and I. Especially preferred halogens include Cl, Br, and F. The term "haloalkyl" as used herein refers to a C₁-C₄ alkyl radical bearing at least one halogen substituent, for example, chloromethyl, fluoroethyl or trifluoromethyl and the like.

The term "C₁-C_n alkyl" wherein n is an integer, as used herein,
20 represents a branched or linear alkyl group having from one to the specified number of carbon atoms. Typically C₁-C₆ alkyl groups include, but are not limited to, methyl, ethyl, n-propyl, iso-propyl, butyl, iso-butyl, sec-butyl, tert-butyl, pentyl, hexyl and the like.

The term "C₂-C_n alkenyl" wherein n is an integer, as used herein,
25 represents an olefinically unsaturated branched or linear group having from 2 to the specified number of carbon atoms and at least one double bond. Examples of such groups include, but are not limited to, 1-propenyl, 2-propenyl, 1,3-butadienyl, 1-butenyl, hexenyl, pentenyl, and the like.

The term "C₂-C_n alkynyl" wherein n is an integer refers to an
30 unsaturated branched or linear group having from 2 to the specified number of carbon atoms and at least one triple bond. Examples of such groups include, but are not limited to, 1-propynyl, 2-propynyl, 1-butynyl, 2-butynyl, 1-pentynyl, and the like.

The term "C₃-C_n cycloalkyl" wherein n = 8, represents cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl.

As used herein, the term "optionally substituted" refers to from zero to four substituents, wherein the substituents are each independently selected. Each of
5 the independently selected substituents may be the same or different than other substituents.

As used herein the term "aryl" refers to a mono- or bicyclic carbocyclic ring system having one or two aromatic rings including, but not limited to, phenyl, benzyl, naphthyl, tetrahydronaphthyl, indanyl, indenyl, and the like. "Optionally substituted aryl" includes aryl compounds having from zero to four substituents, and
10 "substituted aryl" includes aryl compounds having one to three substituents, wherein the substituents, including alkyl, halo or amino substituents. The term (C₅-C₈ alkyl)aryl refers to any aryl group which is attached to the parent moiety via the alkyl group.

15 The term "heterocyclic group" refers to a mono- or bicyclic carbocyclic ring system containing from one to three heteroatoms wherein the heteroatoms are selected from the group consisting of oxygen, sulfur, and nitrogen.

As used herein the term "heteroaryl" refers to a mono- or bicyclic carbocyclic ring system having one or two aromatic rings containing from one to three
20 heteroatoms and includes, but is not limited to, furyl, thienyl, pyridyl and the like.

The term "bicyclic" represents either an unsaturated or saturated stable 7- to 12-membered bridged or fused bicyclic carbon ring. The bicyclic ring may be attached at any carbon atom which affords a stable structure. The term includes, but is not limited to, naphthyl, dicyclohexyl, dicyclohexenyl, and the like.

25 The terms 16:0, 18:0, 18:1, 20:4 or 22:6 hydrocarbon refers to a branched or straight alkyl or alkenyl group, wherein the first integer represents the total number of carbons in the group and the second integer represent the number of double bonds in the group.

As used herein, an "S1P modulating agent" refers a compound or
30 composition that is capable of inducing a detectable change in S1P receptor activity *in vivo* or *in vitro* (e.g., at least 10% increase or decrease in S1P activity as measured by a given assay such as the bioassay described in Example 2).

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As used herein, the term "EC₅₀ of an agent" refers to that concentration of an agent at which a given activity, including binding of sphingosine or other ligand of an S1P receptor and/or a functional activity of a S1P receptor (e.g., a signaling activity), is 50% maximal for that S1P receptor. Stated differently, the EC₅₀ is the concentration of agent that gives 50% activation, when 100% activation is set at the amount of activity of the S1P receptor which does not increase with the addition of more ligand/agonist and 0% is set at the amount of activity in the assay in the absence of added ligand/agonist.

As used herein, the term "phosphate analog" and "phosphonate analog" comprise analogs of phosphate and phosphonate wherein the phosphorous atom is in the +5 oxidation state and one or more of the oxygen atoms is replaced with a non-oxygen moiety, including for example, the phosphate analogs phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, boronophosphates, and the like, including associated counterions, e.g., H, NH₄, Na, and the like if such counterions are present.

The S1P analogs of the present invention contain one or more asymmetric centers in the molecule. In accordance with the present invention a structure that does not designate the stereochemistry is to be understood as embracing all the various optical isomers, as well as racemic mixtures thereof.

The Invention

The present invention is directed to S1P analogs that have activity as modulators of S1P receptor activity. Modulators of S1P activity include agents that have agonist and antagonist activity at the S1P receptor as well as analogs of those compounds that have been modified to resist enzymatic modification (i.e. block modification of the compounds by phosphohydrolases, sphingosine lyases or sphingosine kinases), or provide a suitable substrate for sphingosine kinases to convert an administered form into a more active form.

The structure of S1P can be described as a combination of three regions: the phosphate head group, the linker region, and the fatty acid tail. Through structure activity relationships (SAR) of the closely related lysophospholipid,

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lysophosphatidic acid (LPA), it has been determined that the presence of a phosphate head group is an important feature to allow binding of S1P to its S1P receptors.

However, there are exceptions to the requirement for a phosphate head group. In particular a phosphonate, hydroxyl, phosphate or phosphonate group can be

5 substituted for the phosphate head group while retaining activity at the S1P receptor.

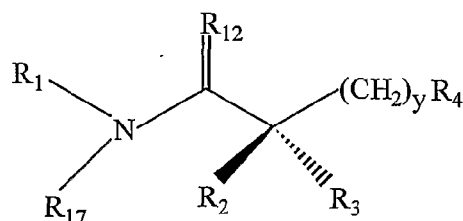
Based on the SAR of LPA, the linker region of S1P is anticipated to be the area of the molecule that can best accommodate change. Again using the SAR of LPA as a lead, it is presumed that presence of a hydrogen bond donor 5 bonds away from the phosphate is important to binding. From a retrosynthetic standpoint, the linker region may be seen as a functionalized derivative of L-Serine.

Due to the long fatty acid chain and charged phosphate head group, S1P has an amphipathic nature that makes it extremely insoluble in organic solvents. Manipulation of the saturation of the fatty acid chain may compromise aggregate formation of the molecule, thereby increasing solubility. The most important aspect of the long chain, however, is the length. GTP γ S studies that have been completed thus far have demonstrated that an 18 carbon backbone, as is the case in S1P, displays optimal activity compared to 16 and 20 carbon backbones.

It is also anticipated that the S stereochemistry of the C-2 amine may have an effect on binding as one would expect from a receptor. Hydrogen bonds from the phosphate head group and the C-2 amine to adjacent arginine and glutamic acid residues on the model receptor have been demonstrated to be important to S1P-receptor binding.

In accordance with one embodiment an S1P receptor modulating compound is provided wherein the compound has the general structure:

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wherein R₁ is selected from the group consisting of alkyl, alkenyl, alkynyl, alkyl(optionally substituted aryl), alkyl(optionally substituted cycloalkyl), arylalkyl, and arylalkyl(optionally substituted)aryl;

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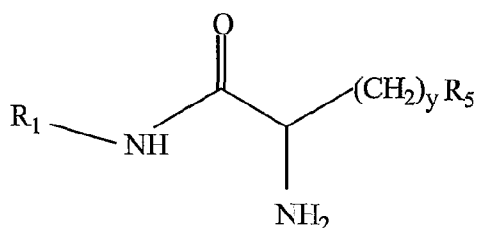
R_{12} is O, or R_1 and R_{12} taken together form an optionally substituted heteroaryl;

R_{17} is H, C_1 - C_4 alkyl or $(CH_2)_n$ aryl;

R_2 and R_3 are independently selected from the group consisting of H, NH_2 ,
 5 OH, C_1 - C_6 alkyl, $-(C_1-C_4 \text{ alkyl})OH$, and $-(C_1-C_4 \text{ alkyl})NH_2$; y is an integer from 1-10,
 and R_4 is selected from the group consisting of hydroxyl, phosphate, methylene
 phosphonate, α -substituted methylene phosphonate, phosphate analogs and
 phosphonate analogs or a pharmaceutically acceptable salt thereof. In one
 embodiment one of the R_2 and R_3 substituents of Formula I is NH_2 . Examples of
 10 pharmaceutically acceptable salts of the compounds of the formula (I) include salts
 with inorganic acids, such as hydrochloride, hydrobromide and sulfate, salts with
 organic acids, such as acetate, fumarate, maleate, benzoate, citrate, malate,
 methanesulfonate and benzenesulfonate salts, and when a carboxy group is present,
 salts with metals such as sodium, potassium, calcium and aluminium, salts with
 15 amines, such as triethylamine and salts with dibasic amino acids, such as lysine. The
 compounds and salts of the present invention encompass hydrate and solvate forms.

In one embodiment, an S1P modulating compound is provided having the general structure:

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wherein R_1 is selected from the group consisting of C_8 - C_{22} alkyl, C_8 - C_{22} alkenyl, C_8 - C_{22} alkynyl and $-(CH_2)_n-Z-R_6$;

R_5 is selected from the group consisting of hydroxyl, phosphonate, α -substituted methylene phosphonate, phosphate analogs and phosphonate analogs;

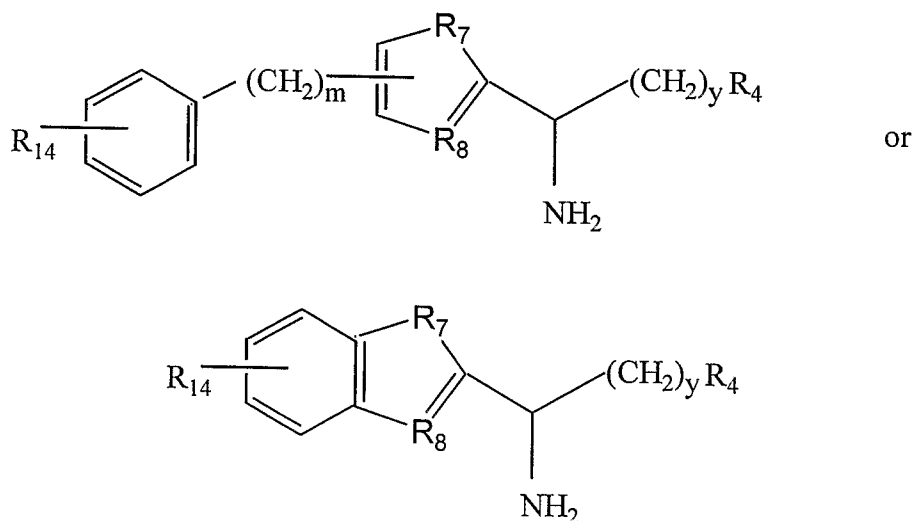
y is an integer ranging from 1 to 4;

n is an integer ranging from 0 to 10;

5 Z is selected from the group consisting of cycloalkyl, aryl and heteroaryl; and

R_6 is selected from the group consisting of H, C_1 - C_{12} alkyl, C_1 - C_{20} alkoxy, C_1 - C_{20} alkylthio, and C_1 - C_{20} alkylamino. In one embodiment, R_1 is C_8 - C_{22} alkyl, and more preferably C_{12} - C_{16} alkyl, y is 1 or 2 and R_5 is hydroxy, phosphate or phosphonate. Alternatively, in one embodiment, R_1 is $-(CH_2)_n-Z-R_6$, wherein n is an integer ranging from 1-4, Z is aryl and R_6 is C_1 - C_{10} alkyl; more preferably, Z is phenyl, R_5 is hydroxy, phosphate or phosphonate, and R_6 is C_6 - C_{10} alkyl. In another embodiment R_1 is C_8 - C_{22} alkyl or $-(CH_2)_n-Z-R_6$, y is 1, R_{17} is H, Z is aryl and R_6 is C_1 - C_{10} alkyl or C_1 - C_{20} alkoxy.

15 In another embodiment of the present invention an S1P modulating compound is provided having the general structure:



wherein R_{14} is selected from the group consisting of H, hydroxy, NH_2 , C_8 - C_{22} alkyl, C_8 - C_{22} alkenyl, C_8 - C_{22} alkynyl and $-(CH_2)_n-Z-R_6$;

30 R_4 is selected from the group consisting of hydroxyl, phosphate, phosphonate, α -substituted methylene phosphonate, phosphate analogs and phosphonate analogs;

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y is an integer ranging from 1 to 4;

m is an integer ranging from 0 to 4;

n is an integer ranging from 0 to 10;

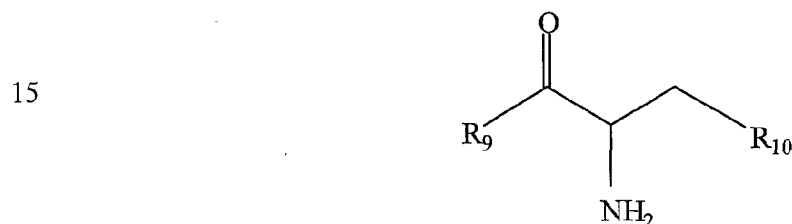
Z is selected from the group consisting of cycloalkyl, aryl and heteroaryl; and

5 R_6 is selected from the group consisting of H, C_1 - C_{12} alkyl, C_1 - C_{20} alkoxy, C_1 - C_{20} alkylthio, and C_1 - C_{20} alkylamino; and

R_7 and R_8 are independently selected from the group consisting of O, S and N.

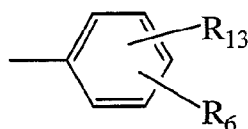
In one embodiment R_1 is selected from the group consisting of C_8 - C_{22} alkyl, C_8 - C_{22} alkenyl and C_8 - C_{22} alkynyl, R_4 is hydroxyl, phosphate or phosphonate, y is 1 or 2, m is
10 0 or 1 and either R_7 or R_8 is N; more preferably, R_1 is C_4 - C_{10} alkyl, R_4 is hydroxyl or phosphate, y is 1, m is 0 and R_7 and R_8 are both N.

The present invention also encompasses compounds of the general structure:



wherein R_9 is selected from the group consisting of $-NR_1$, and $-OR_1$;

20 R_1 is selected from the group consisting of C_8 - C_{22} alkyl and

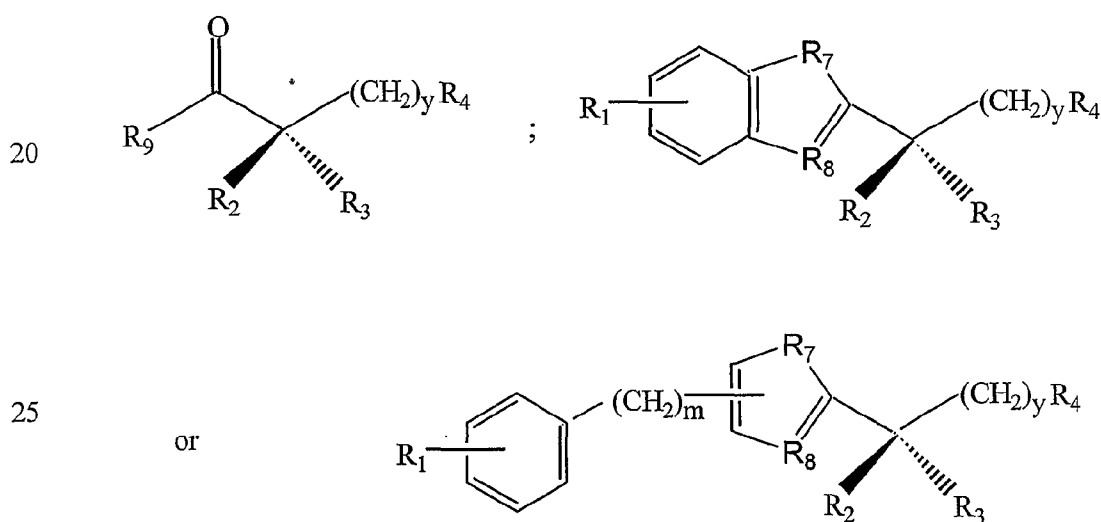


25 wherein R_6 and R_{13} are independently selected from the group consisting of H, C_1 - C_{10} alkyl and C_1 - C_{20} alkoxy and R_{10} is hydroxy, phosphonate, methylene phosphonate or phosphate, with the proviso that when R_9 is $-NR_1$, R_{10} is not phosphate. In one preferred embodiment, R_9 is $-NR_1$, R_6 is C_1 - C_{10} alkyl, R_{13} is H and R_{10} is hydroxy, phosphonate, or methylene phosphonate.

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A GTP[γ ³⁵S] binding assay was developed to analyze directly the activation of individual S1P receptors, and thus allow the identification of S1P receptor agonists and antagonists as well as determine the relative efficacies and potencies at each receptor in a common system. The same results were obtained
 5 regardless of whether the recombinant receptor used exogenous G proteins (HEK293T cells) or endogenous G proteins (RH7777 cells). In addition, insect Sf9 cells infected with recombinant baculovirus encoding receptors (e.g. LPA and S1P receptors) and G proteins can also serve as the source of membranes for the broken cells used in the GTPgammaS-35 binding assays. The Sf9 cell and HEK293T cell membranes gave
 10 similar results. Furthermore, the activities measured in the broken cell assay predicted the responses seen in whole cell assays. Thus the primary assay used in the present invention for determining compound potency and efficacy is a valid measure of activity at the S1P receptors.

The GTP[γ ³⁵S] binding assay has revealed that the compounds of the present invention have the ability to modulate S1P receptor activity (See Examples 2 and 3). More particularly, compounds having the general structure:



wherein R₉ is selected from the group consisting of -NR₁, and -OR₁;

R₁ is selected from the group consisting of alkyl, alkenyl, alkynyl,
 30 optionally substituted aryl, optionally substituted heteroaryl, alkyl(optionally substituted aryl), arylalkyl and arylalkyl(optionally substituted)aryl;

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R_2 and R_3 are independently selected from the group consisting of H, NH_2 , OH, C_1-C_6 alkyl, $-(C_1-C_4 \text{ alkyl})OH$, $-(C_1-C_4 \text{ alkyl})NH_2$, $-(C_1-C_4 \text{ alkyl})aryl(C_0-C_4 \text{ alkyl})$ and $-(C_1-C_4 \text{ alkyl})aryloxyaryl(C_0-C_4 \text{ alkyl})$;

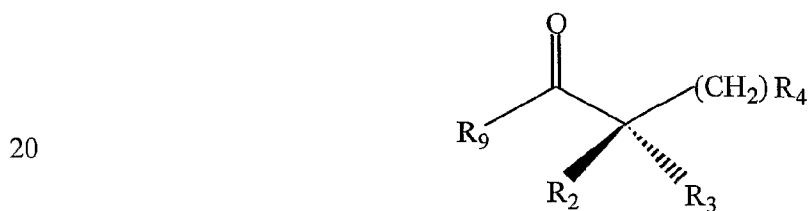
y is an integer from 1-10;

5 R_4 is selected from the group consisting of hydroxyl, phosphate, phosphonate, methylene phosphonate, α -substituted methylene phosphonate, phosphate analogs and phosphonate analogs;

m is an integer ranging from 0 to 4; and

10 R_7 and R_8 are independently selected from the group consisting of O, S and N display activity as modulators of S1P activity. In one embodiment R_1 is selected from the group consisting of C_8-C_{22} alkyl, C_8-C_{22} alkenyl, C_8-C_{22} alkynyl and $-(CH_2)_n-Z-R_6$, wherein n is an integer ranging from 0 to 10, Z is selected from the group consisting of aryl and heteroaryl and R_6 is selected from the group consisting of H, C_1-C_{10} alkyl, C_1-C_{20} alkoxy, C_1-C_{20} alkylthio, and C_1-C_{20} alkylamino, and at least one of R_2 and R_3 is NH_2 .

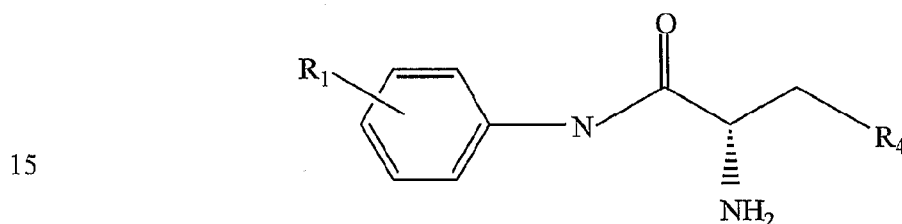
As described in Example 2 compounds having the general structure



wherein R_9 is selected from the group consisting of $-NR_1$, and $-OR_1$, R_1 is C_8-C_{22} alkyl, R_2 and R_3 are independently selected from the group consisting of H and NH_2 , wherein at least one of R_2 and R_3 is NH_2 and R_4 is phosphate all display significant agonist activity at the S1P receptors tested (S1P1, S1P2, S1P3, S1P5), although none were as potent as S1P itself (See Table 1 of Example 2). However, one compound, VPC22135 (wherein R_2 is H, R_3 is NH_2 , R_4 is phosphate and R_9 is $-N(CH_2)_{13}CH_3$), approached the potency of S1P at both the human S1P1 and human S1P3 receptors. In accordance with one embodiment of the present invention compound VPC22135 is used as a selective agonist of human S1P1 and human S1P3 receptors. Curiously, this compound has the amino group in the unnatural (R)

configuration. Its enantiomer, VPC22053, was more than 1 log order less potent at both the S1P1 and S1P3 receptors.

An additional series of compounds have shown activity in modulating S1P receptor activity, however these compounds also displayed selectivity for certain S1P receptor subtypes (See Example 3 and Figures 1-5). Each of these compounds (VPC 23019, 23031, 23065, 23069, 23087, 23089, 23075, 23079) are inactive at the S1P2 receptor. Compounds VPC23031, 23019, 23089 are inverse agonists (antagonists of the S1P3) receptor, but this inverse agonism becomes agonism when the alkyl chain length is 9 carbons (VPC23079) or 10 (VPC23069). In accordance with one embodiment of the present invention an antagonist of S1P activity is provided. In particular, a compound having the general structure:



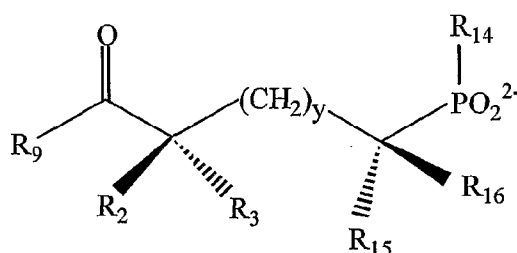
wherein R_1 is C_4 - C_8 alkyl and R_4 is phosphate has antagonist activity at the S1P3 receptor. In accordance with one embodiment the R_1 substituent is located in the ortho or meta position on the phenyl ring, and in one embodiment the R_1 substituent is located in the meta position on the phenyl ring.

Compounds VPC23065, VPC23087 and VPC23075 are primary alcohols, i.e. R_4 of formula I is hydroxy. These compounds demonstrates significant agonist activity at various S1P receptors. In particular, the S1P4 receptor binds to the primary alcohol S1P analogs with an EC_{50} within a log order of the phosphorylated compounds. Since S1P4 is present on lymphocytes, the use of the primary alcohol analogs may be used for immuno-suppression. In addition, it is also hypothesized that the hydroxy moiety of the primary alcohols may be converted to phosphates *in vivo*. Therefore the primary alcohol S1P analogs of the present invention may also serve as prodrug forms of active S1P receptor modulating compounds.

S1P is metabolized by a variety of conceivable routes including phosphatases, esterases or transported into cells. The S1P signal at receptors might be prolonged if the routes of degradation could be evaded or inhibited by S1P structural analogs. The S1P analogs of the present invention can be used, in accordance with one embodiment, to inhibit or evade endogenous S1P metabolic pathways including phosphatases, esterases, transporters and S1P acyl transferases. For example those S1P analogs of Formula I that lack an ester bond would be resistant to degradation by endogenous esterases. One embodiment of the present invention is directed to compounds that function as a S1P receptor agonists and antagonists that are resistant to hydrolysis by lipid phosphate phosphatases (LPPs) or are sub-type selective inhibitors of LPPs, and in particular are resistant to hydrolysis by sphingosine 1-phosphate phosphohydrolase. Previously described S1P mimetics contain a phosphate group, and thus are likely susceptible to hydrolysis by LPPs.

Alpha hydroxy phosphonates are well known phosphate mimetics. For example, the compounds used clinically to treat osteoporosis (pamidronate, alendronate) are alpha hydroxy bisphosphonates that are analogs of pyrophosphate. S1P analogs can be prepared wherein the phosphate moiety is replaced by an alpha hydroxy phosphonate. Accordingly, one aspect of the present invention is directed to lipid phosphate phosphatase resistant S1P analogs having the general structures:

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wherein R_9 is selected from the group consisting of $-NR_1$, and $-OR_1$;

R_1 is selected from the group consisting of C_8 - C_{22} alkyl, C_8 - C_{22} alkenyl, C_8 - C_{22} alkynyl and $-(CH_2)_n-Z-R_6$, wherein n is an integer ranging from 0 to 10, Z is selected from the group consisting of aryl and heteroaryl and R_6 is selected

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from the group consisting of H, C₁-C₁₀ alkyl, C₁-C₂₀ alkoxy, C₁-C₂₀ alkylthio, and C₁-C₂₀ alkylamino;

R₂ and R₃ are independently selected from the group consisting of H, NH₂, OH, C₁-C₆ alkyl, -(C₁-C₄ alkyl)OH, -(C₁-C₄ alkyl)NH₂, -(C₁-C₄ alkyl)aryl(C₀-C₄ alkyl) and -(C₁-C₄ alkyl)aryloxyaryl(C₀-C₄ alkyl);

y is an integer from 0-10;

R₁₄ is selected from the group consisting of O, S and NH; and

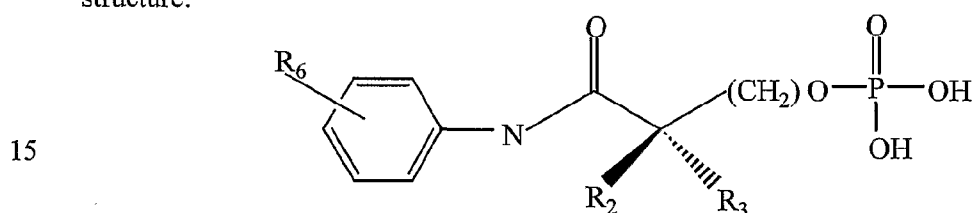
R₁₅ and R₁₆ are independently selected from the group consisting of H, hydroxyl, amino, COOH, halo, -PO₃; or R₁₅ and R₁₆ taken together form a keto group or a methylene group. In one preferred embodiment, R₉ is -NR₁, wherein R₁ is C₈-C₂₂ alkyl or -(CH₂)_n-Z-R₆, one of R₂ and R₃ is H and the other NH₂, y is 0 or 1, R₁₅ and R₁₆ are independently H or hydroxyl, and R₁₄ is O.

Lysophospholipids sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) stimulate cellular proliferation and affect numerous cellular functions by signaling through G protein-coupled endothelial differentiation gene-encoded (S1P) receptors. Accordingly, the S1P receptor agonists disclosed in the present invention are anticipated to have utility in a variety of clinical settings including but not limited to the acceleration of wound healing (including corneal wounds), the promotion of myelination (oligodendrocyte cell function) and for immuno-modulation. In particular, LPA has been reported (Balazs et al. *Am J Physiol Regul Integr Comp Physiol*, 2001 280(2):R466-472) as having activity in accelerating wound closing and increasing neopithelial thickness.

In accordance with one embodiment of the present invention a pharmaceutical composition comprising one or more of the S1P receptor agonists of the present invention is administered to a mammalian species (including humans) to enhance wound repair, improve neuronal function or enhance an immune response of that species. It has also been reported that S1P inhibits fibrosis in various organs. Accordingly, the S1P receptor agonists of the present invention can be used to prevent/treat diseases associated with fibrosis of organs such as pulmonary fibrosis, interstitial pneumonia, chronic hepatitis, hepatic cirrhosis, chronic renal insufficiency or kidney glomerular sclerosis. In one embodiment a composition comprising an S1P receptor agonist of the present invention is used to treat wounds, including burns,

cuts, lacerations, surgical incisions, bed sores, and slow-healing ulcers such as those seen in diabetics. Typically the composition is administered locally as a topical formulation, however other standard routes of administration are also acceptable.

In an another embodiment, the S1P receptor modulating compounds of the present invention are administered to a subject to treat or prevent a disorder of abnormal cell growth and differentiation as well as inflammatory diseases. These disorders include, but are not limited to, Alzheimer's disease, aberrant corpus luteum formation, osteoarthritis, osteoporosis, anovulation, Parkinson's disease, multiple sclerosis, rheumatoid arthritis and cancer. In accordance with one embodiment an S1P antagonist is administered to a patient to treat a disease associated with abnormal growth. In one embodiment a composition comprising a compound of the general structure:



wherein R_6 is selected from the group consisting of C_1 - C_8 alkyl and R_2 and R_3 are independently selected from the group consisting of H, and NH_2 is administered to treat a patient suffering from a disease associated with abnormal cell growth.

20 In addition it is believed that the S1P analogs of the present invention mobilize lymphocytes and increase their homing to secondary lymphoid tissues. Thus the present analogs can be used to direct lymphocytes away from transplanted organs (allografts) or healthy cells (e.g. pancreatic islets (type I diabetes), myelin sheathing (multiple sclerosis)), or other tissues that may be subjected to an undesirable immune response and thus decrease damage to such tissues from the immune system.

25 In accordance with one embodiment the S1P analogs of the present invention are used for immunomodulation. As used herein the term immunomodulation refers to an affect on the functioning of the immune system, and includes both the enhancement of an immune response as well as suppression of the immune response. In one embodiment of the present invention the S1P analogs of the present invention are used as immunomodulators to suppress the immune system and prevent damage to healthy tissue that would otherwise occur in autoimmune diseases

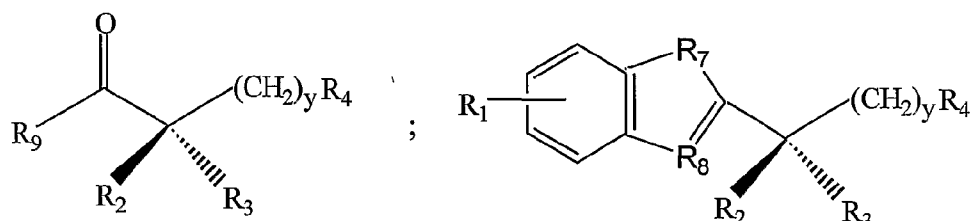
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and in organ transplantation. In particular, the compounds can be administered to patients as part of the treatment associated with organ transplantation, including pancreas, pancreatic islets, kidney, heart and lung transplantations. The S1P analogs can be administered alone or in combo with known immunosuppressants such as cyclosporine, tacrolimus, azathioprine, desoxymetasone, cyclophosphamide, cortisol, cortisone, betametasone, FK 506 (a fungal macrolide immunosuppressant), desametasone, flunisolide, prednisolone, prednisone, amcinomide desonide, methylprednisolone, triamcinolone, alclometasone and methotrexate.

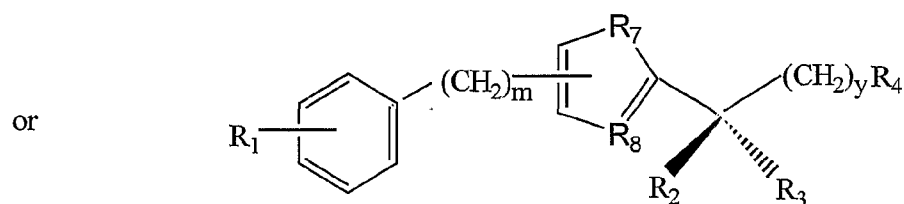
Additionally the S1P analogs of the present invention can be administered to patients suffering from an autoimmune disease to treat that disease. Examples of diseases considered to be autoimmune in nature are: type I diabetes, systemic lupus erythematosus, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease including colitis and Crohn's disease, glomerulonephritis, Hashimoto's thyroiditis, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenic purpura, autoimmune hepatitis and Wegner's granuloma.

In accordance with one embodiment an immunomodulation therapy is provided for treating mammals, including humans, in need thereof. The method comprises the steps of administering to said mammal an effective amount of a compound of the general formula:

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wherein R_3 is selected from the group consisting of $-NR_1$, and $-OR_1$;

R_1 is selected from the group consisting of alkyl, alkenyl, alkynyl, optionally substituted aryl, optionally substituted heteroaryl, alkyl(optionally substituted aryl), arylalkyl and arylalkyl(optionally substituted)aryl;

5 R_2 and R_3 are independently selected from the group consisting of H, NH_2 , OH, C_1-C_6 alkyl, $-(C_1-C_4 \text{ alkyl})OH$, $-(C_1-C_4 \text{ alkyl})NH_2$, $-(C_1-C_4 \text{ alkyl})aryl(C_0-C_4 \text{ alkyl})$ and $-(C_1-C_4 \text{ alkyl})aryloxyaryl(C_0-C_4 \text{ alkyl})$;

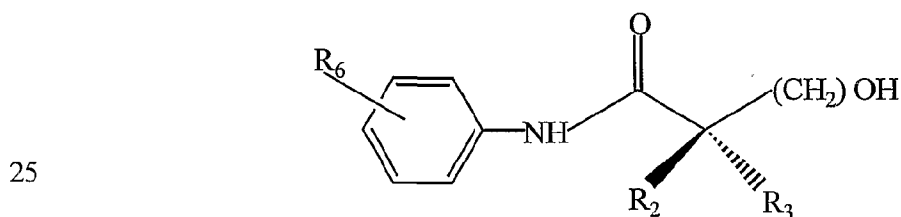
y is an integer from 1-10;

10 R_4 is selected from the group consisting of hydroxyl, phosphate, phosphonate, methylene phosphonate, α -substituted methylene phosphonate, phosphate analogs and phosphonate analogs;

m is an integer ranging from 0 to 4; and

15 R_7 and R_8 are independently selected from the group consisting of O, S and N display activity as modulators of S1P activity. In one embodiment, R_1 is selected from the group consisting of C_8-C_{22} alkyl, C_8-C_{22} alkenyl, C_8-C_{22} alkynyl and $-(CH_2)_n-Z-R_6$, wherein n is an integer ranging from 0 to 10, Z is selected from the group consisting of aryl and heteroaryl and R_6 is selected from the group consisting of H, C_1-C_{10} alkyl, C_1-C_{20} alkoxy, C_1-C_{20} alkylthio, and C_1-C_{20} alkylamino and at least one of R_2 and R_3 is NH_2 .

20 In one embodiment the immunomodulating compound has the general structure:



wherein R_6 is selected from the group consisting of C_1-C_{10} alkyl and R_2 and R_3 are independently selected from the group consisting of H, and NH_2 with the proviso that R_2 and R_3 are not the same, and in one embodiment R_2 is H and R_3 is NH_2 .

30 The dosage to be used is, of course, dependent on the specific disorder to be treated, as well as additional factors including the age, weight, general state of health, severity of the symptoms, frequency of the treatment and whether additional

pharmaceuticals accompany the treatment. The dosages are in general administered several times per day and preferably one to three times per day. The amounts of the individual active compounds are easily determined by routine procedures known to those of ordinary skill in the art

5 S1P also acts as a survival factor in many cell types. In particular S1P receptor agonists are anticipated to have activity in protecting cells and tissues from hypoxic conditions. In accordance with one embodiment the S1P antagonists of the present invention are administered to treat cells and tissues exposed to hypoxic conditions, including injury sustained as a result of ischemia. In accordance with one
10 embodiment the S1P analogs exhibiting S1P receptor antagonist activity can be used to treat ischemia reperfusion type injury. Interference with the supply of oxygenated blood to tissues is defined as ischemia. The effects of ischemia are known to be progressive, such that over time cellular vitality continues to deteriorate and tissues become necrotic. Total persistent ischemia, with limited oxygen perfusion of tissues,
15 results in cell death and eventually in coagulation-induced necrosis despite reperfusion with arterial blood. A substantial body of evidence indicates that a significant proportion of the injury associated with ischemia is a consequence of the events associated with reperfusion of ischemic tissues, hence the term reperfusion injury.

20 The present invention is also directed to pharmaceutical compositions comprising the S1P receptor modulating compounds of the present invention. More particularly, such S1P receptor agonists and antagonists can be formulated as pharmaceutical compositions using standard pharmaceutically acceptable carriers, fillers, solubilizing agents and stabilizers known to those skilled in the art.

25 Pharmaceutical compositions comprising the S1P receptor agonists and/or antagonists are administered to an individual in need thereof by any number of routes including, but not limited to, topical, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means. The oral route
30 is typically employed for most conditions requiring the compounds of the invention. Preference is given to intravenous injection or infusion for the acute treatments. For maintenance regimens the oral or parenteral, e.g. intramuscular or subcutaneous, route

is preferred. In accordance with one embodiment a composition is provided that comprises an S1P analog of the present invention and albumin, more particularly, the composition comprises an S1P analog of the present invention, a pharmaceutically acceptable carrier and 0.1-1.0% albumin. Albumin functions as a buffer and improves the solubility of the compounds.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. In accordance with one embodiment a kit is provided for treating a patient in need of immunomodulation. In this embodiment the kit comprises one or more of the S1P analogs of the present invention and may also include one or more known immunosuppressants. These pharmaceuticals can be packaged in a variety of containers, *e.g.*, vials, tubes, microtiter well plates, bottles, and the like. Other reagents can be included in separate containers and provided with the kit; *e.g.*, positive control samples, negative control samples, buffers, cell culture media, etc. Preferably, the kits will also include instructions for use.

The present invention is also directed to methods for discovering agonists and antagonists of the interaction between S1P and the S1P receptor. Such compounds are identified by using an assay for detecting S1P receptor activity (such as the [γ -³⁵S]GTP binding assay) and assaying for activity in the presence of S1P and the test compound. More particularly, in the method described by Traynor and Nahorski, 1995, *Mol. Pharmacol.* 47: 848-854, incorporated herein by reference, G-protein coupling to membranes can be evaluated by measuring the binding of labeled GTP.

For example, samples comprising membranes isolated from cells expressing an S1P polypeptide can be incubated in a buffer promoting binding of the polypeptide to ligand (i.e. S1P), in the presence of radiolabeled GTP and unlabeled GDP (*e.g.*, in 20 mM HEPES, pH 7.4, 100 mM NaCl, and 10 mM MgCl₂, 80 pM ³⁵S-GTP γ S and 3 μ M GDP), with and without a candidate modulator. The assay mixture is incubated for a suitable period of time to permit binding to and activation of the receptor (*e.g.*, 60 minutes at 30^o C), after which time unbound labeled GTP is removed (*e.g.*, by filtration onto GF/B filters). Bound, labeled GTP can be measured by liquid scintillation counting. A decrease of 10% or more in labeled GTP binding

as measured by scintillation counting in a sample containing a candidate modulator, relative to a sample without the modulator, indicates that the candidate modulator is an inhibitor of S1P receptor activity.

5 A similar GTP-binding assay can be performed without the presence of the ligand (i.e. S1P) to identify agents that act as agonists. In this case, ligand-stimulated GTP binding is used as a standard. An agent is considered an agonist if it induces at least 50% of the level of GTP binding induced by S1P when the agent is present at 10 uM or less, and preferably will induce a level which is the same as or higher than that induced by ligand.

10 GTPase activity can be measured by incubating cell membrane extracts containing an S1P receptor with $\gamma^{32}\text{P}$ -GTP. Active GTPase will release the label as inorganic phosphate, which can be detected by separation of free inorganic phosphate in a 5% suspension of activated charcoal in 20 mM H_3PO_4 , followed by scintillation counting. Controls would include assays using membrane extracts isolated from cells
15 not expressing an S1P receptor (e.g., mock-transfected cells), in order to exclude possible non-specific effects of the candidate modulator. In order to assay for the effect of a candidate modulator on S1P-regulated GTPase activity, cell membrane samples can be incubated with a ligand (e.g., S1P), with and without the modulator, and a GTPase assay can be performed as described above. A change (increase or
20 decrease) of 10% or more in the level of GTP binding or GTPase activity relative to samples without modulator is indicative of S1P modulation by a candidate modulator.

Identified S1P receptor agonists and antagonists can be used to treat a variety of human diseases and disorders, including, but not limited to the treatment of infections such as bacterial, fungal, protozoan and viral infections, particularly
25 infections caused by HIV-1 or HIV-2; pain; cancers; diabetes, obesity; anorexia; bulimia; asthma; Parkinson's disease; acute heart failure; hypotension; hypertension; urinary retention; osteoporosis; angina pectoris; myocardial infarction; stroke; ulcers; asthma; allergy; benign prostatic hypertrophy; migraine; vomiting; psychotic and neurological disorders, including anxiety, schizophrenia, manic depression,
30 depression, delirium, dementia, and severe mental retardation

Example 1**Chemical Syntheses of S1P Analogs**

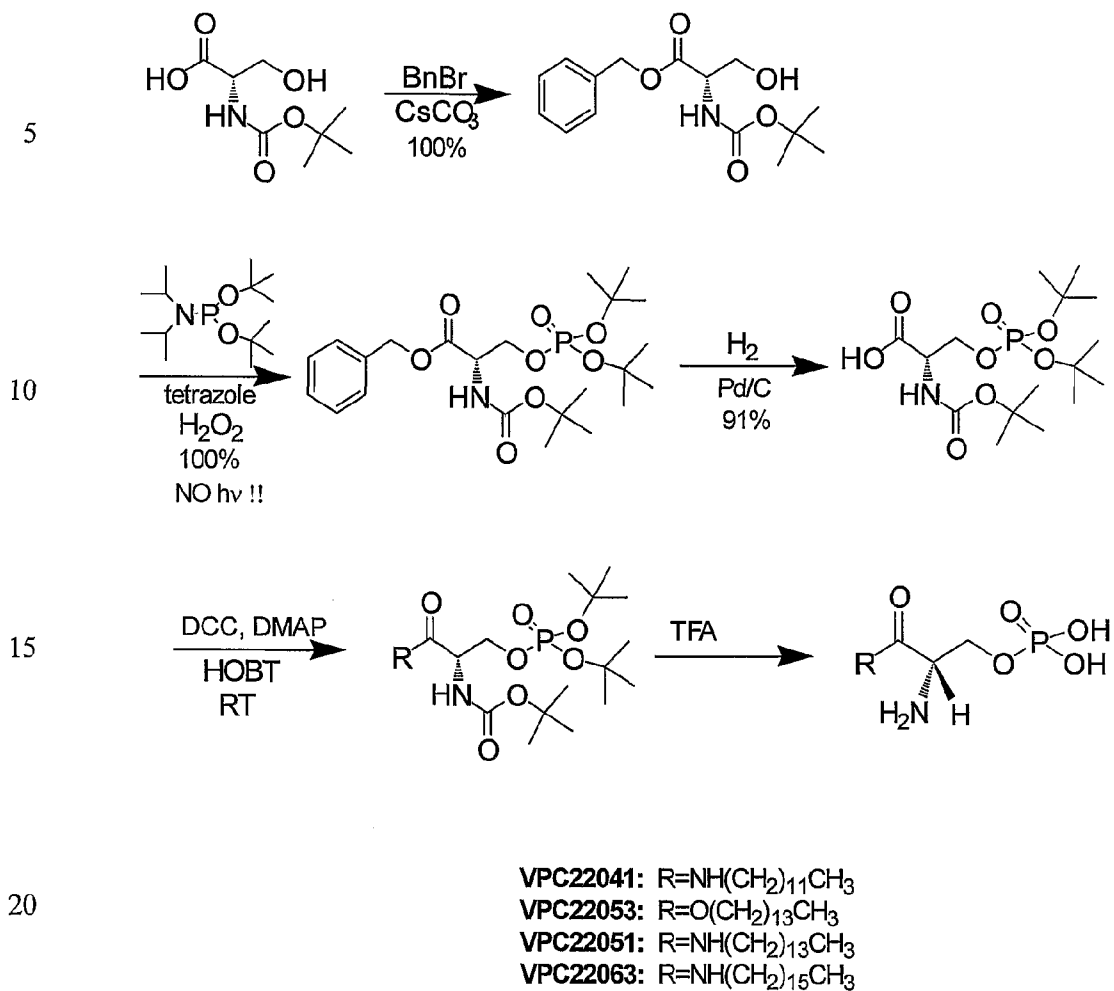
To develop good mimetics for S1P, a synthetic route was designed that had several aspects in mind (Scheme 1). First, butoxycarbonyl protected L-serine was chosen as starting material primarily because it retrosynthetically resembled the linker region of S1P. In addition, the starting material is a cheap and commercially available protected amino acid. Secondly, chemodivergence was taken into consideration. Coupling of the long chain was performed late in the synthesis so that several chain lengths could be prepared from a common intermediate. Another important issue to address was the overwhelming insolubility of the final compounds. Due to this insolubility, the target molecules could not be purified by chromatography or crystallization methods, nor could they tolerate a simple workup. It was therefore necessary to design a final step that quantitatively generated only the target product, and allowed for removal of excess reagents under vacuum. This was accomplished by employing trifluoroacetic acid deprotection at the end of the route.

The syntheses of all of the S1P analogs were accomplished using solvents purified by filtration through alumina (activity I). All reactions were performed under an inert atmosphere and all products were purified using 230-400 mesh silica gel. Each product was analyzed by thin layer chromatography (single spot) and spectroscopic methods including ^1H NMR, ^{13}C NMR, and mass spectrometry. The assigned structures of the S1P analogs were consistent with all spectral data obtained. All final products were obtained as the TFA salts.

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Synthesis of (2S) S1P Analogs VPC22041, 51, 53, and 63



Scheme 1

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% Yields

Compound	R	A	B	C	D	E
VPC22041	n-C ₁₂ H ₂₅ NH	100	100	91	33	100
VPC22051	n-C ₁₄ H ₂₉ NH	100	100	91	41	96
VPC22053	n-C ₁₄ H ₂₉ O	100	100	91	15	100
VPC22063	n-C ₁₆ H ₃₃ NH	100	100	91	26	100

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Benzyl protection of N-Boc serine. To a stirring solution of N-Boc-(L)-Serine (4.87 mmol) in DMF (100 mL) was added cesium carbonate (5.11 mmol) and stirring was continued 30 min. Benzyl bromide (5.84 mmol) was then added and the resulting solution was stirred 12 h. The reaction mixture was then diluted with ethyl acetate (25 mL), washed with lithium bromide (3 x 15 mL), sodium bicarbonate (2 x 15 mL), and brine (2 x 15 mL). The organic layer was dried over sodium sulfate. The solvent was then removed under reduced pressure and the resulting tan oil was purified by flash chromatography, using 1:1 petroleum ether/diethyl ether, to afford the product (100%) as a white solid. $R_f = 0.26$ (1:1 petroleum ether/diethyl ether).

Phosphorylation of resulting alcohol. For phosphorylation, reaction is performed in the absence of light, work up and columns are completed with as little light as possible. To a solution of the benzyl protected serine (1.98 mmol) in 1:1 $\text{CH}_2\text{Cl}_2/\text{THF}$ (50 mL) was added tetrazole (3.96 mmol) and the resulting mixture was stirred 30 min. Di-tert-butyl-di-isopropylphosphoramidite (3.96 mmol) was then added and the resulting reaction mixture was stirred 15h. Hydrogen peroxide (7.92 mmol) was then added and the resulting mixture was stirred 3h, cooled to 0°C , and quenched by addition of aqueous $\text{Na}_2\text{S}_2\text{O}_5$. The resulting solution was diluted with ethyl acetate (100 mL) and extracted with 50% aqueous $\text{Na}_2\text{S}_2\text{O}_5$ (2 x 20 mL). The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure to afford a tan oil. Flash chromatography, using 90:10 $\text{CHCl}_3/\text{acetone}$, provided the product (97%) as a clear oil. $R_f = 0.67$ (90:10 $\text{CHCl}_3/\text{acetone}$).

Debenzylation of phosphorylated serine. To a solution of the phosphorylated serine (1.55 mmol) in 200 proof ethanol (25 mL) was added a catalytic amount of palladium on activated carbon. To the resulting solution was applied a positive pressure of hydrogen gas and the reaction mixture was stirred 12h. The reaction mixture was then filtered through a plug of celite eluting with methanol and the solvent was removed under reduced pressure to yield the product (91%) as a slightly yellow oil. $R_f = 0$ (90:10 $\text{CHCl}_3/\text{methanol}$).

Coupling of long chain amine with phosphorylated acid. A solution of the acid (0.252 mmol), a catalytic amount of 4-dimethylaminopyridine, 1-hydroxybenzotriazole hydrate (0.277 mmol), the long chain amine or alcohol (0.252

-26-

mmol), and 15 mL of CH_2Cl_2 was cooled to 0°C with stirring. To the resulting solution at 0°C was added dicyclohexylcarbodiimide (0.277 mmol) and the mixture was allowed to return to rt. with stirring continuing for 12h. The reaction mixture was then recooled to 0°C and filtered. The filtrate was washed with sodium bicarbonate (3 x 10 mL), ammonium chloride (3 x 10 mL), and the organic layers were dried over sodium sulfate. The solvent was then removed under reduced pressure and the resulting yellow oil was purified by flash chromatography to afford the product.

- VPC22041: 33%, white solid, $R_f = 0.78$ (90:10 CHCl_3 /methanol).
10 VPC22051: 41%, white solid, $R_f = 0.80$ (90:10 CHCl_3 /methanol).
VPC22053: 15%, white solid, $R_f = 0.20$ (95:5 CHCl_3 /acetone).
VPC22061: 26%, white solid, $R_f = 0.79$ (90:10 CHCl_3 /methanol).

Deprotection of N-Boc and phosphate groups. To a stirred solution of the protected final product (0.072 mmol) in CH_2Cl_2 (1 mL) was added trifluoroacetic acid (12.98 mmol) and stirring was continued 4h. Under reduced pressure, solvent and excess trifluoroacetic acid were removed affording a brown oil. The oil was rinsed with ether and the solvent was removed under vacuum 5 times to afford the product.

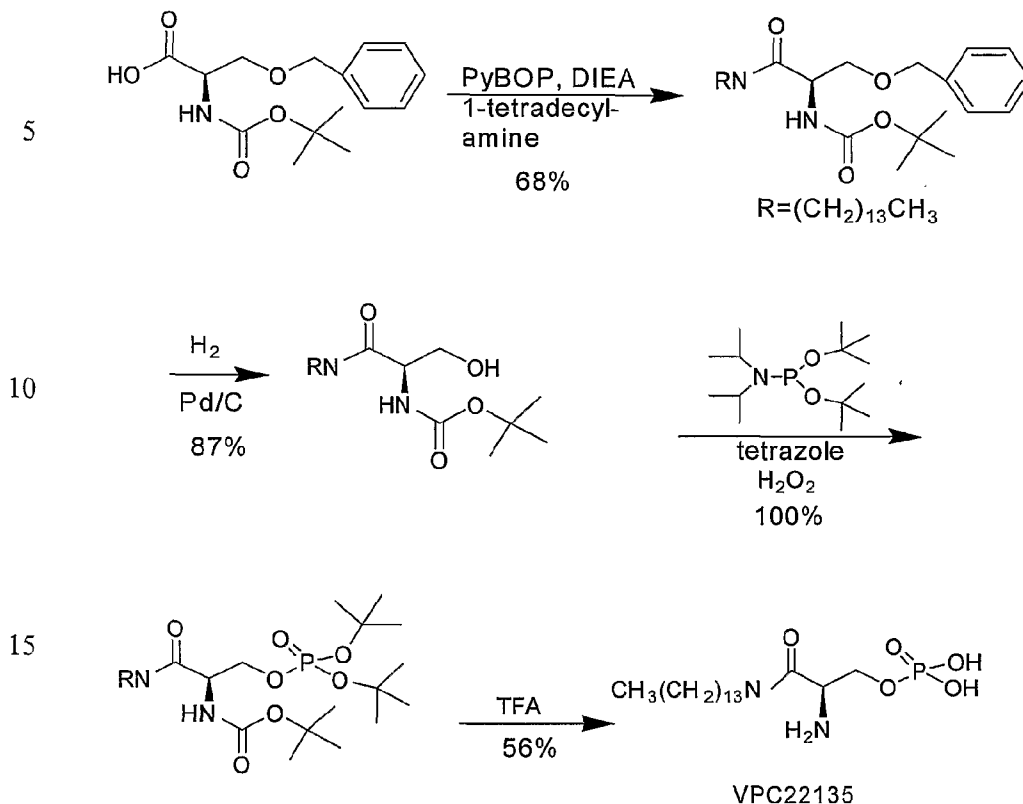
- 20 VPC22041: 100%, white solid, $R_f = 0$ (90:10 CHCl_3 /methanol).
VPC22051: 96%, white solid, $R_f = 0$ (90:10 CHCl_3 /methanol).
VPC22053: 100%, white solid, $R_f = 0$ (90:10 CHCl_3 /methanol).
VPC22061: 100%, white solid, $R_f = 0$ (90:10 CHCl_3 /methanol).

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For S1P analog VPC22051 the PyBOP coupling procedure (as used in VPC22135) was used in place of DCC coupling. The product was obtained in 15% yield as a clear oil.

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Synthesis of (2R) S1P Analog VPC22135



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Scheme 2

Coupling of long chain amine with protected serine. To a stirring solution of N-Boc-(D)-Serine-OBn (0.847 mmol) in CH₂Cl₂ (20 mL) was added PyBOP (0.847 mmol) followed by diisopropylethylamine (0.847 mmol). After 5 min.

25 of stirring, 1-tetradecylamine (0.847 mmol) was added and stirring was continued for 1 h after which time more 1-tetradecylamine was added (0.254 mmol). Stirring was continued for another 3 h and then the reaction mixture was diluted with ethyl acetate (20 mL) and washed with sodium bicarbonate (3 x 15 mL), ammonium chloride (2 x 15mL), and the organic layer was dried over sodium sulfate. Solvents were removed

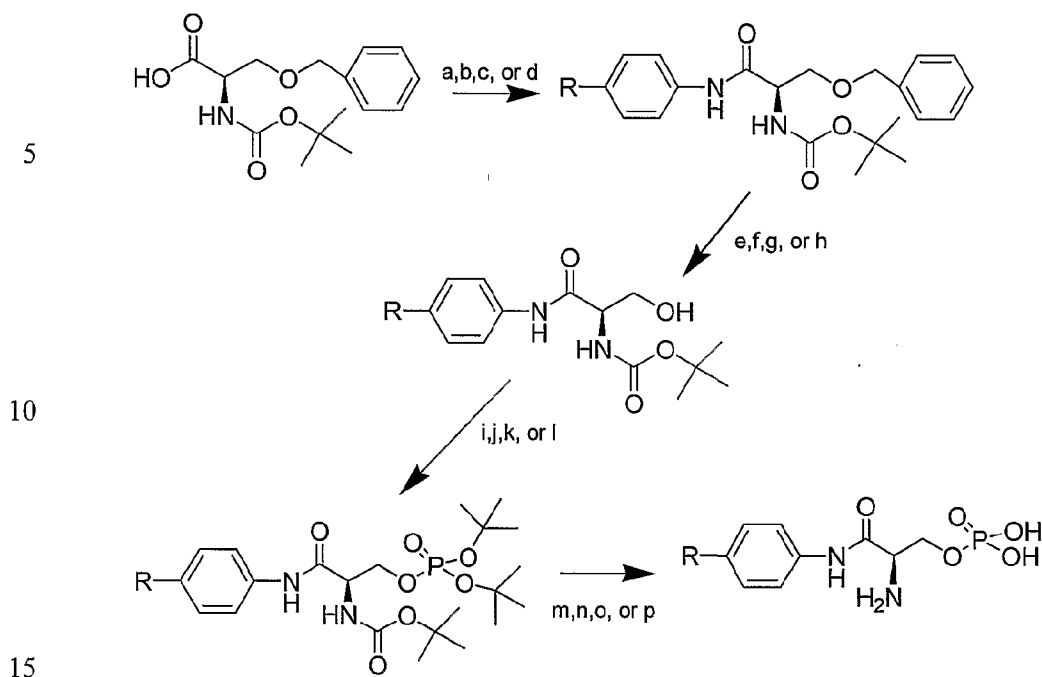
30 under reduced pressure to afford a clear gelatinous solid, which was purified by flash chromatography, using 95:5 CHCl₃/methanol, to afford the product (68%) as a white solid. R_f = 0.78 (95:5 CHCl₃/methanol).

Benzyl deprotection of coupled product. To a solution of the coupled product (0.579 mmol) in 200 proof ethanol (15 mL) was added a catalytic amount of palladium on activated carbon. To the resulting solution was applied a positive pressure of hydrogen gas and the reaction mixture was stirred 12h. The reaction
5 mixture was then filtered through a plug of celite eluting with methanol and then the solvent was removed under reduced pressure to yield the product (87%) as a clear oil. $R_f = 0.5$ (95:5 CHCl_3 /methanol).

Phosphorylation of resulting alcohol. For phosphorylation, reaction is performed in the absence of light, work up and columns are completed with as little
10 light as possible. To a solution of the alcohol (0.474 mmol) in 1:1 CH_2Cl_2 /THF (20 mL) was added tetrazole (0.948 mmol) and the resulting mixture was stirred 30 min. Di-tert-butyl-di-isopropylphosphoramidite (0.948 mmol) was then added and the resulting reaction mixture was stirred 15h. Hydrogen peroxide (1.896 mmol) was then added and the resulting mixture was then stirred 24h, cooled to 0°C , and
15 quenched by addition of aqueous $\text{Na}_2\text{S}_2\text{O}_5$. The resulting solution was diluted with ethyl acetate (50 mL) and washed with sodium bicarbonate (2 x 15 mL), water (1 x 15 mL), and finally brine (1 x 15mL). The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure to afford a clear oil. Flash chromatography, using 90:10 CHCl_3 /acetone, provided the product (100%) as a clear
20 oil. $R_f = 0.23$ (90:10 CHCl_3 /acetone).

Deprotection of N-Boc and phosphate groups. To a stirred solution of the protected product (0.071 mmol) in CH_2Cl_2 (1 mL) was added trifluoroacetic acid (12.98 mmol) and stirring was continued 4h. Under reduced pressure, solvent and excess trifluoroacetic acid were removed affording a brown oil. Rinsed oil with ether
25 and removed under vacuum 5 times to afford the product (56%) as a white solid. $R_f = 0$ (90:10 CHCl_3 /methanol).

Synthesis of (2R) S1P Analog VPC22157, 173, 199, and 211



Scheme 3

- 20
- a) PyBOP, DIEA, 4-hexylaniline, 77%
 b) PyBOP, DIEA, 4-octylaniline, 73%
 c) PyBOP, DIEA, 4-decylaniline, 65%
 d) PyBOP, DIEA, 4-dodecylaniline, 71%
- e) Di^tBu-diⁱPr-phosphoramidite, tetrazole, H₂O₂, 85%
 f) Di^tBu-diⁱPr-phosphoramidite, tetrazole, H₂O₂, 60%
 g) Di^tBu-diⁱPr-phosphoramidite, tetrazole, H₂O₂, 70%
 h) Di^tBu-diⁱPr-phosphoramidite, tetrazole, H₂O₂, 9%
- i) H₂, Pd/C, 84%
 j) H₂, Pd/C, 96%
 k) H₂, Pd/C, 87%
 l) H₂, Pd/C, 90%
- 25
- m) TFA, 100%
 n) TFA, 58%
 o) TFA, 75%
 p) TFA, 100%

VPC22157: R=(CH₂)₅CH₃ (a,e,i,m)

VPC22173: R=(CH₂)₇CH₃ (b,f,j,n)

VPC22199: R=(CH₂)₉CH₃ (c,g,k,o)

VPC22211: R=(CH₂)₁₁CH₃ (d,h,l,p)

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Coupling of long chain aniline with protected serine. To a stirring solution of N-Boc-(D)-Serine-OBn (0.339 mmol) in CH₂Cl₂ (10 mL) was added PyBOP (0.339 mmol) followed by diisopropylethylamine (0.339 mmol). After 5 min.

-30-

of stirring, the aniline (0.339 mmol) was added and stirring was continued for 4 h. The reaction mixture was then diluted with ethyl acetate (10 mL) and washed with sodium bicarbonate (3 x 10 mL), ammonium chloride (2 x 10mL), and the organic layer was dried over sodium sulfate. Solvents were removed under reduced pressure to afford a clear gelatinous solid, which was purified by flash chromatography to afford the product.

VPC22157: 77%, white solid, $R_f = 0.80$ (90:10 CHCl_3 /acetone).

VPC22173: 73%, white solid, $R_f = 0.78$ (90:10 CHCl_3 /acetone).

10 VPC22199: 65%, white solid, $R_f = 0.79$ (90:10 CHCl_3 /acetone).

VPC22211: 71%, white solid, $R_f = 0.80$ (90:10 CHCl_3 /acetone).

Benzyl deprotection of coupled product. To a solution of the coupled product (0.260 mmol) in 200 proof ethanol (10 mL) was added a catalytic amount of palladium on activated carbon. To the resulting solution was applied a positive pressure of hydrogen gas and the reaction mixture was stirred 12h. The reaction mixture was then filtered through a plug of celite eluting with methanol and then the solvent was removed under reduced pressure to yield the product.

20 VPC22157: 85%, clear oil, $R_f = 0.50$ (95:5 CHCl_3 /methanol).

VPC22173: 60%, clear oil, $R_f = 0.55$ (95:5 CHCl_3 /methanol).

VPC22199: 70%, clear oil, $R_f = 0.48$ (95:5 CHCl_3 /methanol).

VPC22211: 9%, clear oil, $R_f = 0.53$ (95:5 CHCl_3 /methanol).

25 Phosphorylation of resulting alcohol. For phosphorylation, reaction is performed in the absence of light, work up and columns are completed with as little light as possible. To a solution of the alcohol (0.220 mmol) in 1:1 CH_2Cl_2 /THF (10 mL) was added tetrazole (0.400 mmol) and the resulting mixture was stirred 30 min. Di-tert-butyl-di-isopropylphosphoramidite (0.400 mmol) was then added and the resulting reaction mixture was stirred 15h. Hydrogen peroxide (0.800 mmol) was then added and the resulting mixture was then stirred 24h, cooled to 0°C, and quenched by addition of aqueous $\text{Na}_2\text{S}_2\text{O}_5$. The resulting solution was diluted with

30

ethyl acetate (25 mL) and washed with sodium bicarbonate (2 x 10 mL), water (1 x 10 mL), and finally brine (1 x 10mL). The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure to afford a clear oil. Flash chromatography provided the product as a clear oil.

5

VPC22157: 84%, clear oil, $R_f = 0.23$ (90:10 CHCl_3 /acetone).

VPC22173: 96%, clear oil, $R_f = 0.30$ (90:10 CHCl_3 /acetone).

VPC22199: 87%, clear oil, $R_f = 0.72$ (80:20 CHCl_3 /acetone).

VPC22211: 90%, clear oil, $R_f = 0.58$ (80:20 CHCl_3 /acetone).

10

Deprotection of N-Boc and phosphate groups. To a stirred solution of the protected product (0.162 mmol) in CH_2Cl_2 (2 mL) was added trifluoroacetic acid (25.96 mmol) and stirring was continued 4h. Under reduced pressure, solvent and excess trifluoroacetic acid were removed affording a brown oil. Rinsed oil with ether and removed under vacuum 5 times to afford the product.

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VPC22157: 100%, white solid, $R_f = 0$ (90:10 CHCl_3 /methanol).

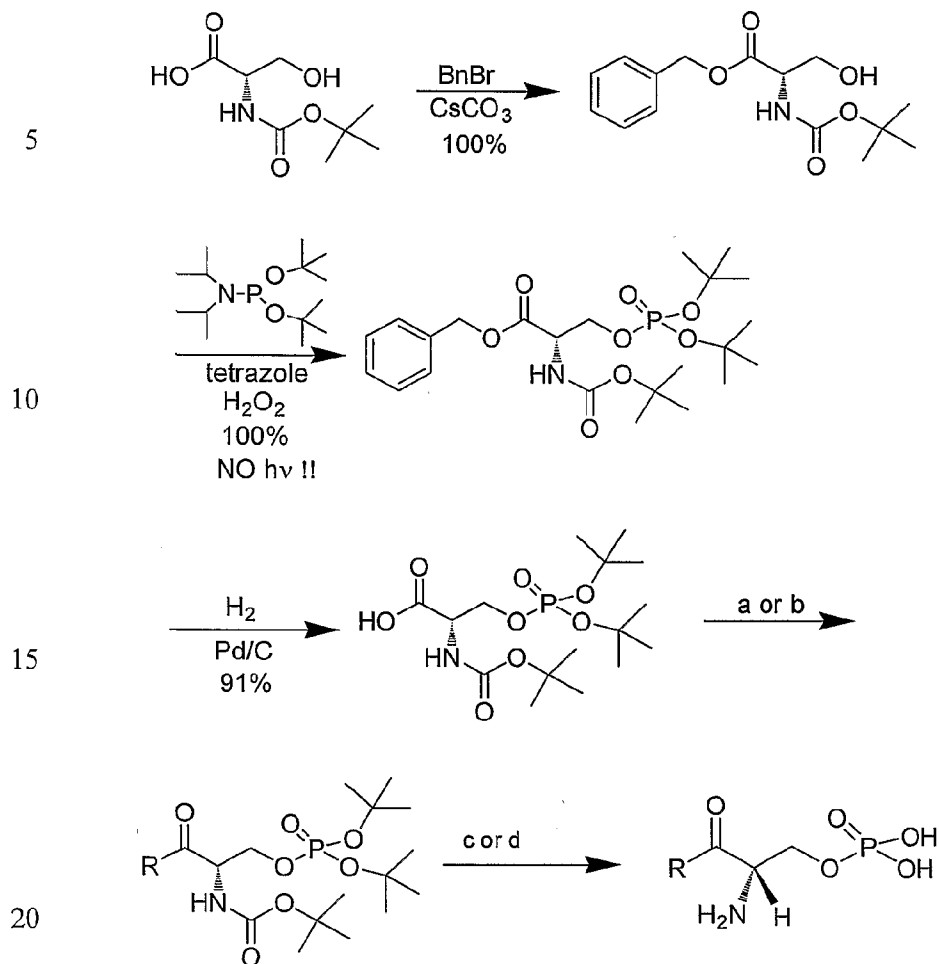
VPC22173: 58%, white solid, $R_f = 0$ (90:10 CHCl_3 /methanol).

VPC22199: 75%, white solid, $R_f = 0$ (90:10 CHCl_3 /methanol).

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VPC22211: 100%, white solid, $R_f = 0$ (90:10 CHCl_3 /methanol).

Synthesis of (2S) SIP Analogs VPC22179 and 181



Scheme 4

- 25
- a) PyBOP, DIEA, 4-hexylaniline, 43%
 - b) PyBOP, DIEA, 4-octylaniline, 60%
 - c) TFA, 100%
 - d) TFA, 100%

VPC22179: R=NHPH(CH₂)₅CH₃ (a,c)

VPC22181: R=NHPH(CH₂)₇CH₃ (b,d)

30 Benzyl protection of N-Boc serine. To a stirring solution of N-Boc-(L)-Serine (2.44 mmol) in DMF (50 mL) was added cesium carbonate (2.56 mmol) and stirring was continued 30 min. Benzyl bromide (2.92 mmol) was then added and the resulting solution was stirred 12 h. The reaction mixture was then diluted with

ethyl acetate (15 mL), washed with lithium bromide (3 x 10 mL), sodium bicarbonate (2 x 10 mL), and brine (2 x 10 mL). The organic layer was dried over sodium sulfate. The solvent was then removed under reduced pressure and the resulting tan oil was purified by flash chromatography, using 1:1 petroleum ether/diethyl ether, to afford
5 the product (100%) as a white solid. $R_f = 0.26$ (1:1 petroleum ether/diethyl ether).

Phosphorylation of resulting alcohol. For phosphorylation, reaction is performed in the absence of light, work up and columns are completed with as little light as possible. To a solution of the benzyl protected serine (2.22 mmol) in 1:1 $\text{CH}_2\text{Cl}_2/\text{THF}$ (100 mL) was added tetrazole (4.43 mmol) and the resulting mixture was
10 stirred 30 min. Di-tert-butyl-di-isopropylphosphoramidite (4.43 mmol) was then added and the resulting reaction mixture was stirred 15h. Hydrogen peroxide (8.86 mmol) was then added and the resulting mixture was stirred 3h, cooled to 0°C , and quenched by addition of aqueous $\text{Na}_2\text{S}_2\text{O}_5$. The resulting solution was diluted with ethyl acetate (100 mL) and extracted with 50% aqueous $\text{Na}_2\text{S}_2\text{O}_5$ (2 x 20 mL). The
15 organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure to afford a tan oil. Flash chromatography, using 90:10 $\text{CHCl}_3/\text{acetone}$, provided the product (97%) as a clear oil. $R_f = 0.67$ (90:10 $\text{CHCl}_3/\text{acetone}$).

Debenzylation of phosphorylated serine. To a solution of the
20 phosphorylated serine (1.55 mmol) in 200 proof ethanol (25 mL) was added a catalytic amount of palladium on activated carbon. To the resulting solution was applied a positive pressure of hydrogen gas and the reaction mixture was stirred 12h. The reaction mixture was then filtered through a plug of celite eluting with methanol and the solvent was removed under reduced pressure to yield the product (91%) as a
25 slightly yellow oil. $R_f = 0$ (90:10 $\text{CHCl}_3/\text{methanol}$).

Coupling of long chain aniline with phosphorylated acid. To a stirring solution of the phosphorylated acid (0.252 mmol) in CH_2Cl_2 (10 mL) was added PyBOP (0.252 mmol) followed by diisopropylethylamine (0.252 mmol). After 5 min. of stirring, the aniline (0.252 mmol) was added and stirring was continued for 4 h.
30 The reaction mixture was then diluted with ethyl acetate (10 mL) and washed with sodium bicarbonate (3 x 10 mL), ammonium chloride (2 x 10mL), and the organic

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layer was dried over sodium sulfate. Solvents were removed under reduced pressure to afford the product.

VPC22179: 43%, white solid, $R_f = 0.40$ (90:10 CHCl_3 /acetone).

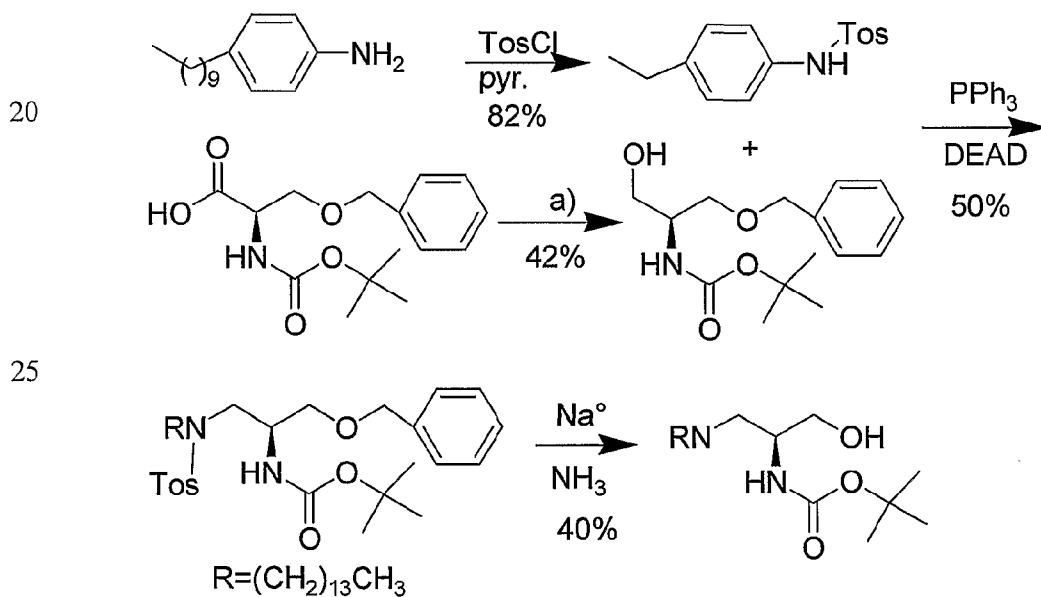
5 VPC22181: 60%, white solid, $R_f = 0.35$ (90:10 CHCl_3 /acetone).

Deprotection of N-Boc and phosphate groups. To a stirred solution of the protected final product (0.117 mmol) in CH_2Cl_2 (1.5 mL) was added trifluoroacetic acid (19.48 mmol) and stirring was continued 4h. Under reduced
10 pressure, solvent and excess trifluoroacetic acid were removed affording a brown oil. The oil was rinsed with ether and the solvent was removed under vacuum 5 times to afford the product.

VPC22179: 100%, white solid, $R_f = 0$ (90:10 CHCl_3 /methanol).

15 VPC22181: 100%, white solid, $R_f = 0$ (90:10 CHCl_3 /methanol).

Synthesis of (2R) S1P Analog VPC22277



with ethyl acetate (15 mL) and the aqueous layer was discarded. The organic layer was then washed with sat. ammonium chloride (3 X 10 mL), sat. sodium bicarbonate (3 X 10 mL) and finally brine (1 X 10 mL). The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure to yield the crude product as a white solid. The crude product was purified by flash chromatography, using 80:20 CHCl₃/acetone, to afford the product (42%) as a white solid. R_f = 0.48 (80:20 CHCl₃/acetone).

Coupling of aniline with alcohol. To a stirring solution of the aniline (0.209 mmol) in THF (3 mL) under an inert atmosphere was added triphenylphosphine (0.254 mmol), the alcohol (0.105 mmol), and finally DEAD (0.209 mmol). The reaction mixture was stirred 12 h and then concentrated to a clear oil. Petroleum ether was added to the clear oil and solid triphenylphosphine oxide was allowed to settle on the bottom of the flask. The clear petroleum ether layer was then pipetted off and concentrated to a clear oil. The crude product was then subjected to flash chromatography, using 1:1 petroleum ether/ether, to afford the final product (50%) as a white solid. R_f = 0.83 (1:1 petroleum ether/ether).

Tosyl deprotection of the coupled product. Ammonia (20 mL) was condensed in a 2-neck round bottom flask equipped with a stirbar and cold finger that was cooled to -70°C under an inert atmosphere. Sodium metal (4.27 mmol) was then added to the reaction mixture followed by the tosyl protected amine (0.427 mmol) in THF (8 mL). The dark blue reaction mixture was stirred for 1 h at -70°C and was then quenched with ethanol until the solution was clear/white and the reaction mixture was then stirred at r.t. overnight. The reaction mixture was then diluted with ethyl acetate (20 mL) and washed with sat. ammonium chloride (3 X 20 mL), sat. sodium bicarbonate (3 X 20 mL), and finally brine (1 X 20 mL). The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure to yield the crude product as a clear oil. The crude product was purified by flash chromatography, using 1:1 ethyl acetate/hexanes, to afford the product (40%) as a white solid. R_f = 0.42 (1:1 ethyl acetate/hexanes).

Phosphorylation of resulting alcohol. For phosphorylation, reaction is performed in the absence of light, work up and columns are completed with as little light as possible. To a solution of the alcohol (0.130 mmol) in 1:1 CH₂Cl₂/THF (5

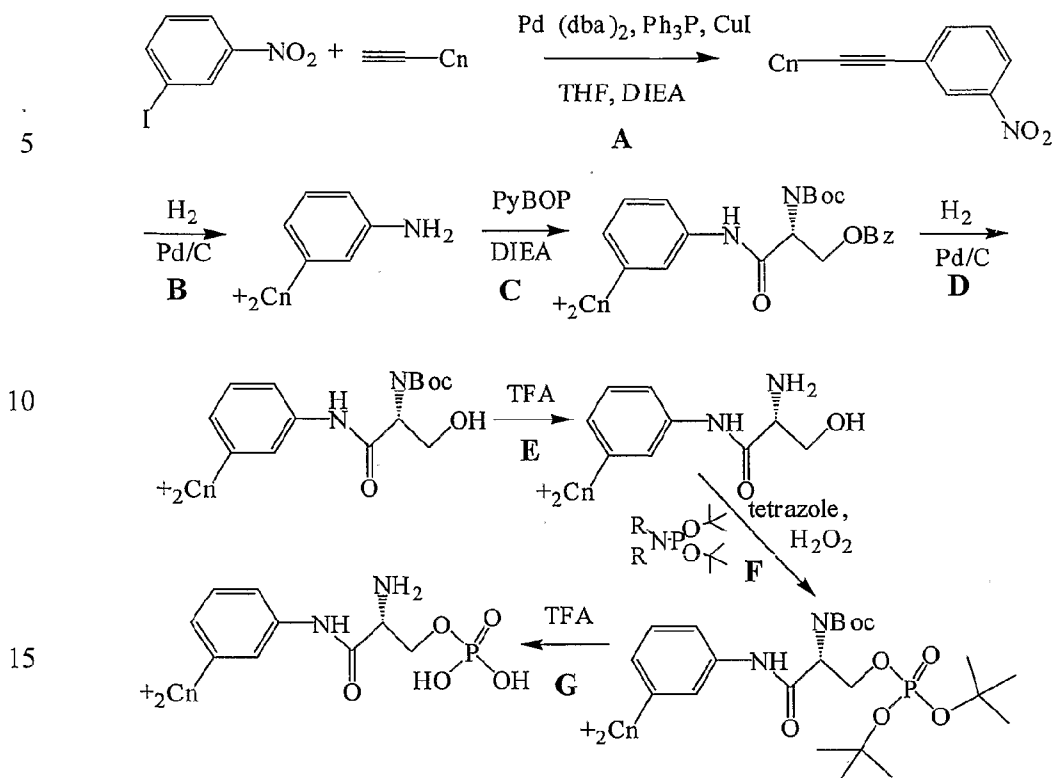
-37-

mL) was added tetrazole (0.130 mmol) and the resulting mixture was stirred 30 min. Di-tert-butyl-di-isopropylphosphoramidite (0.130 mmol) was then added and the resulting reaction mixture was stirred 15h. Hydrogen peroxide (30%, 0.044 mL) was then added and the resulting mixture was then stirred 24h, cooled to 0°C, and
5 quenched by addition of aqueous $\text{Na}_2\text{S}_2\text{O}_5$. The resulting solution was diluted with ethyl acetate (10 mL) and washed with sodium bicarbonate (2 x 10 mL), water (1 x 10 mL), and finally brine (1 x 10mL). The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure to afford a clear oil. Flash chromatography, using 1:1 ethyl acetate/hexanes, provided the product (12%) as a
10 clear oil. $R_f = 0.41$ (1:1 ethyl acetate/hexanes).

Deprotection of N-Boc and phosphate groups. To a stirred solution of the protected final product (0.016 mmol) in CH_2Cl_2 (0.5 mL) was added trifluoroacetic acid (6.49 mmol) and stirring was continued 4h. Under reduced pressure, solvent and excess trifluoroacetic acid were removed affording a brown oil.
15 The oil was rinsed with ether and the solvent was removed under vacuum 5 times to afford the product (100%) as a white solid. $R_f = 0$ (90:10 CHCl_3 /methanol).

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Synthesis of (2R) S1P Analog VPC23031, 19, 65, 69, 75 and 79



Scheme 6

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% Yields

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Compound(s)	n	A	B	C	D	E	F	G
VPC23031	4	24	66	52	100	X	90	100
VPC23019	6	100	85	90	95	X	56	92
VPC23065, 69	8	34	84	84	89	100	89	86
VPC23075, 79	7	66	100	100	27	93	77	100

Coupling of aryl halide with terminal alkyne. All starting materials were thoroughly flushed with nitrogen before the reaction. To a stirring solution of the aryl halide (2.01 mmol), bis(dibenzylideneacetone) palladium (0.04 mmol), triphenylphosphine (0.10 mmol), and copper iodide (0.04 mmol) in THF (10 mL) under inert atmosphere was added the terminal alkyne (2.21 mmol) followed by diisopropylethylamine (8.04 mmol). The reaction mixture was then stirred at r.t. for 12 h. The reaction mixture was then diluted with ethyl acetate (15 mL) and washed

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with sodium bicarbonate (3 x 15 mL), ammonium chloride (3 x 15 mL) and finally brine (1 X 15 mL). The organic layer was then dried over sodium sulfate. Solvents were removed under reduced pressure to afford a tan oil. Flash chromatography provided the final product.

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VPC23031: 24%, yellow oil, $R_f = 0.61$ (90:10 hexanes/ether).

VPC23019: 100%, yellow oil, $R_f = 0.55$ (90:10 hexanes/ether).

VPC23065, 69: 66%, yellow oil, $R_f = 0.75$ (90:10 hexanes/ether).

VPC23075, 79: 34%, yellow oil, $R_f = 0.75$ (90:10 hexanes/ether).

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Reduction of the coupled product. To a solution of the coupled product (1.68 mmol) in 200 proof ethanol (10 mL) was added a catalytic amount of palladium on activated carbon. To the resulting solution was applied a positive pressure of hydrogen gas and the reaction mixture was stirred 12h. The reaction mixture was then filtered through a plug of celite eluting with methanol and then the solvent was removed under reduced pressure to yield the crude product.

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VPC23031: 66%, yellow solid, $R_f = 0.53$ (95:5 CHCl_3 /acetone).

VPC23019: 85%, yellow solid, $R_f = 0.55$ (95:5 CHCl_3 /acetone).

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VPC23065, 69: 84%, yellow solid, $R_f = 0.79$ (95:5 CHCl_3 /acetone).

VPC23075, 79: 100%, yellow solid, $R_f = 0.80$ (95:5 CHCl_3 /acetone).

Coupling of long chain aniline with protected serine. To a stirring solution of N-Boc-(D)-Serine-OBn (0.740 mmol) in CH_2Cl_2 (20 mL) was added PyBOP (0.740 mmol) followed by diisopropylethylamine (0.740 mmol). After 5 min. of stirring, the aniline (0.740 mmol) was added and stirring was continued for 4 hours. The reaction mixture was then diluted with ethyl acetate (20 mL) and washed with 1 N HCl (3 X 20 mL), sodium bicarbonate (3 X 20 mL), and finally brine (1 X 20 mL), and the organic layer was dried over sodium sulfate. Solvents were removed under reduced pressure to afford a clear oil, which was purified by flash chromatography to afford the product.

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VPC23031: 52%, clear oil, $R_f = 0.35$ (dichloromethane).

VPC23019: 90%, clear oil, $R_f = 0.61$ (70:30 hexanes/ethyl acetate).

VPC23065, 69: 84%, clear oil, $R_f = 0.82$ (90:10 CHCl_3 /acetone).

VPC23075, 79: 100%, clear oil, $R_f = 0.92$ (90:10 CHCl_3 /acetone).

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Benzyl deprotection of coupled product. To a solution of the coupled product (0.667 mmol) in 200 proof ethanol (15 mL) was added a catalytic amount of palladium on activated carbon. To the resulting solution was applied a positive pressure of hydrogen gas and the reaction mixture was stirred 12h. The reaction mixture was then filtered through a plug of celite eluting with methanol and then the solvent was removed under reduced pressure to yield the product.

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VPC23031: 100%, clear oil, $R_f = 0.27$ (70:30 hexanes/ethyl acetate).

VPC23019: 95%, clear oil, $R_f = 0.28$ (70:30 hexanes/ethyl acetate).

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VPC23065, 69: 89%, clear oil, $R_f = 0.62$ (1:1 hexanes/ethyl acetate).

VPC23075, 79: 27%, clear oil, $R_f = 0.43$ (1:1 hexanes/ethyl acetate).

Deprotection to afford free alcohol. To a stirred solution of the N-Boc protected alcohol (0.143 mmol) in CH_2Cl_2 (2 mL) was added trifluoroacetic acid (25.96 mmol) and stirring was continued 4h. Under reduced pressure, solvent and excess trifluoroacetic acid were removed affording a brown oil. The oil was rinsed with ether and the solvent was removed under vacuum 5 times to afford the product.

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VPC23065: 100%, white solid, $R_f = 0.2$ (90:10 CHCl_3 /methanol).

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VPC23075: 93%, white solid, $R_f = 0.2$ (90:10 CHCl_3 /methanol).

Phosphorylation of N-Boc protected alcohol. For phosphorylation, the reaction is performed in the absence of light, work up and columns are completed with as little light as possible. To a solution of the alcohol (0.247 mmol) in 1:1 CH_2Cl_2 /THF (15 mL) was added tetrazole (0.495 mmol) and the resulting mixture was stirred 30 min. Di-tert-butyl-di-isopropylphosphoramidite (0.495 mmol) was then added and the resulting reaction mixture was stirred 15h. Hydrogen peroxide (0.989

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mmol) was then added and the resulting mixture was then stirred 24h, cooled to 0°C, and quenched by addition of aqueous Na₂S₂O₅. The resulting solution was diluted with ethyl acetate (25 mL) and washed with sodium bicarbonate (3 x 15 mL), ammonium chloride (3 x 15 mL), and finally brine (1 x 15 mL). The organic layer
5 was dried over sodium sulfate and the solvent was removed under reduced pressure to afford a clear oil. Flash chromatography provided the product.

VPC23031: 90%, clear oil, R_f = 0.80 (80:20 ether/ethyl acetate).

VPC23019: 56%, clear oil, R_f = 0.82 (80:20 ether/ethyl acetate).

10 VPC23069: 89%, clear oil, R_f = 0.85 (90:10 ether/ethyl acetate).

VPC23079: 77%, clear oil, R_f = 0.85 (90:10 ether/ethyl acetate).

Deprotection of N-boc and phosphate groups. To a stirred solution of the protected product (0.162 mmol) in CH₂Cl₂ (2 mL) was added trifluoroacetic acid
15 (25.96 mmol) and stirring was continued 4h. Under reduced pressure, solvent and excess trifluoroacetic acid were removed affording a brown oil. Rinsed oil with ether and removed under vacuum 5 times to afford the product.

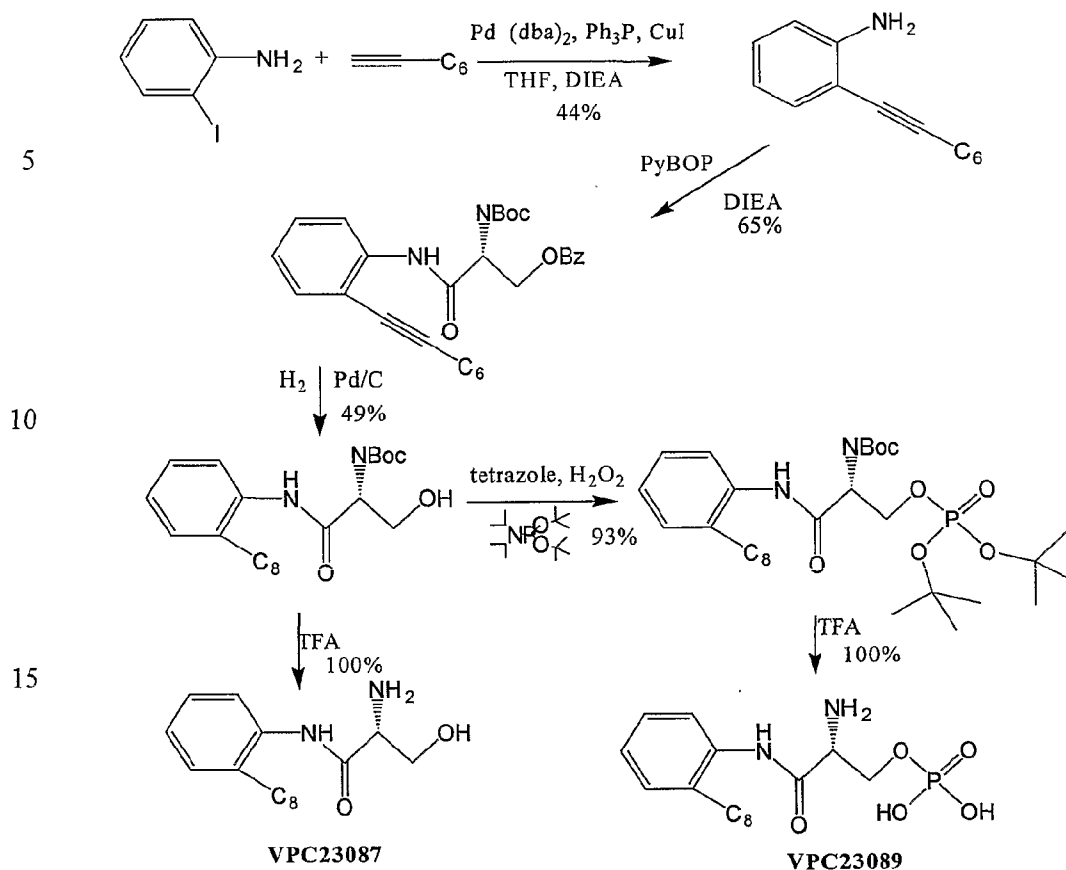
VPC23031: 100%, clear oil, R_f = 0 (90:10 CHCl₃/methanol).

20 VPC23019: 92%, clear oil, R_f = 0 (90:10 CHCl₃/methanol).

VPC23069: 86%, clear oil, R_f = 0 (90:10 CHCl₃/methanol).

VPC23079: 100%, clear oil, R_f = 0 (90:10 CHCl₃/methanol).

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Synthesis of (2R) S1P Analog VPC23087 and 89:**Scheme 7**

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Coupling of aryl halide with terminal alkyne. All starting materials were thoroughly flushed with nitrogen before the reaction. To a stirring solution of the aryl halide (2.01 mmol), bis(dibenzylideneacetone) palladium (0.04 mmol), triphenylphosphine (0.10 mmol), and copper iodide (0.04 mmol) in THF (10 mL) under inert atmosphere was added the terminal alkyne (2.21 mmol) followed by diisopropylethylamine (8.04 mmol). The reaction mixture was then stirred at r.t. for 12 h. The reaction mixture was then diluted with ethyl acetate (15 mL) and washed with sodium bicarbonate (3 x 15 mL), ammonium chloride (3 x 15 mL) and finally brine (1 X 15 mL). The organic layer was then dried over sodium sulfate. Solvents were removed under reduced pressure to afford a tan oil. Flash chromatography, using 70:30 hexanes/ethyl acetate provided the final product (44%) as a yellow solid. $R_f = 0.79$ (70:30 hexanes/ethyl acetate).

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Coupling of long chain aniline with protected serine. To a stirring solution of N-boc-(D)-Serine-OBn (0.288 mmol) in CH_2Cl_2 (10 mL) was added PyBOP (0.288 mmol) followed by diisopropylethylamine (0.288 mmol). After 5 min. of stirring, the aniline (0.288 mmol) was added and stirring was continued for 4 hours.

5 The reaction mixture was then diluted with ethyl acetate (10 mL) and washed with 1 N HCl (3 X 10 mL), sodium bicarbonate (3 X 10 mL), and finally brine (1 X 10 mL), and the organic layer was dried over sodium sulfate. Solvents were removed under reduced pressure to afford a clear oil. Flash chromatography, using 70:30

10 hexanes/ethyl acetate provided the final product (65%) as a clear oil. $R_f = 0.64$ (70:30 hexanes/ethyl acetate).

Benzyl deprotection and reduction of coupled product. To a solution of the coupled product (0.188 mmol) in 200 proof ethanol (10 mL) was added a catalytic amount of palladium on activated carbon. To the resulting solution was applied a positive pressure of hydrogen gas and the reaction mixture was stirred 12h.

15 The reaction mixture was then filtered through a plug of celite eluting with methanol and then the solvent was removed under reduced pressure to yield the crude product as a clear oil. Flash chromatography, using 1:1 hexanes/ethyl acetate provided the final product (49%) as a clear oil. $R_f = 0.51$ (1:1 hexanes/ethyl acetate).

Deprotection to afford free alcohol. To a stirred solution of the N-Boc protected alcohol (0.025 mmol) in CH_2Cl_2 (1 mL) was added trifluoroacetic acid (12.98 mmol) and stirring was continued 4h. Under reduced pressure, solvent and excess trifluoroacetic acid were removed affording a brown oil. The oil was rinsed with ether and the solvent was removed under vacuum 5 times to afford the product (100%) as a white solid. $R_f = 0.2$ (90:10 CHCl_3 /methanol).

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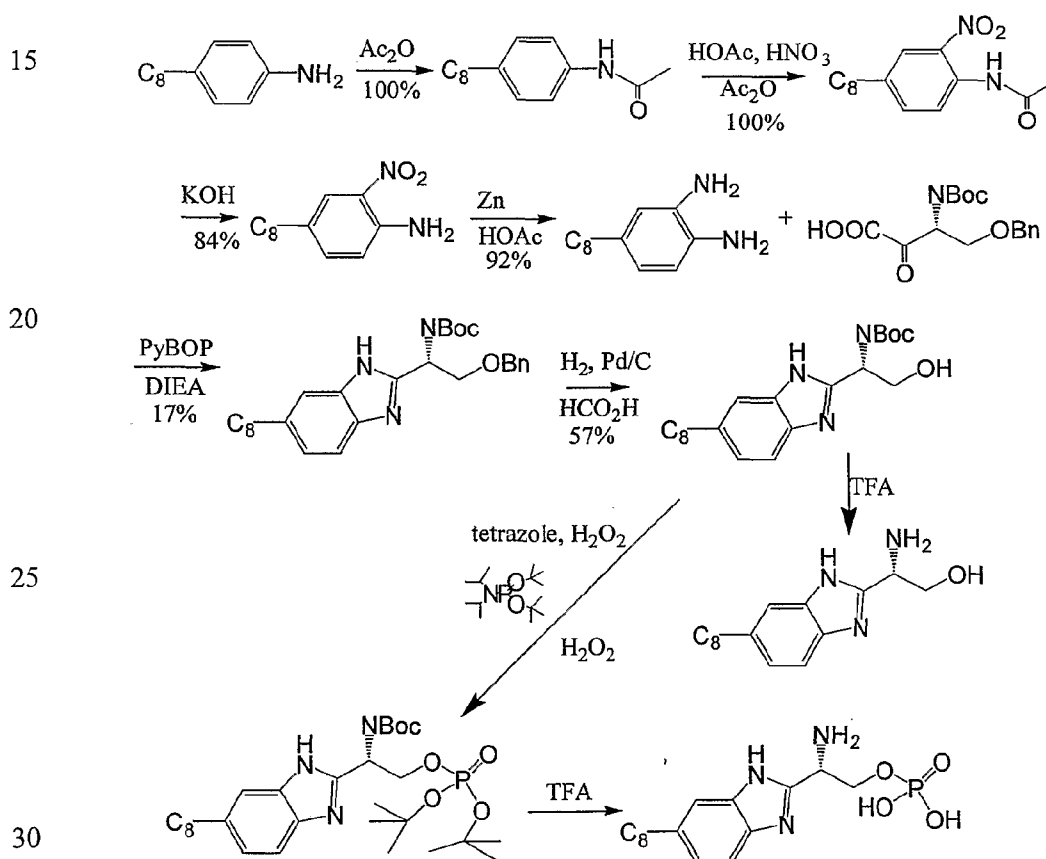
Phosphorylation of N-Boc protected alcohol. For phosphorylation, reaction is performed in the absence of light, work up and columns are completed with as little light as possible. To a solution of the alcohol (0.092 mmol) in 1:1 CH_2Cl_2 /THF (10 mL) was added tetrazole (0.183 mmol) and the resulting mixture was stirred 30 min. Di-tert-butyl-di-isopropylphosphoramidite (0.183 mmol) was then

30 added and the resulting reaction mixture was stirred 15h. Hydrogen peroxide (0.367 mmol) was then added and the resulting mixture was then stirred 24h, cooled to 0°C , and quenched by addition of aqueous $\text{Na}_2\text{S}_2\text{O}_5$. The resulting solution was diluted

with ethyl acetate (15 mL) and washed with sodium bicarbonate (3 x 15 mL), ammonium chloride (3 x 15 mL), and finally brine (1 x 15 mL). The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure to afford a clear oil. Flash chromatography, using 90:10 ethyl acetate/ether provided the
5 final product (93%) as a clear oil. $R_f = 0.85$ (90:10 ethyl acetate/ether).

Deprotection of N-Boc and phosphate groups. To a stirred solution of the protected product (0.063 mmol) in CH_2Cl_2 (2 mL) was added trifluoroacetic acid (25.96 mmol) and stirring was continued 4h. Under reduced pressure, solvent and excess trifluoroacetic acid were removed affording a brown oil. The oil was rinsed
10 with ether and the solvent was removed under vacuum 5 times to afford the product (100%) as a white solid. $R_f = 0$ (90:10 CHCl_3 /methanol).

Synthesis of (2R) benzimidazole compound:



Scheme 8

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Acetylation of the aniline. To a stirring solution of acetic anhydride (10 mL) under inert atmosphere was added octyl aniline (0.738 mmol) and stirring was continued for 1 h. Sat. aqueous sodium bicarbonate was then added to neutralize and acetic acid present. The aqueous solution was then extracted with ethyl acetate (3 X 15 mL) and the combined organic extracts were dried over sodium sulfate and concentrated to afford the final product (100%) as a yellow solid that was used without further purification. $R_f = 0.48$ (90:10 CHCl_3 /acetone).

Nitration of the acetylated aniline. To a stirring solution of acetic acid (1.08 mL), acetic anhydride (0.73 mL), and nitric acid (0.20 mL) at -15°C under an inert atmosphere was added the acetylated aniline (0.91 mmol) in approx. 1 mL of acetic acid over a period of 3 h. Reaction mixture was periodically warmed to 0°C to avoid freezing. The reaction mixture was stirred for an additional hour and was then diluted with ethyl acetate (10 mL) and neutralized using 1M NaOH and sat. aqueous sodium bicarbonate. The organic layer was removed and the aqueous portion was washed twice more with ethyl acetate (10 mL each). The organic layers were combined and dried over sodium sulfate and then concentrated to a yellow solid. Flash chromatography, using 95:5 CHCl_3 /acetone provided the final product (100%) as a yellow solid. $R_f = 0.68$ (95:5 CHCl_3 /acetone).

Deacetylation of the aniline. To a stirring solution of the nitrated, acetylated aniline (0.62 mmol) in ethanol (2.5 mL) under an inert atmosphere was added 40% KOH (0.13 mL). The reaction mixture was then heated to reflux for 1 h. The solution was then cooled in ice and brought to pH = 6 using conc. HCl. This mixture was then concentrated to an orange solid and redissolved in ether (10 mL) and washed with sat. aqueous sodium bicarbonate (2 X 10 mL) and brine (1 X 10 mL). The organic layer was then dried over sodium sulfate and concentrated to afford the final product (84%) as an orange solid that was used without further purification. $R_f = 0.82$ (95:5 CHCl_3 /acetone).

Reduction of the nitro group. To a stirring solution of the nitrated aniline (0.248 mmol) in acetic acid (5 mL) was added a catalytic amount of zinc dust and stirring was continued overnight under an inert atmosphere. The reaction mixture was then diluted with ether and filtered through a plug of celite under an inert atmosphere using ether to elute. Care was taken not to expose the ether solution to

air. The solution was then concentrated to afford the final product (92%) as a reddish-brown oil which was used directly in the next step without further purification. $R_f = 0.05$ (95:5 $\text{CHCl}_3/\text{acetone}$).

Coupling of the diamine with protected Serine. A solution of N-boc-
5 (D)-Serine-OBn (0.999 mmol), PyBOP (0.999 mmol), diisopropylethylamine (0.999 mmol) in CH_2Cl_2 (25 mL) was stirred 5 min. under an inert atmosphere and then cannulated into a flask containing the diamine (0.999 mmol). This reaction mixture was then stirred 12 h. The reaction mixture was then diluted with ethyl acetate (30 mL) and washed with sat. aqueous sodium bicarbonate (3 X 3 mL), ammonium
10 chloride (3 X 30 mL), and finally brine (1 X 30 mL), and the organic layer was dried over sodium sulfate. Solvents were removed under reduced pressure to afford a brown oil. Flash chromatography, using 90:10 $\text{CHCl}_3/\text{acetone}$ provided the final product (17%) as a brown oil. $R_f = 0.52$ (90:10 $\text{CHCl}_3/\text{acetone}$).

Benzyl deprotection of coupled product. To a solution of the coupled
15 product (0.167 mmol) in 200 proof ethanol (10 mL) and a catalytic amount of formic acid was added a catalytic amount of palladium on activated carbon. To the resulting solution was applied a positive pressure of hydrogen gas and the reaction mixture was stirred 12h. The reaction mixture was then filtered through a plug of celite cluting with methanol and then the solvent was removed under reduced pressure to yield the
20 crude product as a tan oil. Prep. plate thin layer chromatography, using 90:10 $\text{CHCl}_3/\text{acetone}$ provided the final product (57%) as a tan/white solid. $R_f = 0.08$ (90:10 $\text{CHCl}_3/\text{acetone}$).

Deprotection to afford free alcohol. To a stirring solution of the N-Boc
25 protected alcohol (0.008 mmol) in CH_2Cl_2 (0.5 mL) was added trifluoroacetic acid (0.5 mL) and stirring was continued 4h. Under reduced pressure, solvent and excess trifluoroacetic acid were removed affording a brown oil. The oil was rinsed with ether and the solvent was removed under vacuum 5 times to afford the product (100%) as a tan solid. $R_f = 0.2$ (90:10 $\text{CHCl}_3/\text{methanol}$).

Phosphorylation of N-Boc protected alcohol. For phosphorylation,
30 reaction is performed in the absence of light, work up and columns are completed with as little light as possible. To a solution of the alcohol (0.085 mmol) in 1:1 $\text{CH}_2\text{Cl}_2/\text{THF}$ (5 mL) was added tetrazole (0.170 mmol) and the resulting mixture was

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stirred 30 min. Di-tert-butyl-di-isopropylphosphoramidite (0.170 mmol) was then added and the resulting reaction mixture was stirred 15h. Hydrogen peroxide (0.340 mmol) was then added and the resulting mixture was then stirred 4h, cooled to 0°C, and quenched by addition of aqueous Na₂S₂O₅. The resulting solution was diluted
5 with ethyl acetate (10 mL) and washed with sodium bicarbonate (3 x 10 mL), ammonium chloride (3 x 10 mL), and finally brine (1 x 10 mL). The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure to afford the product.

Deprotection of N-Boc and phosphate groups. To a stirring solution of
10 the protected product in CH₂Cl₂ was added trifluoroacetic acid and stirring was continued 4h. Under reduced pressure, solvent and excess trifluoroacetic acid were removed affording a brown oil. The oil was rinsed with ether and the solvent was removed under vacuum 5 times to afford the product.

15 **Example 2**

[γ-35 S]GTP Binding Assay for Measuring S1P Activity

Transient Expression in HEK293T Cells.

Human or mouse S1P5 DNA was mixed with an equal amount of DNA encoding a rat G_i2R protein as well as DNAs encoding cow β1 and γ2 proteins and
20 used to transfect monolayers of HEK293T cells using the calcium phosphate precipitate method. After 60 h, cells were harvested, and microsomes were prepared, aliquoted, and stored at -70 °C until use.

[γ-35 S]GTP Binding.

25 Briefly, 5 ug of membranes from S1P expressing HEK293T cells was incubated in 0.1 mL of binding buffer (in mM: HEPES 50, NaCl 100, MgCl₂ 5), pH 7.5, containing 5 ug of saponin, 10 uM GDP, 0.1 nM [γ-35 S]GTP (1200 Ci/mmol), and test lipid. After incubating for 30 min at 30°C, bound radionuclide was separated from free by filtration through Whatman GF/C paper using a Brandel Cell Harvester
30 (Gaithersburg, MD).

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Stable Expression in RH7777 Cells.

Rat hepatoma RH7777 cell monolayers were transfected with human or mouse S1P5/pCR3.1 DNA using the calcium phosphate precipitate method, and clonal populations expressing the neomycin phosphotransferase gene were selected by addition of Ge-neticin (G418) to the culture medium. The RH7777 cells were grown in monolayers at 37 °C in a 5%CO₂/95% air atmosphere in growth medium consisting of 90% MEM, 10% fetal bovine serum, 2 mM glutamine, and 1 mM sodium pyruvate.

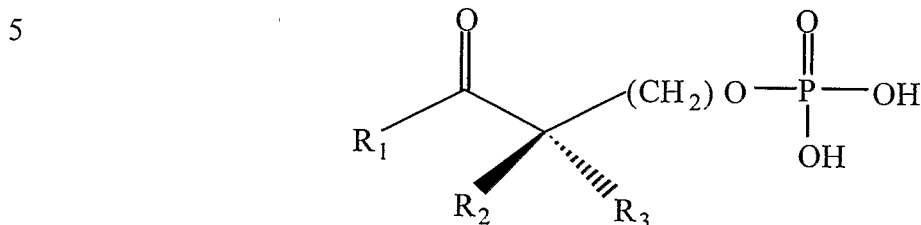
Measurement of cAMP Accumulation.

Assay of cAMP accumulation was performed as described previously (See Im et al., J. Biol. Chem. 275, 14281-14286 (2000), the disclosure of which is incorporated herein). Assays were conducted on populations of 5 x 10⁵ cells stimulated with 1 uM forskolin in the presence of the phosphodiesterase inhibitor isomethylbutylxanthine (IBMX, 1 mM) for 15 min. cAMP was measured by automated radioimmunoassay. The GTPγS studies were performed using zebrafish S1P1 overexpressed rat RH-7777 and human hS1P1, hS1P2, hS1P3 and hS1P5 overexpressed human HEK293 cells. Table 1 shows the EC₅₀ values for each of the S1P analogs at S1P receptors: S1P1, S1P2, S1P3 and S1P5. In addition to testing the human S1P receptors (hS1P1, hS1P2, hS1P3 and hS1P5), a zebrafish S1P receptor (zS1P1) and mouse S1P (mS1P5) were also tested.

Table 1: EC₅₀ Values (nM) for S1P Analogues at Recombinant S1P Receptors

	zS1P ₁	hS1P ₁	hS1P ₃	hS1P ₂	hS1P ₅	mS1P ₅
S1P	54.6	0.9	1.1	2.9	43.9	12.7
VPC22041	2053.0	598.4	845.4	973.2	645.5	>5000
VPC22051	>5000	322.1	601.9	2760.0	>5000	>5000
VPC22053	>5000	397.0	862.4	2685.0	1606.0	2006.0
VPC22063	>5000	1805.0	878.6	>5000	1220.0	1326.0
VPC22135	1625.0	12.7	50.8	2107.0	>5000	1821.0

S1P increases GTP γ S binding significantly (2-5-fold) at each receptor with EC₅₀ values from 1 to 55 nM. The synthetic series consisted of five dihydro S1P of the general formula:



10 wherein

VPC22041 (2S): R₁ is NH(CH₂)₁₁CH₃, R₂ is NH₂ and R₃ is H;

VPC22053 (2S): R₁ is O(CH₂)₁₃CH₃, R₂ is NH₂ and R₃ is H;

VPC22051 (2S): R₁ is NH(CH₂)₁₃CH₃, R₂ is NH₂ and R₃ is H;

VPC22063 (2S): R₁ is NH(CH₂)₁₅CH₃, R₂ is NH₂ and R₃ is H; and

15 VPC22135 (2R): R₁ is NH(CH₂)₁₃CH₃, R₂ is H and R₃ is NH₂.

The amide-containing compounds contained alkyl chains of 12 (VPC22041), 14 (VPC22053), or 16 (VPC22063) carbons, and the 2'-amino group was in the natural configuration (S), except for VPC22135, wherein the 2'-amino was

20 in the (R) configuration. VPC22053 and VPC22135 are an enantiomeric pair, while VPC22051 is the ester-containing equivalent of VPC22053 (see Scheme 4). All of these compounds had significant agonist activity at each of the S1P receptors, although none were as potent as S1P itself (see Table 1). In particular, on the S1P5 transfected HEK293 cells, the five mimetics showed EC₅₀'s of approximately 1 μ M,

25 where as the EC₅₀ of S1P itself on the same cells is closer to 10 nM. However, one compound, VPC22135, approached the potency of S1P at both the human S1P1 and human S1P3 receptors. Curiously, this compound has the amino group in the unnatural (R) configuration. Its enantiomer, VPC22053, was more than 1 log order less potent at both the S1P1 and S1P3 receptors. The results obtained for the S1P1

30 transfected RH-7777 cells showed a preference for binding with the 18 carbon

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backbone mimetic compounds (identical to S1P) over the 16 and 20 carbon backbone mimetic compounds.

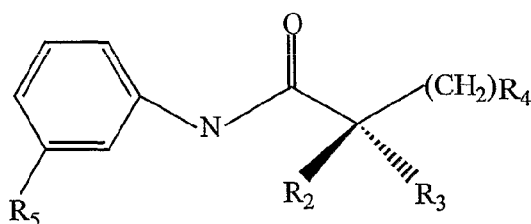
Example 3

5 Biological Assay of the Synthesized Mimetics

An additional series of compounds was tested using the GTP γ S binding assay described in Example 2 and in Im et al., J. Biol. Chem. 275, 14281-14286 (2000), the disclosure of which is incorporated herein). The compounds tested for binding at

10 human S1P receptors (hS1P1, hS1P2, hS1P3, hS1P4 and hS1P5) have the general

structure:

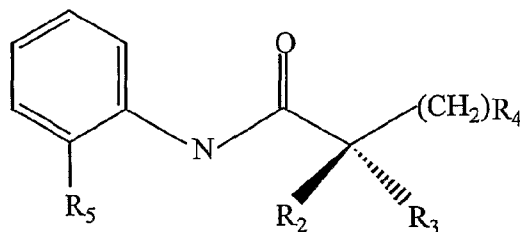


15

wherein for VPC23019: R_5 is $(CH_2)_7CH_3$, R_2 is H, R_3 is NH_2 and R_4 is phosphate;
 VPC23031: R_5 is $(CH_2)_7CH_3$, R_2 is H, R_3 is NH_2 and R_4 is phosphate;
 VPC23065: R_5 is $(CH_2)_9CH_3$, R_2 is H, R_3 is NH_2 and R_4 is hydroxy;
 VPC23069: R_5 is $(CH_2)_9CH_3$, R_2 is H, R_3 is NH_2 and R_4 is phosphate;
 20 VPC23075: R_5 is $(CH_2)_8CH_3$, R_2 is H, R_3 is NH_2 and R_4 is hydroxy;
 VPC23079: R_5 is $(CH_2)_8CH_3$, R_2 is H, R_3 is NH_2 and R_4 is phosphate;

or have the general structure:

25



30

wherein for VPC23087: R_5 is $(CH_2)_7CH_3$, R_2 is H, R_3 is NH_2 and R_4 is hydroxy;
 VPC23089: R_5 is $(CH_2)_7CH_3$, R_2 is H, R_3 is NH_2 and R_4 is phosphate;

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Each of the compounds tested (VPC 23019, 23031, 23065, 23069, 23087, 23089, 23075, 23079) failed to show significant activity at the S1P2 receptor. Compounds VPC23065, VPC23087 and VPC23075 are primary alcohols and thus lack the phosphate headgroup. Yet several of these compounds exhibit activity at S1P receptors (See Figs. 2A, 2B, 2C, 3A, 3B, 3C and 4C) and each of these compounds shows good agonist activity at the S1P4 receptor.

The GTP γ S binding assay revealed that VPC23031, VPC23019, VPC23089 are inverse agonists (antagonists) of the S1P3 receptor (See Figs. 1A and 4A), but this inverse agonism becomes agonism when the alkyl chain length is 9 carbons (VPC23079) or 10 (VPC23069), see Figs. 2A and 3A.

VPC23089 and VPC23019 are isomers, with the VPC23089 compound having the alkyl chain ortho and the VPC23019 compound meta; in both cases the alkyl chain has 8 carbons, but surprisingly, when one goes from ortho to meta, antagonism at S1P1 is realized (compare Fig. 1A with the competition curve Fig. SA).

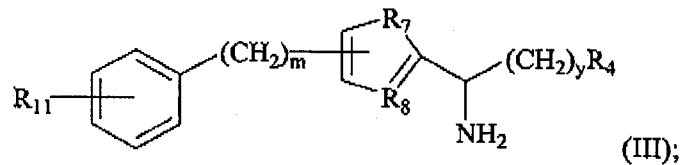
The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A compound having sphingosine-1-phosphate receptor modulating activity, represented by formula (III):



wherein,

R_{11} is selected from the group consisting of H, hydroxy, NH_2 , C_1 - C_{18} alkyl, C_2 - C_{18} alkenyl, C_2 - C_{18} alkynyl and $-(CH_2)_n-Z-R_6$;

R_4 is selected from the group consisting of phosphate, phosphonate, methylene phosphonate, α -substituted methylene phosphonate, phosphate analogs and phosphonate analogs;

y is an integer ranging from 1 to 4;

m is an integer ranging from 0 to 4;

n is an integer ranging from 0 to 10;

Z is selected from the group consisting of aryl and heteroaryl;

R_6 is selected from the group consisting of C_1 - C_{10} alkyl, C_1 - C_{20} alkoxy, C_1 - C_{20} alkylthio, and C_1 - C_{20} alkylamino; and

R_7 and R_8 are independently selected from the group consisting of O, S and N; or a pharmaceutically acceptable salt thereof.

2. The compound of claim 1 wherein

R_4 is selected from the group consisting of phosphate, phosphonate, methylene phosphonate, α -substituted methylene phosphonate, thiophosphate and thiophosphonate;

R_8 is N;

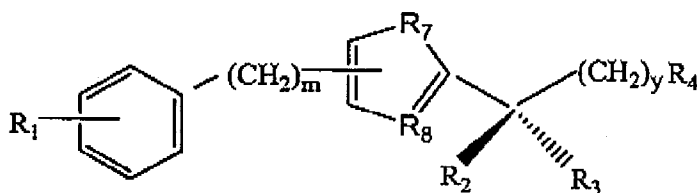
m is 0; and

y is 1.

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3. The compound of claim 2 wherein R_4 is phosphate.
4. A composition comprising a compound of claim 1; and a pharmaceutically acceptable carrier.
5. A method for modulating the activity of an S1P receptor, said method comprising the step of contacting said receptor with a compound of claim 1 or a pharmaceutically acceptable salt thereof.
6. A method of providing immunomodulation to a patient in need thereof, said method comprising the step of administering to said patient a composition comprising a compound of the general formula:



wherein R_9 is selected from the group consisting of $-NR_1R_{17}$, and $-OR_1$;

R_1 is selected from the group consisting of alkyl, alkenyl, alkynyl, optionally substituted aryl, optionally substituted heteroaryl, alkyl(optionally substituted aryl), arylalkyl and arylalkyl(optionally substituted)aryl;

R_2 and R_3 are independently selected from the group consisting of H, NH_2 , OH, C_1-C_6 alkyl, $-(C_1-C_4 \text{ alkyl})OH$, $-(C_1-C_4 \text{ alkyl})NH_2$, $-(C_1-C_4 \text{ alkyl})aryl(C_0-C_4 \text{ alkyl})$ and $-(C_1-C_4 \text{ alkyl})aryloxyaryl(C_0-C_4 \text{ alkyl})$ with the proviso that at least one of R_2 and R_3 is NH_2 ;

y is an integer from 1-10;

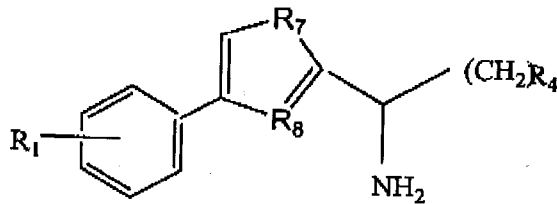
R_4 is selected from the group consisting of phosphate, phosphonate, methylene phosphonate, α -substituted methylene phosphonate, phosphate analogs and phosphonate analogs;

m is an integer ranging from 0 to 4; and

R_7 and R_8 are independently selected from the group consisting of O, S and N or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

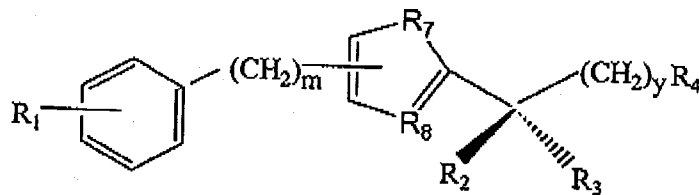
7. The method of claim 6 further comprising the step of administering a second immunomodulatory agent selected from the group consisting of cyclosporine, tacrolimus, azathioprine, prednisolone and prednisone.

8. The method of claim 6 wherein the compound has the general formula:



wherein R_1 is C_1 - C_{12} alkyl.

9. The method of claim 6 wherein the compound has the general formula:



wherein R_1 is alkyl, alkenyl, or alkynyl;

R_2 and R_3 are independently selected from the group consisting of H, NH_2 , OH, C_1 - C_6 alkyl, $-(C_1-C_4 \text{ alkyl})OH$, and $-(C_1-C_4 \text{ alkyl})NH_2$;

y is 1 or 2;

m is 0 or 1; and

either R_7 or R_8 is N;

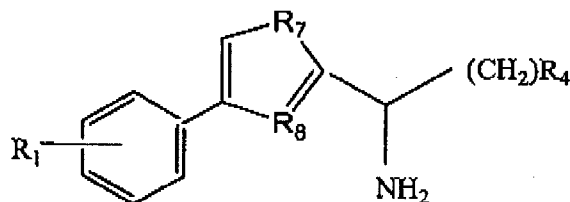
R_4 is selected from the group consisting of phosphate, phosphonate, methylene phosphonate, α -substituted methylene phosphonate, phosphate analogs and phosphonate analogs; and

R_7 and R_8 are independently selected from the group consisting of O, S and N.

10. The composition of claim 4 further comprising albumin.

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11. The composition of claim 4 wherein the compound has the general structure:



wherein R_{11} is C_1 - C_{12} alkyl;

R_4 is selected from the group consisting of phosphate, phosphonate, methylene phosphonate, α -substituted methylene phosphonate, phosphate analogs and phosphonate analogs; and

R_7 and R_8 are independently selected from the group consisting of O, S and N.

12. A compound according to any one of claims 1 to 3, a method according to any one of claims 5 to 9 or a pharmaceutical composition according to any one of claims 4 or 10 to 11 substantially as hereinbefore described with reference to the Figures and/or Examples.

Fig 1A

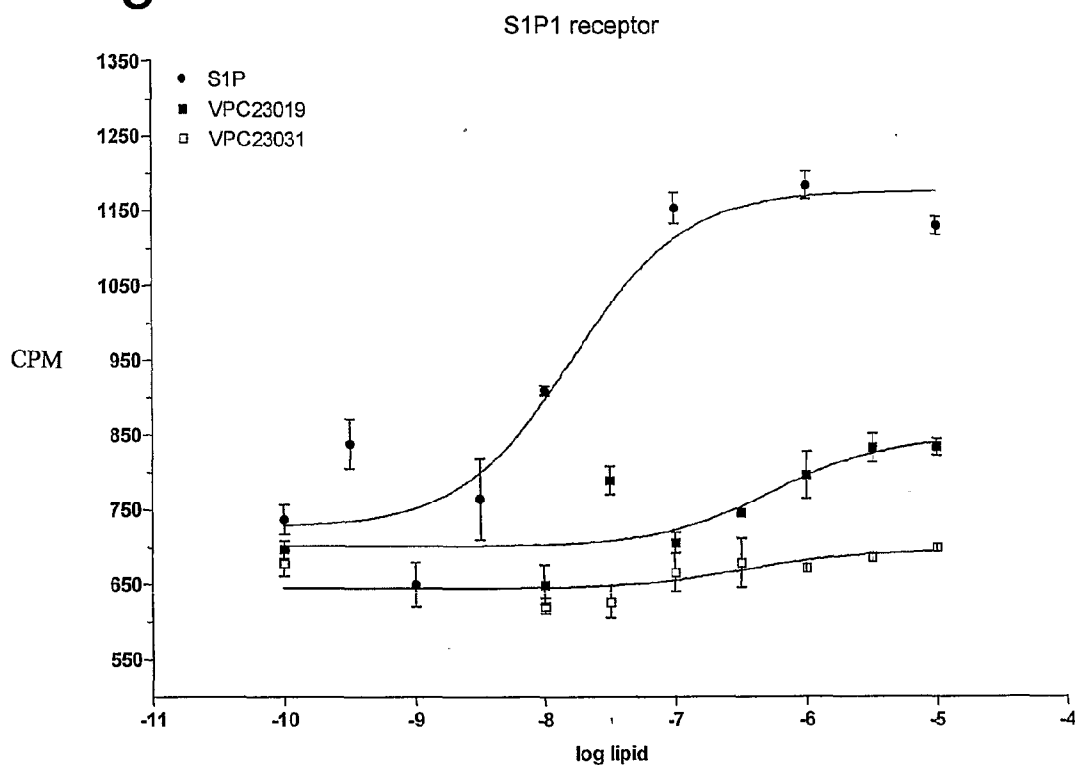


Fig 1B

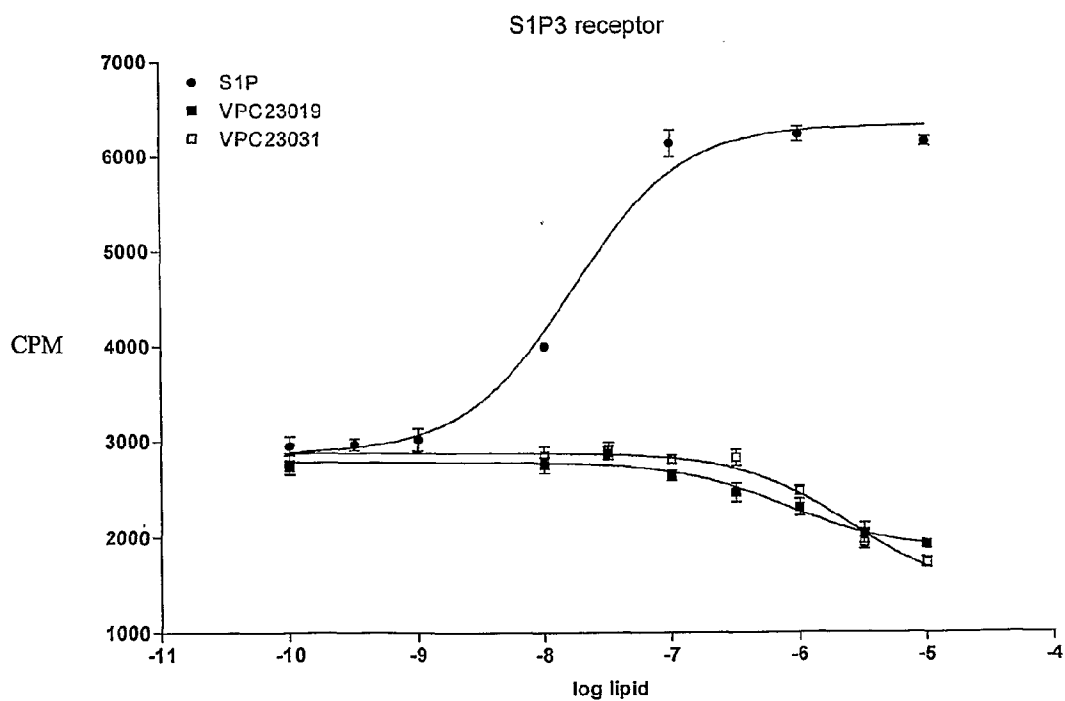


Fig 1C

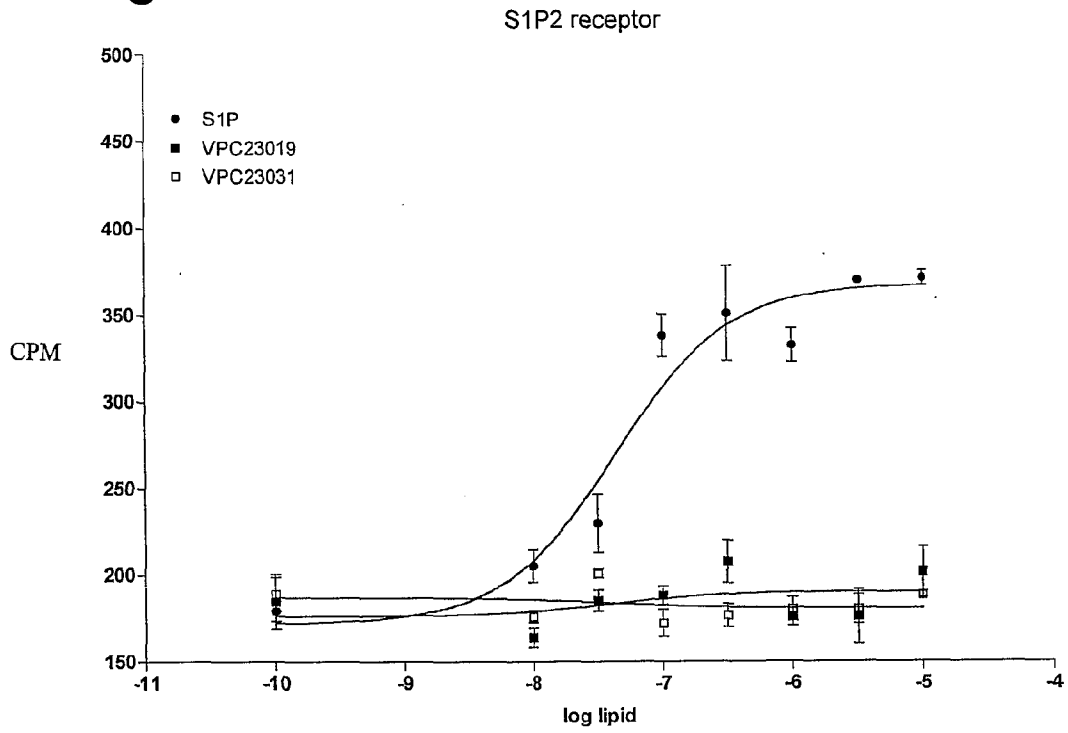
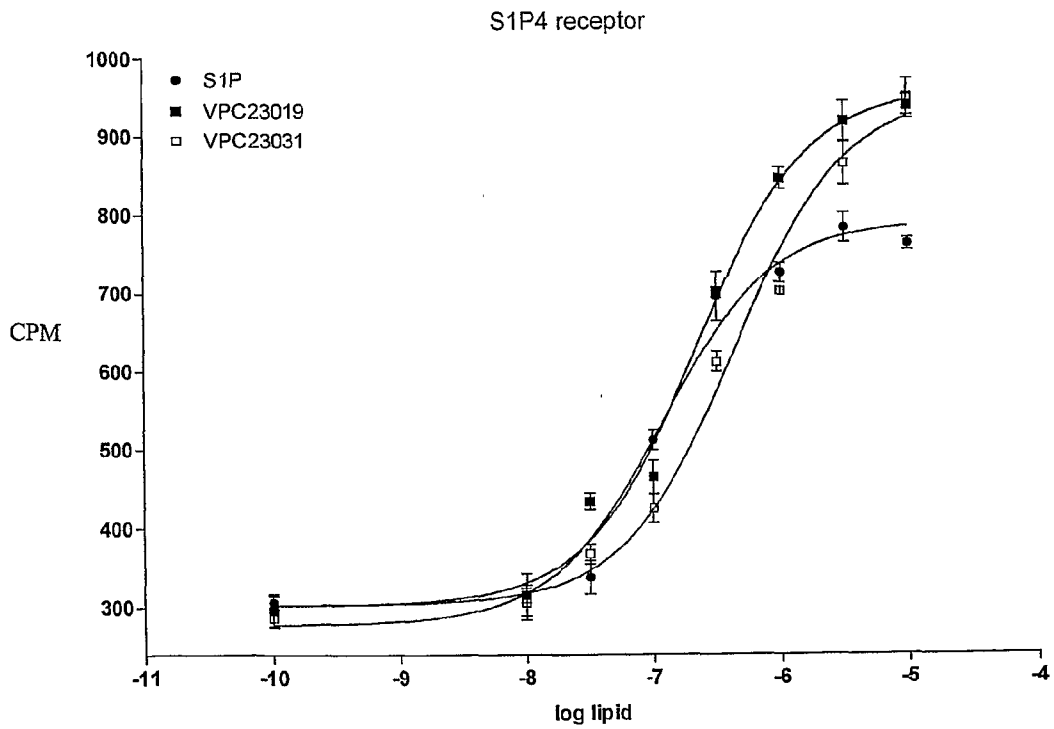


Fig 1D



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Fig 1E

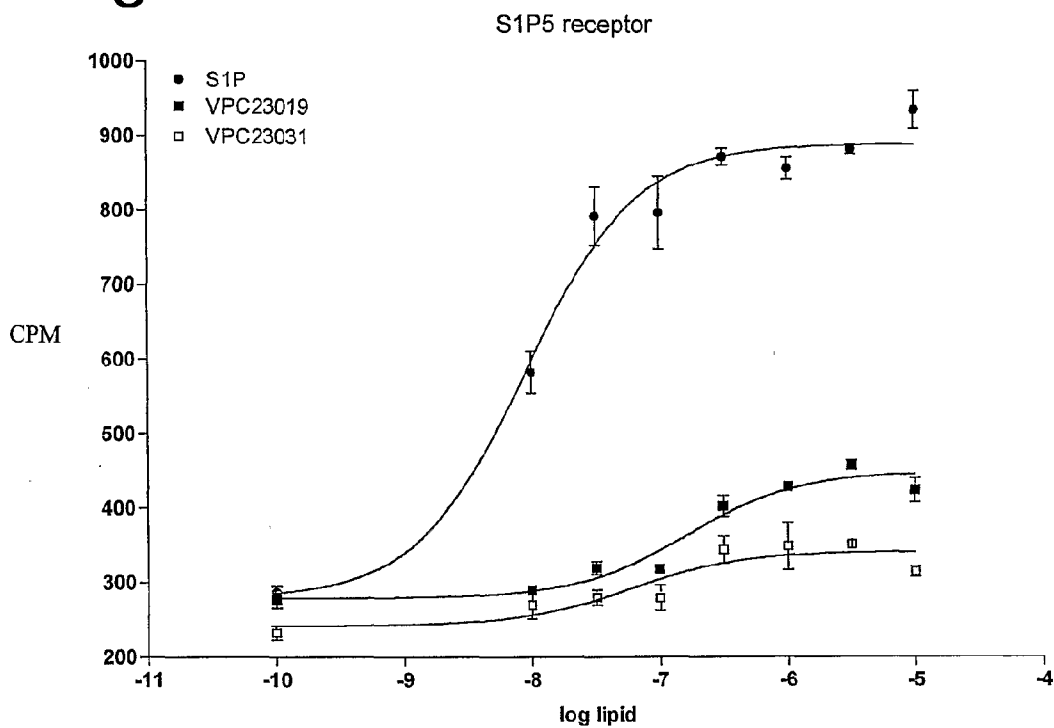


Fig 1F

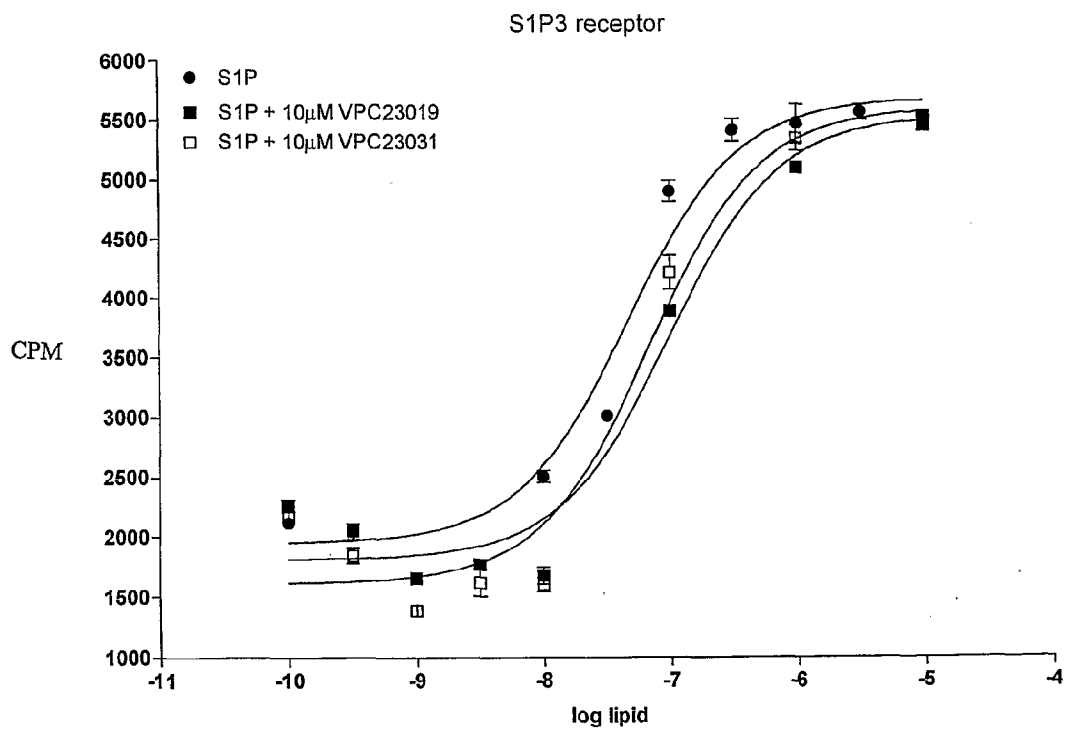


Fig 2A

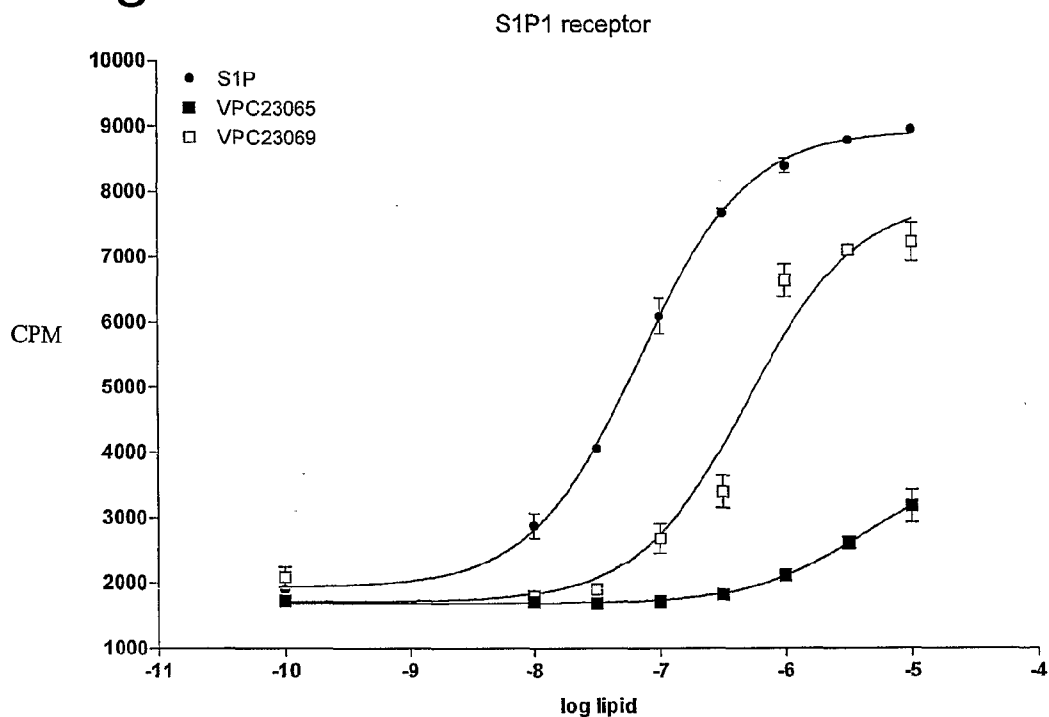


Fig 2B

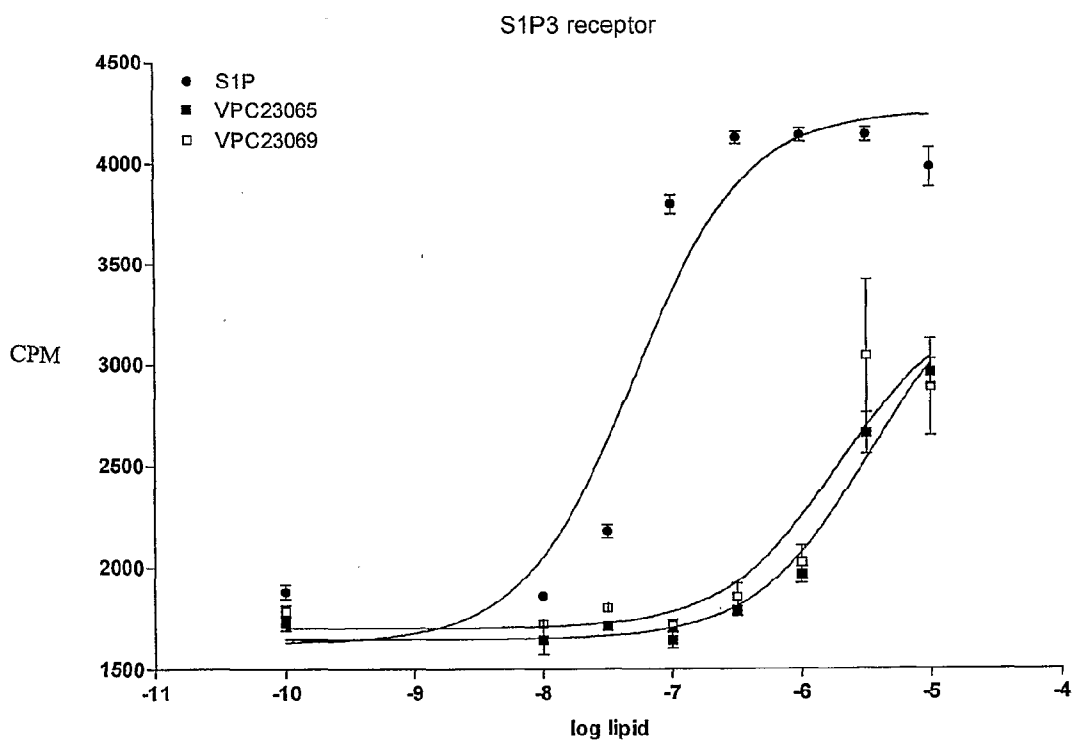


Fig 2C

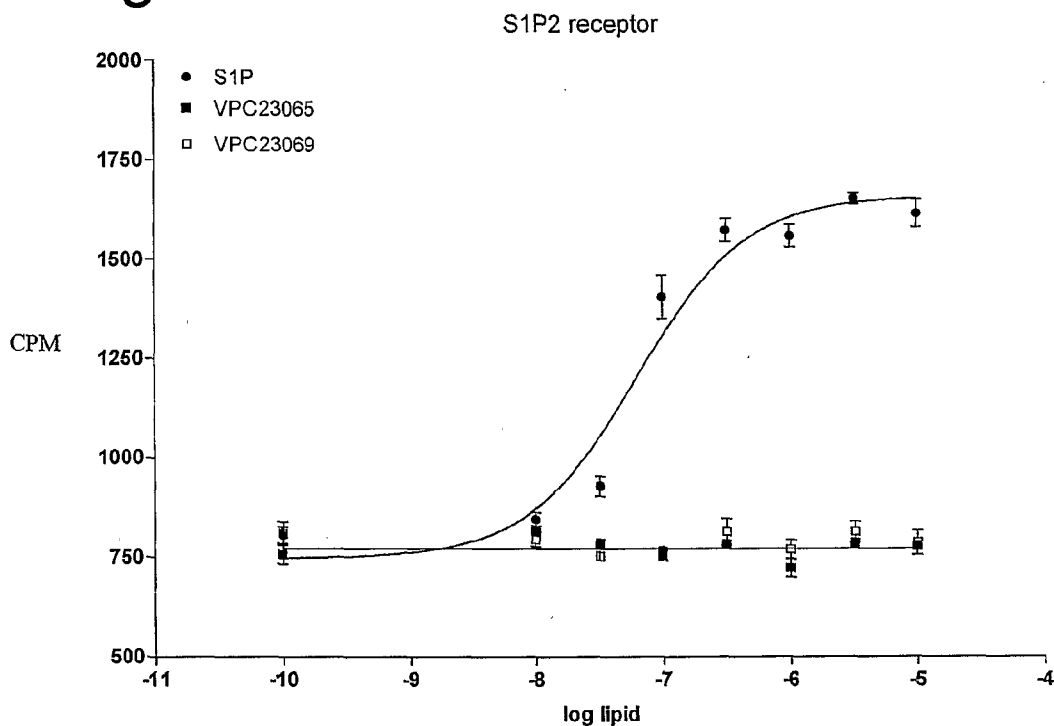


Fig 2D

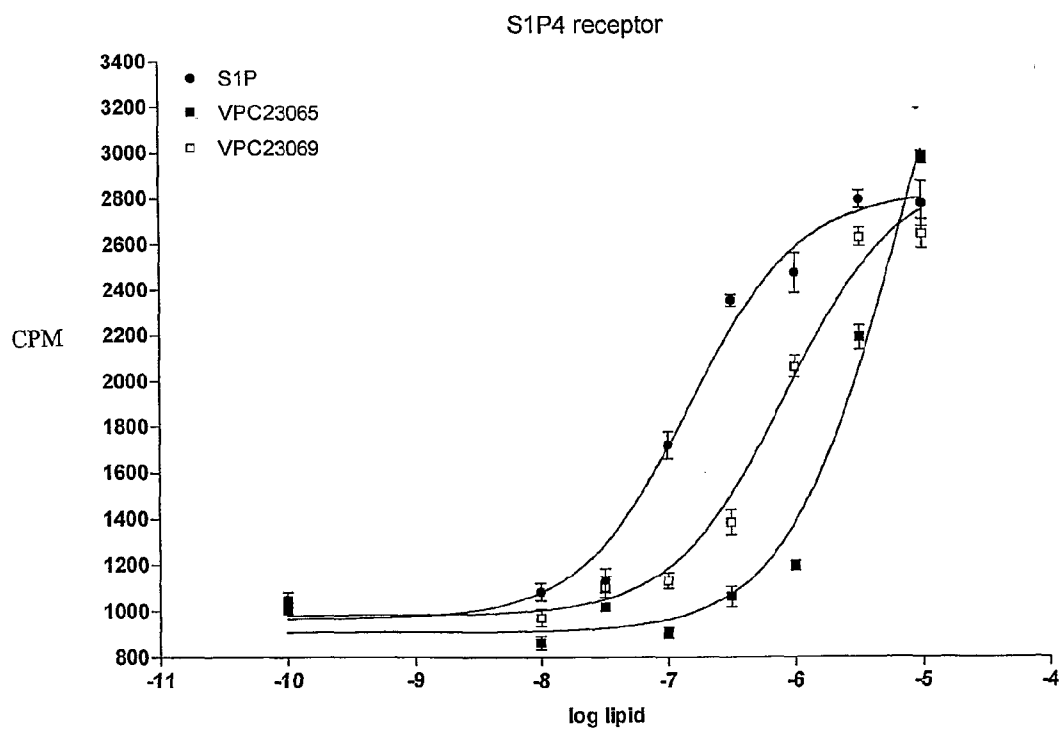


Fig 2E

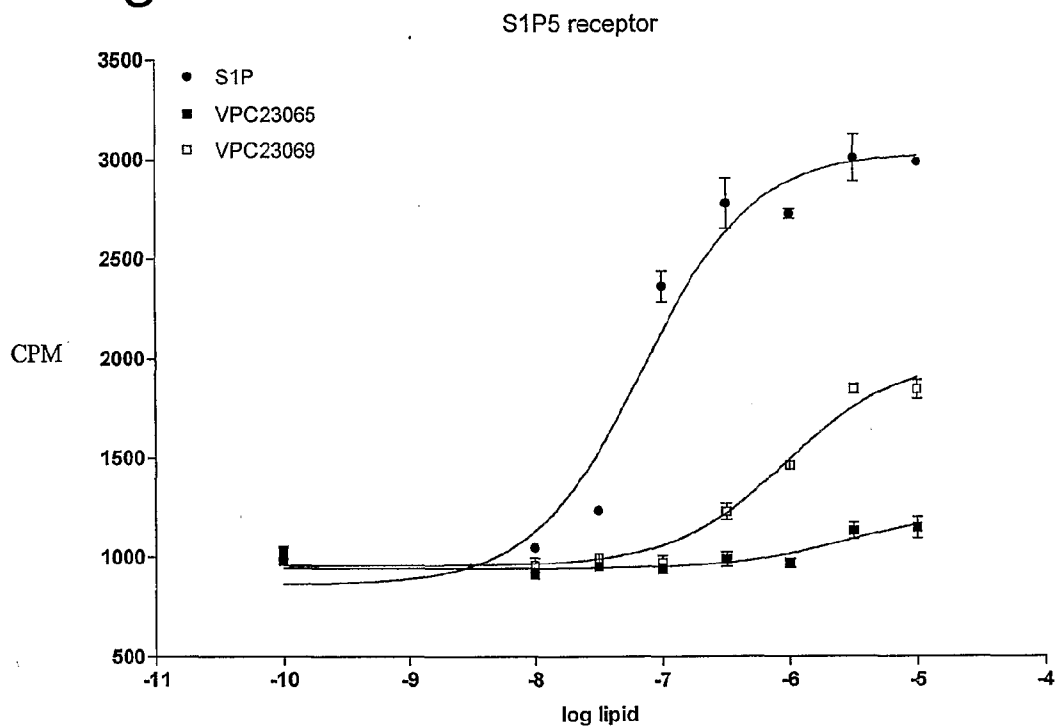
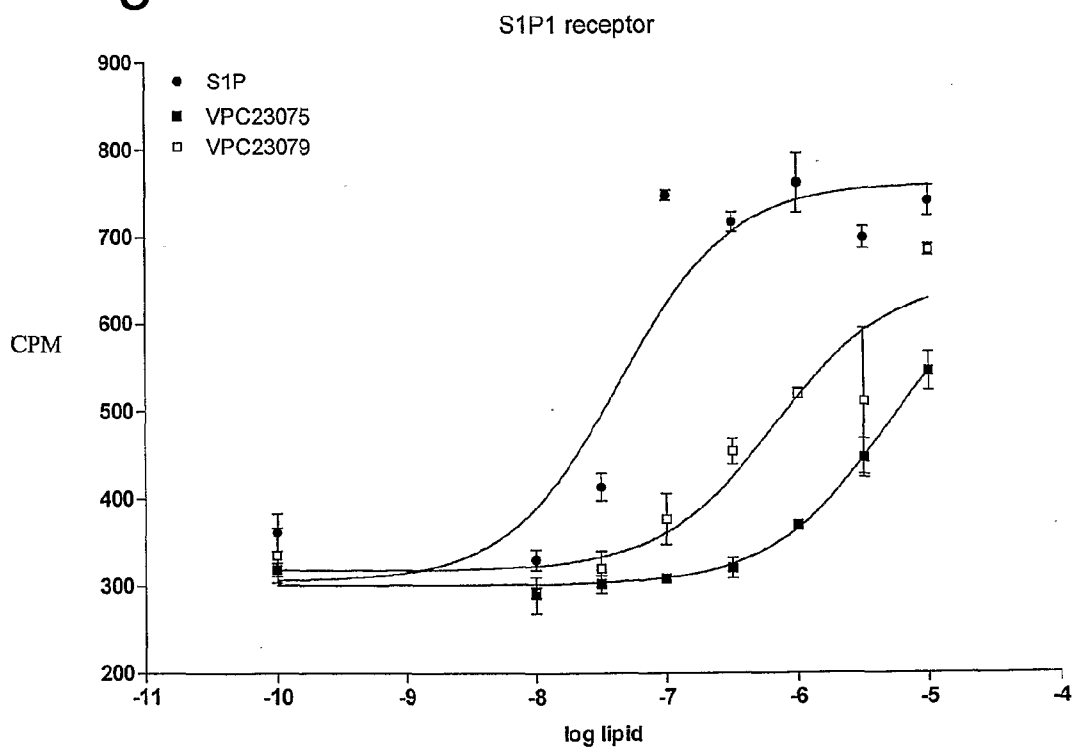


Fig 3A



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Fig 3B

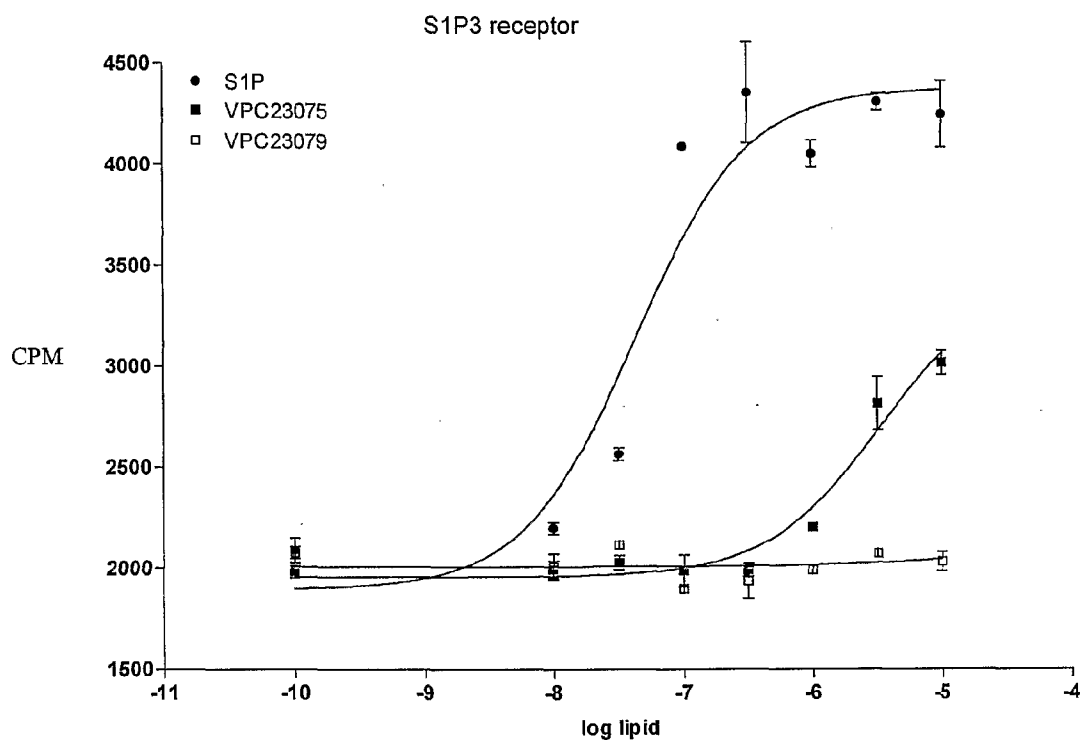
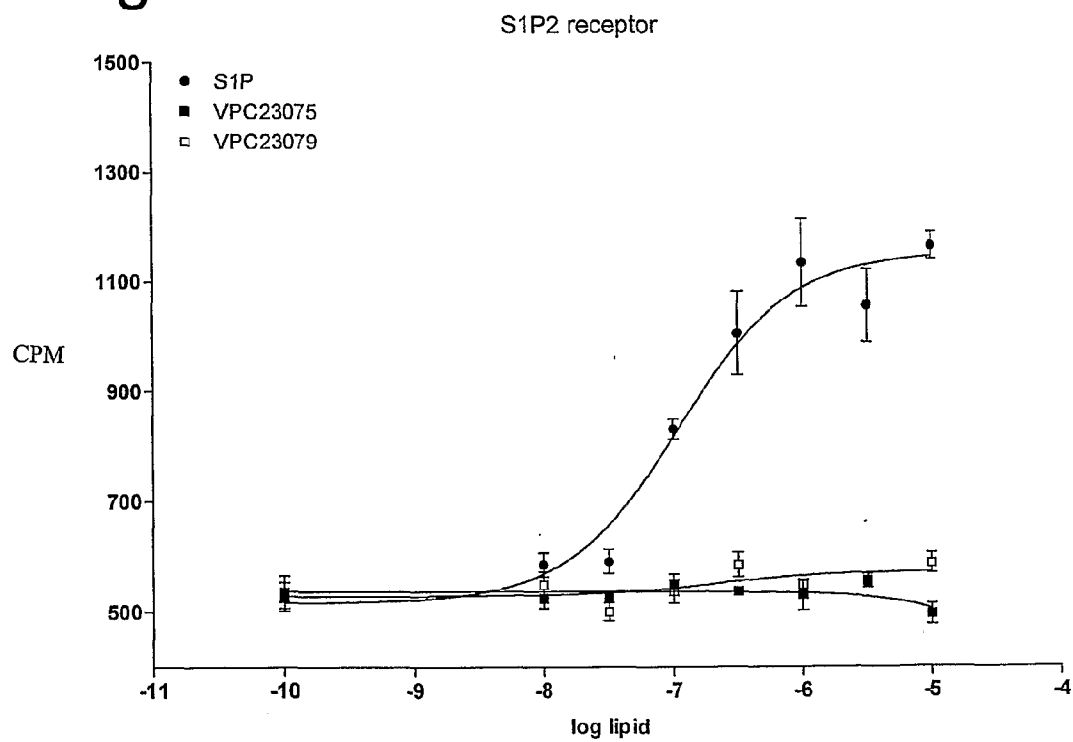


Fig 3C



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Fig 3D

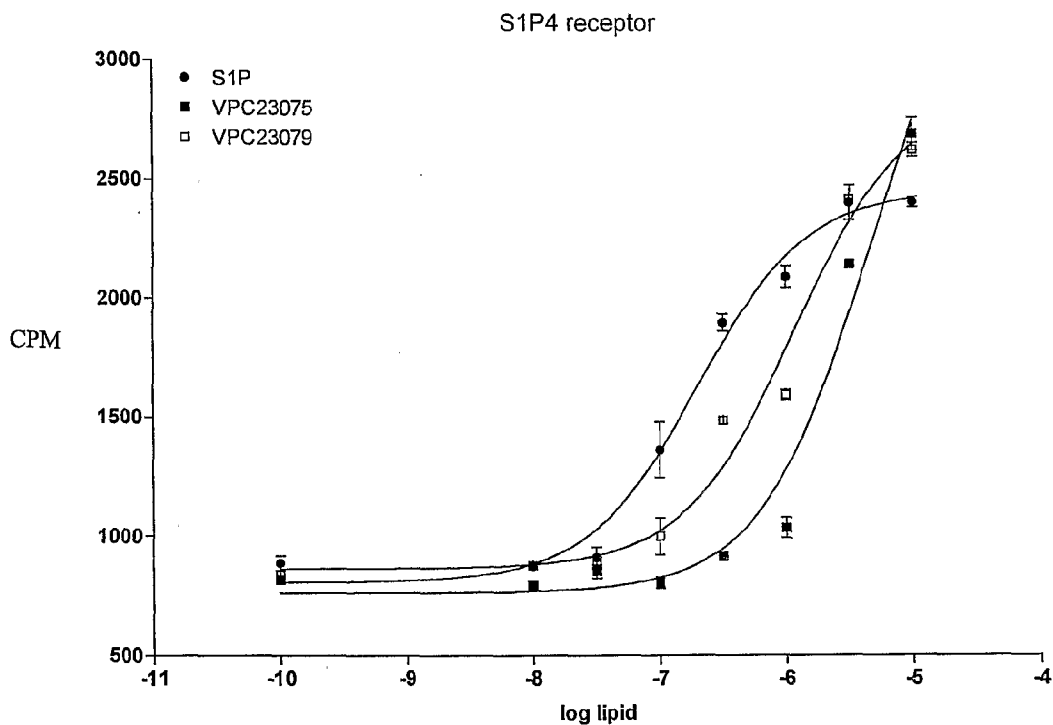
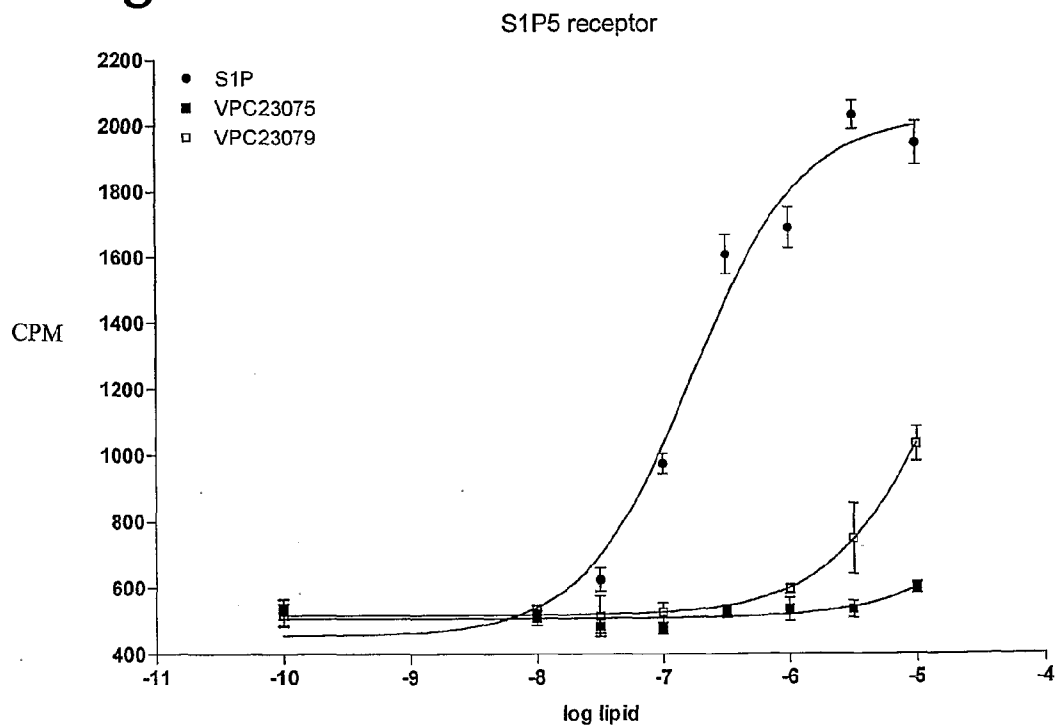


Fig 3E



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Fig 4A

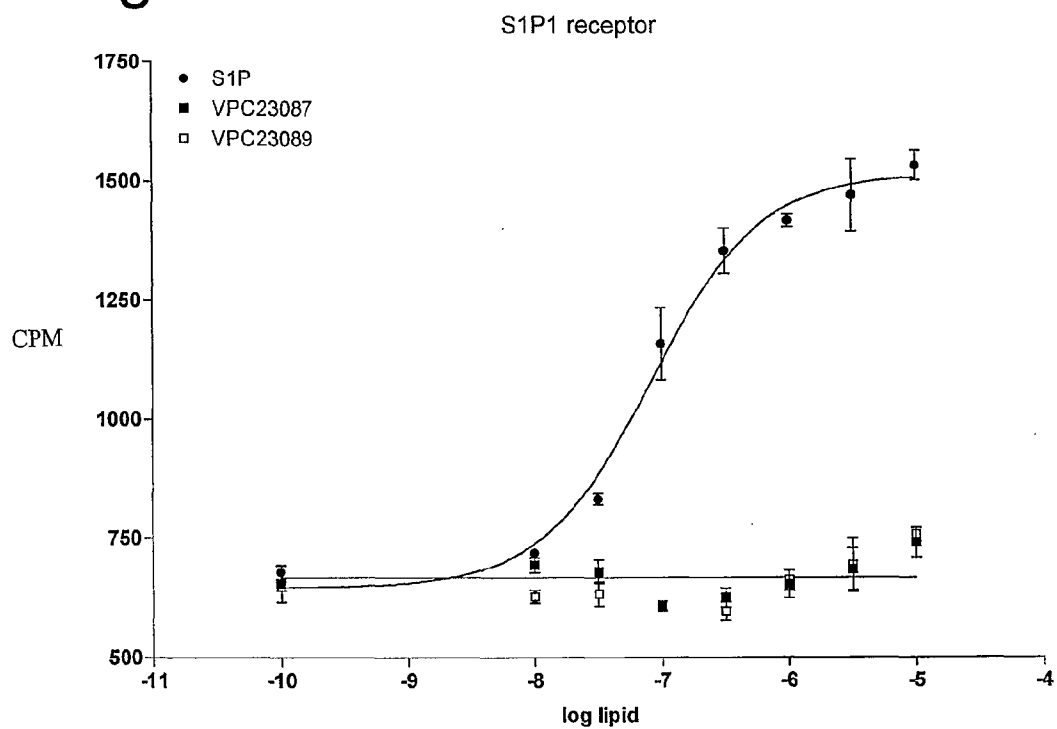
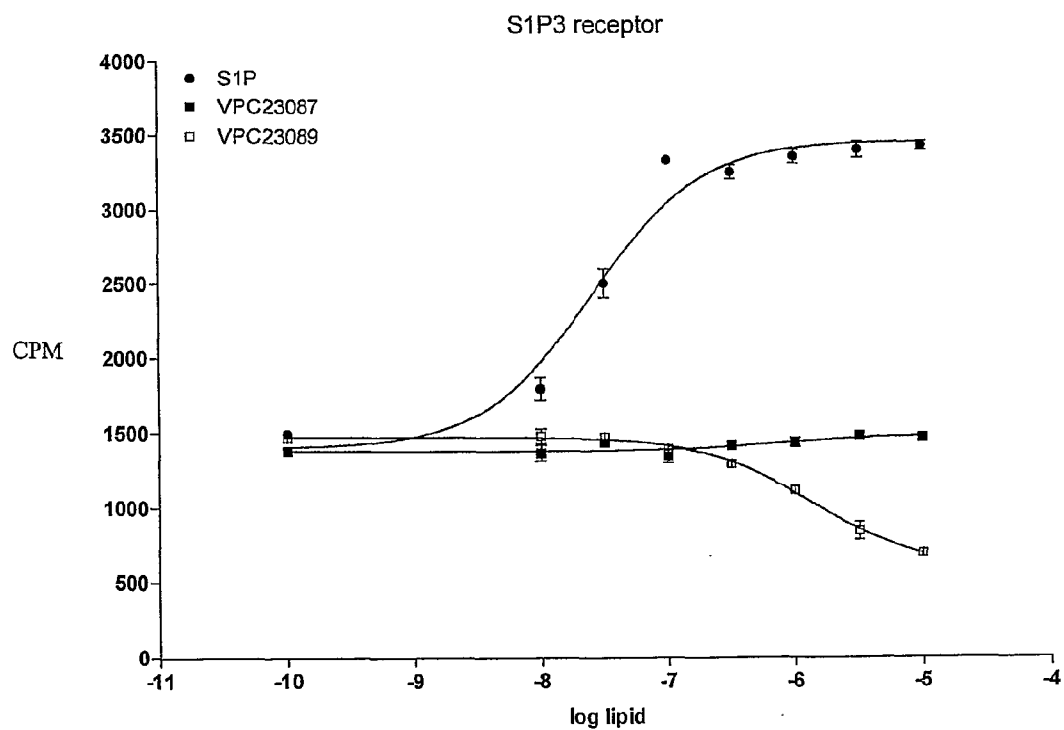


Fig 4B



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Fig 4C

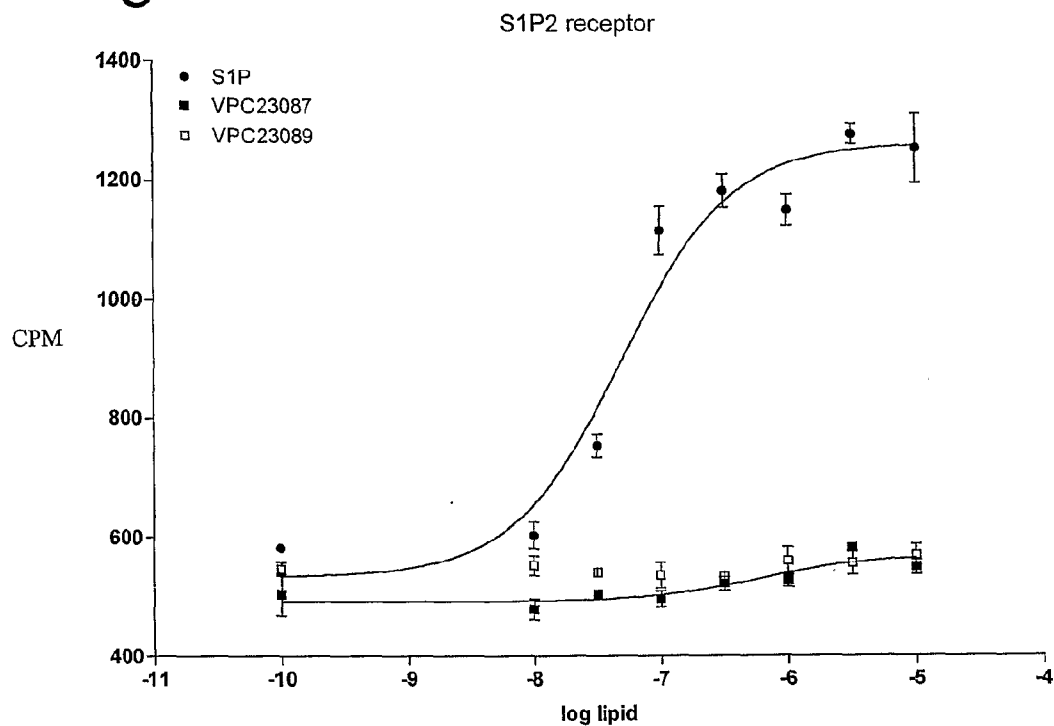
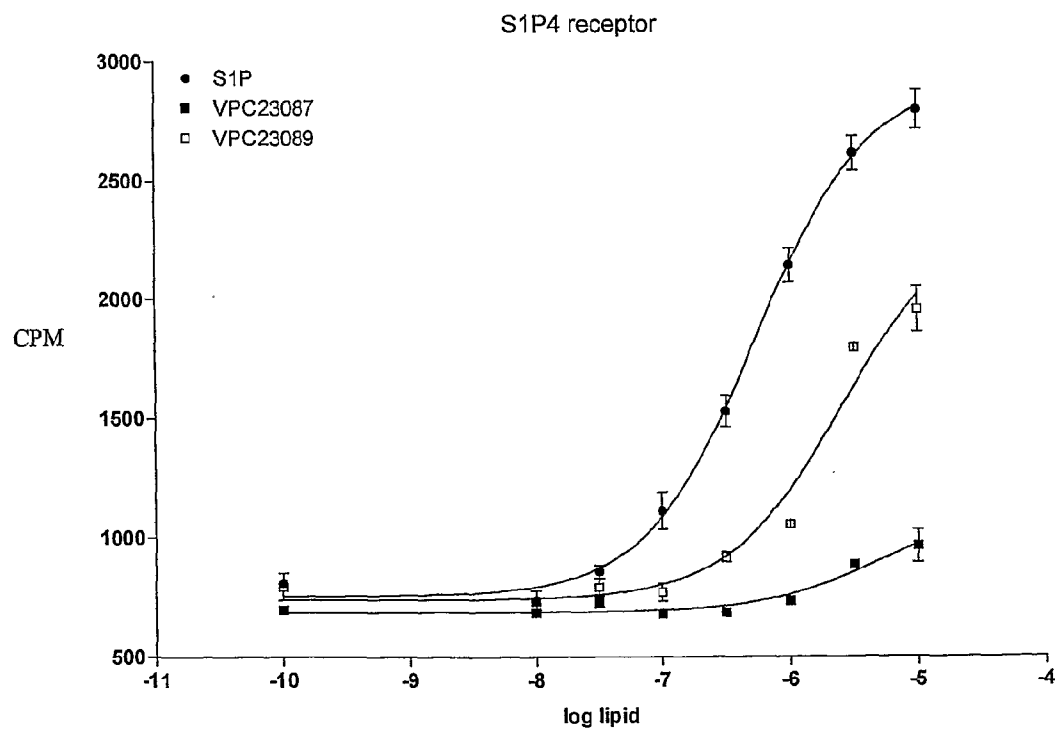


Fig 4D



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Fig 4E

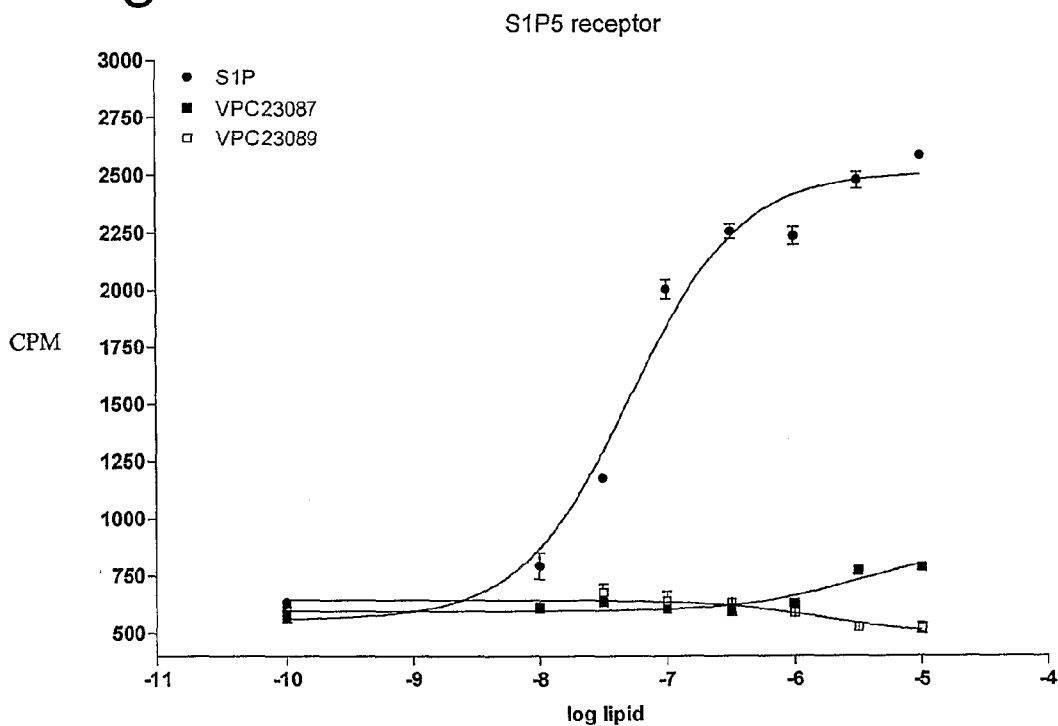
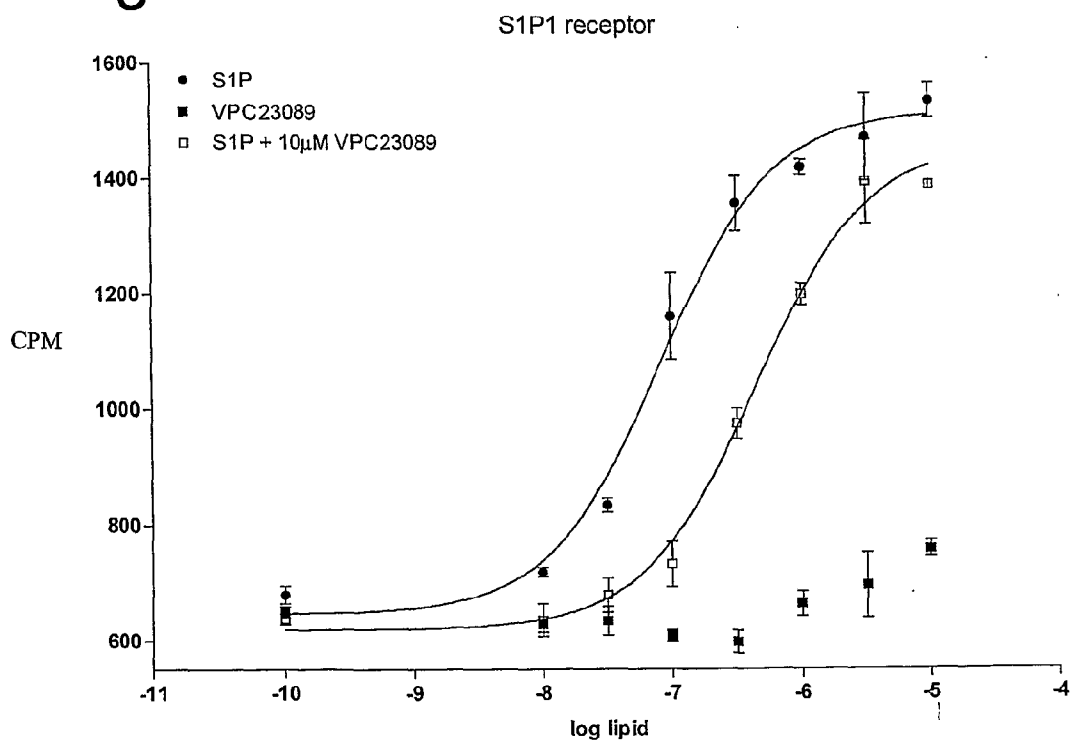


Fig 5A



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Fig 5B

