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Design, synthesis and biological activity of sphingosine kinase 2 selective inhibitors

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ABSTRACT

Sphingosine kinase (SphK) has emerged as an attractive target for cancer therapeutics due to its role in cell survival. SphK phosphorylates sphingosine to form sphingosine 1-phosphate (S1P), which has been implicated in cancer growth and survival. SphK exists as two different isotypes, namely SphK1 and SphK2, which play different roles inside the cell. In this report, we describe SphK inhibitors based on the immunomodulatory drug, FTY720, which is phosphorylated by SphK2 to generate a S1P mimic. Structural modification of FTY720 provided a template for synthesizing new inhibitors. A diversity-oriented synthesis generated a library of SphK inhibitors with a novel scaffold and headgroup. We have discovered subtype selective inhibitors with K_i's in the low micromolar range. This is the first report describing quaternary ammonium salts as SphK inhibitors.

1. Introduction

Sphingosine 1-phosphate (S1P), an intermediate in the sphingomyelin metabolism pathway, is emerging as a potential target in cancer therapeutics.[1] S1P regulates important cellular and physiological processes, including cell motility,[2] invasion,[3] and angiogenesis[4] by functioning as a specific ligand for a family of five G-protein coupled receptors of the rhodopsin family ($S1P_{1-5}$).[5] Recently, histone deacetylase (HDAC) has been identified as a direct intracellular target of nuclear S1P.[6] S1P inhibits HDACs 1 and 2 in repressor complexes, increases histone acetylation and enhances gene transcription of *p21* and *c-fos*. Studies linking S1P to cell proliferation[7] and suppression of apoptosis[8] have generated substantial interest about its role in many diseases.[9] The approval of fingolimod (FTY720, Gilenya™) by the Food and Drug Administration in 2010 for use in treating relapsing-remitting multiple sclerosis[10,11] underscores the potential of regulating the S1P pathway for treating disease.[12] FTY720 is phosphorylated in vivo by SphK2 to FTY720-P,[13] which induces internalization and degradation of the $S1P_1$ receptor resulting in prolonged receptor downregulation.[14] The resultant absence of an S1P signal modulates immune function by influencing lymphocyte trafficking, specifically by decreasing the egress of lymphocytes from secondary lymphoid tissues.

S1P biosynthetic precursors, in particular ceramide, have been identified as inducers of apoptosis.[15] Since these metabolites are interconvertible by the actions of various enzymes, a ceramide/S1P rheostat has been proposed as a determinant of cell fate (Fig. 1).[8] As a result, enzymes in the cell that regulate this pathway are potential targets for cancer therapeutics. Since S1P is the proximal effector, sphingosine kinase (SphK) plays a crucial role in the control of this balance. Two isoforms of SphK, SphK1 and SphK2, have been isolated and characterized.[16] It has been hypothesized that SphK1 and SphK2 might be involved in different cellular functions[17] due to differences in their localization within the cell[18] (i.e., SphK1 is located mainly in the cytoplasm, SphK2 is located in the nucleus and endoplasmic reticulum), differences in selectivity[19] and opposing functions in sphingolipid metabolism.[20] Studies have shown that SphK1 is up-regulated in a variety of solid tumors[21] and promotes cell survival while SphK2 is pro-apoptotic because of its BH3 domain that interacts with $BCLX_L$.[22] However, a recent study suggests an important role for SphK2 in the tumor progression in MCF-7 breast cancer xenografts.[23] These contrasting results demonstrate the need for a deeper understanding of the role of

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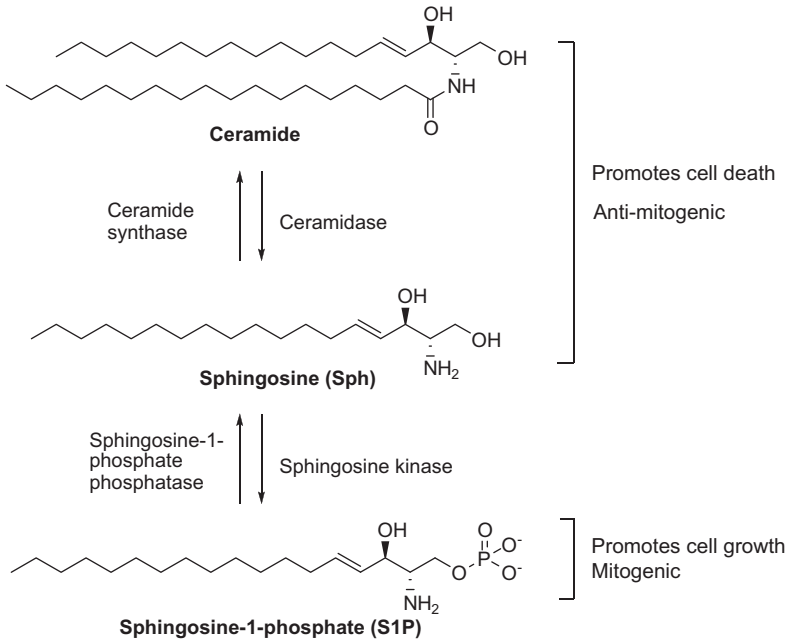


Figure 1. The ceramide/sphingosine-1-phosphate rheostat.

SphK2 in tumor cell models. The widely used inhibitors of SphK, L-*threo*-dihydrosphingosine (DHS) and *N*,*N*-dimethylsphingosine (DMS),[24] are not selective as they also inhibit other enzymes such as protein kinase C[25] and sphingosine-dependent protein kinase (Fig. 2).[26] Indeed, the need for subtype-selective inhibitors has not gone unnoticed. Recently, a water-soluble, isoenzyme-specific inhibitor of SphK1, **SK1-I**, was shown to induce apoptosis in human leukemia cells and reduced growth of AML xenograft tumors.[27] In another study, SK1-I markedly reduced the tumor growth rate of glioblastoma xenografts and induced apoptosis.[28] SphK1 inhibitors based on replacing the aminodiol in sphingosine by a serine amide were reported[29] (**12aa**) and their structure was optimized to increase water solubility and oral bioavailability.[30] The most potent and selective SphK1-selective inhibitors reported to date featured amidine-based groups (**28**).[31,32] While several studies generated SphK1 selective inhibitors, reports of SphK2-selective compounds are scarce. SphK2-selective inhibitors reported in the literature were obtained either via screening of commercial small molecule libraries (**ABC294640**)[33] or by synthesizing analogues of sphingosine (**SG-12**).[34] Recently, the methyl ether of FTY720 ((***R***)-**FTY720-OMe**) was reported as an inhibitor of SphK2 with K_i of 16.5 μM.[35] Unfortunately, rational design of sphingosine kinase inhibitors has been hampered by the lack of crystal structure of either protein. While it has been suggested that the C4 domain is involved in specific recognition of Sph via the interaction of the basic amine in sphingosine with the Asp[177],[36] rational design of SphK inhibitors remains a challenge.

Inhibitors of SphKs with the combination of drug-like properties, potency, and selectivity will be valuable in evaluating these enzymes as therapeutic targets. The lack of potent SphK2-selective inhibitors retards detailed scientific studies exploring the role of SphK2 in many diseases. Herein, we report the discovery of a novel scaffold and structure-activity studies that reveal SphK2-selective inhibitors.

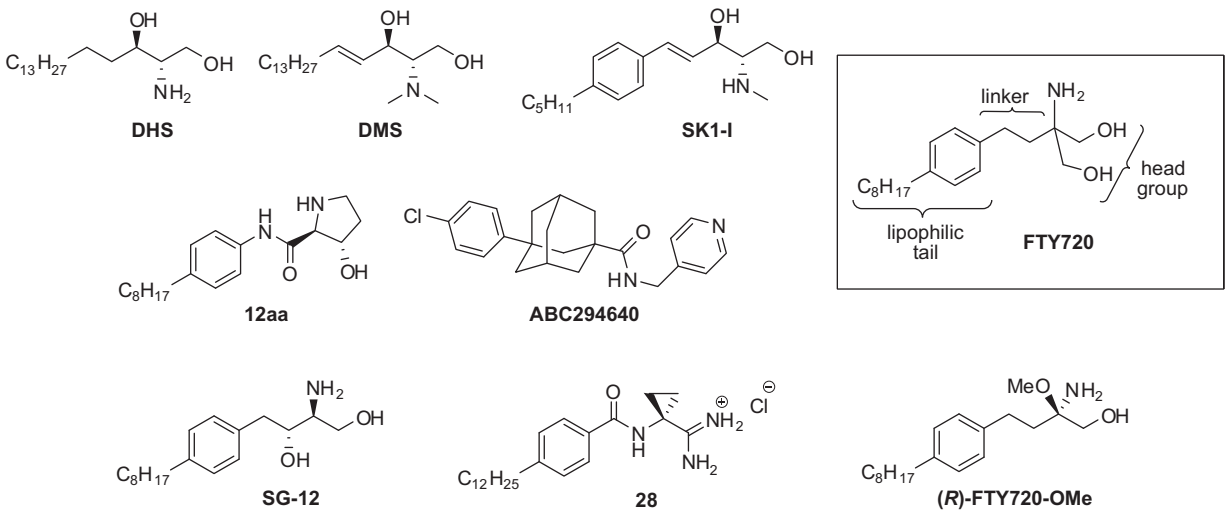


Figure 2. Reported sphingosine kinase inhibitors.

2. Results and discussion

2.1. Design of inhibitors

Fingolimod (FTY720, Fig. 2) has been used as a starting point for the design of S1P receptor agonists prodrugs[37] as well as SphK dual inhibitors.[31] These FTY720 analogues have provided valuable insight into the structural requirements necessary for phosphorylation since FTY720 is a SphK substrate. Synthesis of conformationally biased analogues[38] indicates that SphK shows a high degree of stereoselectivity in substrate recognition.[39] Careful structural analysis of these inhibitors suggests that the highly lipophilic (*p*-octyl)-phenyl backbone is a key characteristic feature for SphK inhibitors and it is noted that inhibitors described in the literature possess a basic amine functionality as the headgroup (primary, secondary, tertiary amine, or an amidine) (Fig. 2). The lipophilic tail and the head group were then connected by a linker (alkyl chain, alkene, amide or adamantane group). In this study, we chose to incorporate the lipophilic (*p*-octyl)phenyl chain, a cyclohexyl linker and various surrogate head groups in our inhibitor design, which provided us a template for the synthesis of inhibitors.

2.2. Synthesis of inhibitors

We examined head groups bearing different functionalities to determine possible lead structures that can be improved. To generate a library with diverse chemical structures, we envisioned a divergent synthesis using substituted cyclohexanone **3** as a key intermediate (Scheme 1). Thus, lithium-halogen exchange of aryl bromide **1** with *n*-butyllithium followed by reaction with 1,4-cyclohexanedione monoethylene ketal afforded tertiary alcohol **2**. Dehydration, hydrogenation of the resulting alkene and deprotection of the spiroketal[40] smoothly provided ketone **3** in 76% yield over three steps. Ketone **3** was the key intermediate that was converted into compounds **4**–**9**, each consisting of different functionalities on the cyclohexane ring (Scheme 1). A Strecker reaction converted the ketone into the corresponding amino-nitrile **4**. A Horner–Wadsworth–Emmons olefination of **3** generated the α,β-unsaturated ester **5** which was subsequently reduced to allylic alcohol **6** using DIBAL-H. Treatment of **3** with nitromethane and sodium ethoxide afforded the addition product **7** while oxime **8** was obtained after coupling with hydroxylamine. Sodium borohydride reduction of **3** yielded the *trans* secondary alcohol **9** as the major product. The synthesis of amine/ammonium functionalized compounds is shown in Scheme 2. Keeping in mind the stereoselective kinase recognition,[39] we hypothesized that *cis* and *trans* isomers would have different activities. We were thus confronted with the stereoselective synthesis of a series of *N*-alkyl-4-(4-octylphenyl)cyclohexanamines. Reductive amination of **3** with various primary amines generated secondary amines **10a–i** predominantly in either *cis* or *trans* form depending on the nature of the reducing agent. Sodium triacetoxyborohydride provided predominantly *cis* isomer whereas sodium cyanoborohydride and lithium borohydride[41] gave predominantly *trans* isomer (Table 1). The stereoselectivity of the reductive amination can be explained based on the steric approach control and torsional strain control (Supplementary Figs. S1 and S2). [42]

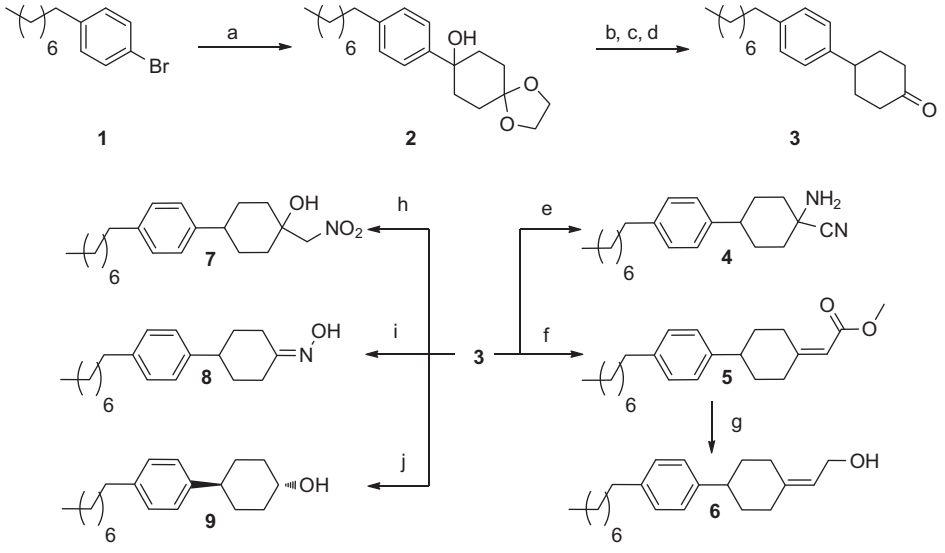
To diversify our inhibitors further, secondary amines **10a–c** were transformed into tertiary methyl amines **11a–c** by an Eschweiler–Clarke reaction. These tertiary amines were further converted into quaternary ammonium salts **12a–c**. The remainder of the active secondary amines were converted directly into the quaternary ammonium salts **12e, 12h**, and **12i** by reaction with methyl iodide in the presence of potassium carbonate.

Scheme 3 illustrates the synthesis of head group analogs linked to basic aromatic moieties. Pyrazine **13** was achieved by boration of **1** followed by Suzuki-Miyaura cross coupling reaction with 5-bromo-2-pyrazineamine and subsequent acidic deprotection. Similarly, piperazine **14** and pyridinone **15** were synthesized using the requisite boronic esters and deprotection procedure.

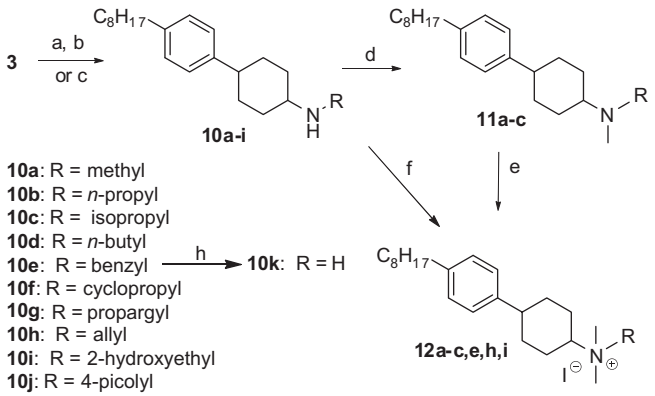
2.3. Biological evaluation

Table 2 lists the results of the in vitro inhibition assay[43] against SphK1 and SphK2 with 5-10 μM sphingosine. The compounds were initially screened at a concentration of 100 μM to identify lead structures that could potentially be improved. Possible inhibitors were defined as those able to inhibit at least 50% at 100 μM concentration.

At the outset, we wanted to test our hypothesis that amine groups are crucial for compounds to function as inhibitors. We note that the amino group in sphingosine is hypothesized to

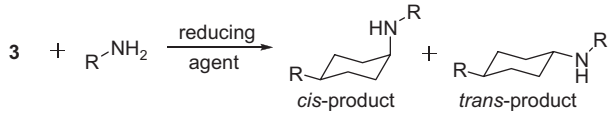


Scheme 1. Reagents and conditions: (a) *n*-BuLi, −78 °C, THF, then 1,4-cyclohexanedione monoketal, 2 h, 75%; (b) TsOH, toluene, 65 °C, 1 h; (c) H_2, Pd/C, EtOH, 20 h; (d) AcOH/H_2O (3:1), 65 °C, 2 h, 76% over 3 steps; (e) KCN, NH_4Cl, MeOH, reflux, 23%; (f) NaO^{t}Bu, methyl diethylphosphonoacetate, CH_2Cl_2, −78 °C to rt, 82%; (g) DIBAL-H (1 M in toluene), CH_2Cl_2, 0 °C to rt, 79%; (h) CH_3NO_2, NaOEt, EtOH, 0 °C to rt, 62%; (i) hydroxylamine hydrochloride, pyridine, 80 °C, 83%; (j) $NaBH_4$, MeOH, rt, 12 h, 72%.



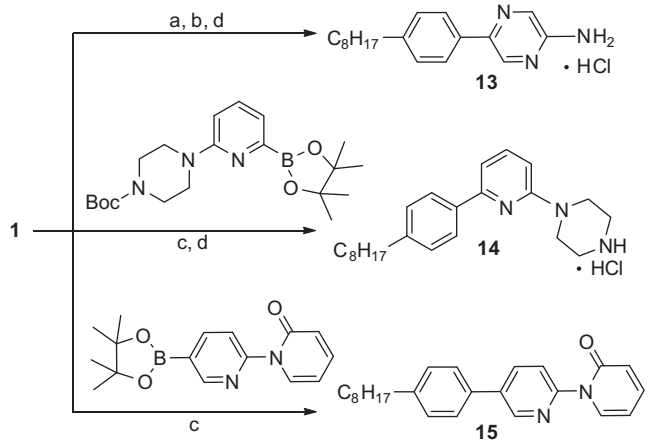
Scheme 2. (a) R-NH_2, $NaBH(OAc)_3$, CH_2Cl_2, rt, 2 h, 63–77%; (b) R-NH_2, $NaBH_3CN$, MeOH, 0 °C to rt, 12 h, 64–82%; (c) R-NH_2, $LiBH_4$, −78 °C to rt, 20 h, 73–82%; (d) $(CH_2O)_n$, HCOOH, MeOH, reflux, 6 h, 66–93%; (e) MeI, CH_3CN, reflux, 2 h, 68–72%; (f) MeI, K_2CO_3, CH_3CN, reflux, 2 h, 39–73%; (h) H_2, 10% Pd/C, MeOH, 84%.

Table 1
Reductive amination of **3** with various reducing agents



Entry	Amine	*cis*:*trans* ratio[a] (yield, %)		
		$NaBH(OAc)_3$[b]	$NaCNBH_3$[b]	$LiBH_4$[c]
10a	CH_3NH_2	71:29 (77)	34:66 (71)	4:96 (99)
10b	$^nPrNH_2$	66:34 (66)	36:64 (69)	8:92 (95)
10c	$^iPrNH_2$	69:31 (64)	41:59 (70)	6:94 (97)
10d	$^nBuNH_2$	64:36 (68)	39:61 (69)	9:91 (99)
10e	$BnNH_2$	69:31 (74)	21:79 (82)	4:96 (88)
10f	Cyclopropyl amine	70:30 (66)	38:62 (70)	5:95 (97)
10g	Propargyl amine	70:30 (75)	32:68 (67)	4:96 (98)
10h	Allylamine	78:22 (63)	37:63 (64)	5:95 (94)

[a] Ratios determined by GC–MS analysis of crude reaction mixture.
[b] Combined isolated yield of *cis* and *trans* products.
[c] GC yield.



Scheme 3. Reagents and conditions: (a) *n*-BuLi, −78 °C, THF, 15 min, then $B(OMe)_3$, rt, 1 h, then 10% aq. HCl, 58%; (b) 5-Bromo-2-pyrazineamine, $Pd(OAc)_2$, K_2CO_3, SPhos, CH_3CN/H_2O (1:1.5), reflux, 6 h, 72%; (c) $Pd(OAc)_2$, K_2CO_3, SPhos, CH_3CN/H_2O (1:1.5 equiv), reflux, 12 h, 81–92%; (d) HCl (g), THF, 1 min., 92–95%.

Table 2
Inhibitory effects of various analogs on SphK1 and SphK2

Entry	Compound	SphK activity level[a] (%)	
		SphK1	SphK2
1	**4**	100.6 ± 3	61.8 ± 4
2	**5**	100.8 ± 1.2	100.9 ± 2.3
3	**6**	100.2 ± 6.1	66.7 ± 2.9
4	**7**	100.4 ± 9	52.7 ± 4.3
5	**8**	130.3 ± 21	56.5 ± 6
6	**9**	113.1 ± 8	70.9 ± 2.3
7	*cis*-**10a**	69.8 ± 6.1	5.4 ± 0.2
8	*trans*-**10a**	15.7 ± 8.1	7.7 ± 3.6
9	*cis*-**10b**	75.6 ± 1	18.3 ± 0.3
10	*trans*-**10b**	15.8 ± 2.9	5.5 ± 0.1
11	*cis*-**10c**	90.0 ± 3	16.5 ± 0.9
12	*trans*-**10c**	24.6 ± 7.1	23.9 ± 3.9
13	*cis*-**10d**	100.2 ± 6.3	60.6 ± 3.1
14	*trans*-**10d**	20.5 ± 4.4	20.1 ± 3.6
15	*cis*-**10e**	98.4 ± 11.2	67.2 ± 6.3
16	*trans*-**10e**	83.9 ± 4	53.3 ± 3.2
17	*cis*-**10f**	113.2 ± 12.3	116.1 ± 14.1
18	*trans*-**10f**	65.0 ± 3.3	77.0 ± 2.3
19	*cis*-**10g**	100.1 ± 5.1	100.7 ± 5.3
20	*trans*-**10g**	108.8 ± 1.2	100.0 ± 2.3
21	*cis*-**10h**	91.8 ± 1	100.1 ± 3.6
22	*trans*-**10h**	19.0 ± 0.2	5.7 ± 0.3
23	*cis*-**10i**	22.4 ± 3.1	8.9 ± 2
24	*trans*-**10i**	21.6 ± 6	8.5 ± 2.1
25	*trans*-**10j**	33 ± 2.7	23 ± 4
26	*trans*-**10k**	47 ± 3	25 ± 3.7
27	*cis*-**11a**	83.9 ± 1.1	50.8 ± 2.2
28	*trans*-**11a**	46.3 ± 2.6	9 ± 0.6
29	*cis*-**11b**	90.7 ± 4.5	65.5 ± 6.2
30	*trans*-**11b**	77.0 ± 1.1	45.9 ± 6.1
31	*cis*-**11c**	112.9 ± 13.1	100.6 ± 11.3
32	*cis*-**12a**	65.8 ± 3.1	8.3 ± 2.2
33	*trans*-**12a**	10.2 ± 1.1	4.0 ± 0.3
34	*cis*-**12b**	25.1 ± 1.1	3.2 ± 0.2
35	*trans*-**12b**	5.3 ± 1	7.2 ± 1.5
36	*cis*-**12c**	100.6 ± 3.2	10.4 ± 1.1
37	*trans*-**12c**	49.1 ± 6.6	11.5 ± 1.3
38	*trans*-**12e**	25.7 ± 2.4	8.3 ± 1.1
39	*trans*-**12h**	30.4 ± 1.1	2.1 ± 0.1
40	*cis*-**12i**	80.9 ± 5.3	32.6 ± 4.4
41	*trans*-**12i**	10.5 ± 2.3	3.0 ± 0.2
42	**13**[b]	89.3 ± 2.1	99 ± 2.0
43	**14**[b]	58 ± 2.1	87.3 ± 3.8
44	**15**[b]	93.7 ± 3.1	93.7 ± 3.8

[a] Values are percent activity of SphK1 or SphK2 with 10 and 5 μM Sph, respectively, in the presence of 100 μM inhibitor. Each value is an average of three experiments. Lower SphK activity level indicates better inhibition.
[b] These compounds were assayed at 10 μM.

electrostatically interact with Asp^{177}.[36] Inhibition studies of various head groups rapidly established that nitrogen-containing compounds are more potent than other functional groups. For example, α,β-unsaturated ester **5**, alcohols **6** and **9**, nitromethyl derivative **7** and oxime **8** were inactive against SphK1 and did not cross the 50% threshold with SphK2 (Table 2). In contrast, cyclohexylamine derivative *trans*-**10k** surpassed the 50% inhibition threshold; further elaborated amine groups were then synthesized. Biological testing and analysis of secondary, tertiary and quaternary ammonium salts revealed interesting structure-activity relationships. The *trans* isomers (**10a–d**, **10f–h**) of secondary amines were significantly more potent inhibitors of SphK1 than the corresponding *cis* isomers. In the case of *trans*-**10e**, both isomers were ineffective while for *trans*-**10i**, both isomers were equally effective. Against SphK2, however, different trends were observed. For compounds **10b**, **10d**, and **10h**, the *trans* isomer was significantly more active than the *cis* isomer. However, for compounds **10a**, **10c**, **10e**, and **10i**, both the *cis* and *trans* isomers were equally effective. Moreover, both isomers of compounds **10f–g** were largely inactive;

the origin of the unfavorable interaction with the cyclopropyl and propargyl groups is currently not clear. Further, we discovered that both *cis* and *trans* isomers of tertiary amines (**11a–c**) were generally not effective as SphK1/2 inhibitors; hence these structures were not pursued. The exception to this is the activity of *trans*-**11a** towards SphK2 (*vide supra*).

During the course of our studies, quaternary ammonium salts were discovered to be effective inhibitors of SphK1/2 comparable to secondary amines. Because quaternary ammonium salts have the desired water solubility and potential cell permeability, a series of compounds (**12**) were synthesized. Consistent with the results with secondary amines, *trans* isomers were more potent than *cis* isomers specifically with SphK1 (compare *trans*- vs *cis*-**12a–c** & **12i**) (Table 2). The data indicate that the *cis* isomers (**12a–c** & **12i**) are selective towards SphK2, but follow-up assays at 10 μM inhibitor concentration revealed moderate inhibition against SphK2 (data not shown).

Additional head group analogs containing pyrazyl or pyridyl rings (**13–15**) were assayed for inhibition but unfortunately the desired activity was not observed. Although the pyridyl group is expected to be protonated at physiological pH, the lack of activity may be attributed to the replacement of the cyclohexyl ring with a flat aromatic ring that displays the important functional group in unfavorable orientation.

To confirm the potencies of select compounds identified as hits in the initial screen (*vide supra*), their K_i values were determined. The K_i values as well as SphK2 selectivity are listed in Table 3. Because SphK1/2 have different K_m values (10 and 5 μM, respectively),[44,45] the selectivity ratios were normalized with the respective K_m values. These data suggest that quaternary ammonium salts are more potent and selective towards SphK2 relative to secondary and tertiary amines (entries 1–4 vs 5–8). As the size of substituents on the amine increase, we found that the potency decreases, presumably as a result of increased steric interaction in the enzyme binding pocket. Among the quaternary ammonium salts tested, *trans*-**12a** and *trans*-**12b** are the most potent (K_i = 8 μM) and selective (∼fourfold for *trans*-**12a** and ∼threefold for *trans*-**12b**) (Table 3). To the best of our knowledge, this is the first demonstration of a SphK inhibitor scaffold containing a quaternary amine and is consistent with the observation that a positive charge is essential in SphK inhibitors.

2.4. Inhibition of SphK2 and Akt/ERK phosphorylation in intact cells

We next decided to investigate the effect of our inhibitors in intact cells. We first measured S1P levels in the presence of *trans*-**12a**/**12b** U937 cells (human histiocytic leukemia cells) using LC/MS[46] and found no change (data not shown). To confirm SphK2 specific inhibition, we added exogenous FTY720 in these cells and monitored the phosphorylation of FTY720 with or without compounds in cell extracts using LC/MS. Because FTY720 is a specific substrate of SphK2, a decrease in FTY720-P concentration would suggest SphK2-selective inhibition. Gratifyingly, both compounds *trans*-**12a** and *trans*-**12b** significantly suppressed the production of FTY720-P (Fig. 3A) and exaggerated the accumulation of FTY720 in cells (Fig. 3B), which suggests that *trans*-**12a**/**12b** inhibit SphK2.

In addition, we assayed for S1P-dependent Akt/ERK phosphorylation status. It was reported recently that treatment with SphK inhibitors resulted in decreased cell survival as a consequence of S1P biosynthesis blockade, a process monitored by the decreased phosphorylation of ERK and Akt.[47,48] When U937 cells were treated with different concentrations of *trans*-**12a** and **12b** for 16 h, a dose-dependent decrease in Akt and ERK phosphorylation was observed (Fig. 3C). Specifically, both *trans*-**12a/12b** quantitatively inhibited ERK phosphorylation; however, *trans*-**12b** appeared to be more potent than *trans*-**12a** as evidenced by the complete disappearance of p-Akt band in the Western blot. Collectively, our data suggest that our inhibitors are cell permeable compounds that may interfere with S1P signaling.

Table 3
K_i Values for select compounds

Entry	Compound	K_i (μM) [a]		SphK2 selectivity [b]
		SphK1	SphK2	
1	*cis*-**10a**	>100	40 ± 6	–
2	*trans*-**10i**	22 ± 2	38 ± 5	1.23
3	*trans*-**10k**	32 ± 4	29 ± 5	0.55
4	*trans*-**11a**	40 ± 7	27 ± 5	0.74
5	*trans*-**12a**	60 ± 6	8 ± 2	3.75
6	*trans*-**12b**	47 ± 4	8 ± 1	2.94
7	*trans*-**12c**	>100	33 ± 7	–
8	*trans*-**12i**	70 ± 8	14 ± 2	2.5

[a] $K_i = [I]/(K'_m/K_m - 1)$; K_m of sphingosine at SphK1 = 10 μM; K_m of sphingosine at SphK2 = 5 μM.
[b] Selectivity = $(K_i/K_m)^{SphK1}/(K_i/K_m)^{SphK2}$.

3. Conclusion

The large number of reports that implicate SphK in various disease states highlights the importance of this enzyme as a key regulator of sphingolipid homeostasis. Although SphK1 and SphK2 share many features, they have been reported to possess different functions. SphK1 promotes cell growth and survival; it is overexpressed in many tumor types such as brain, breast, colon, prostate, skin and others. As a result, selective targeting of SphK1 for the treatment of cancer has caught the attention of the scientific community. SphK2 has been reported to play an important role in tumor progression such as in MCF-7 breast cancer xenografts. On the other hand, some studies suggest SphK2 has the opposite effect—it inhibits cell growth and induces apoptosis which is attributed to the release of its BH3 domain upon proteasomal degradation. These contradictory functions for SphK2 need to be further understood. Clearly, it is still unknown whether selective inhibition of SphK1, SphK2 or both is beneficial. Indeed, selective small molecule inhibitors are key to answering these questions. Unfortunately, these inhibitors are currently lacking—and even more so with SphK2.

In this report, we documented our efforts in developing SphK2-selective inhibitors. We discovered a novel scaffold that afforded compounds with low micromolar inhibitory activities that are moderately SphK2 selective. In general, *trans* isomers bearing small quaternary ammonium salts are good SphK2 inhibitors. Finally, we demonstrated that *trans*-**12a/12b** inhibited Akt/ERK phosphorylation suggesting that these compounds inhibit the SphK-dependent phosphorylation cascade. Current efforts are aimed at modifying several regions of lead compounds to arrive at more potent and selective SphK2 inhibitors.

4. Experimental section

4.1. Chemistry

4.1.1. General procedures

Melting points were recorded using a Büchi B-540 melting point instrument and are uncorrected. ^{1}H NMR spectra were recorded on a JEOL EclipsePlus-500 (500 MHz) or a Varian Inova-400 (400 MHz) spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as an internal standard

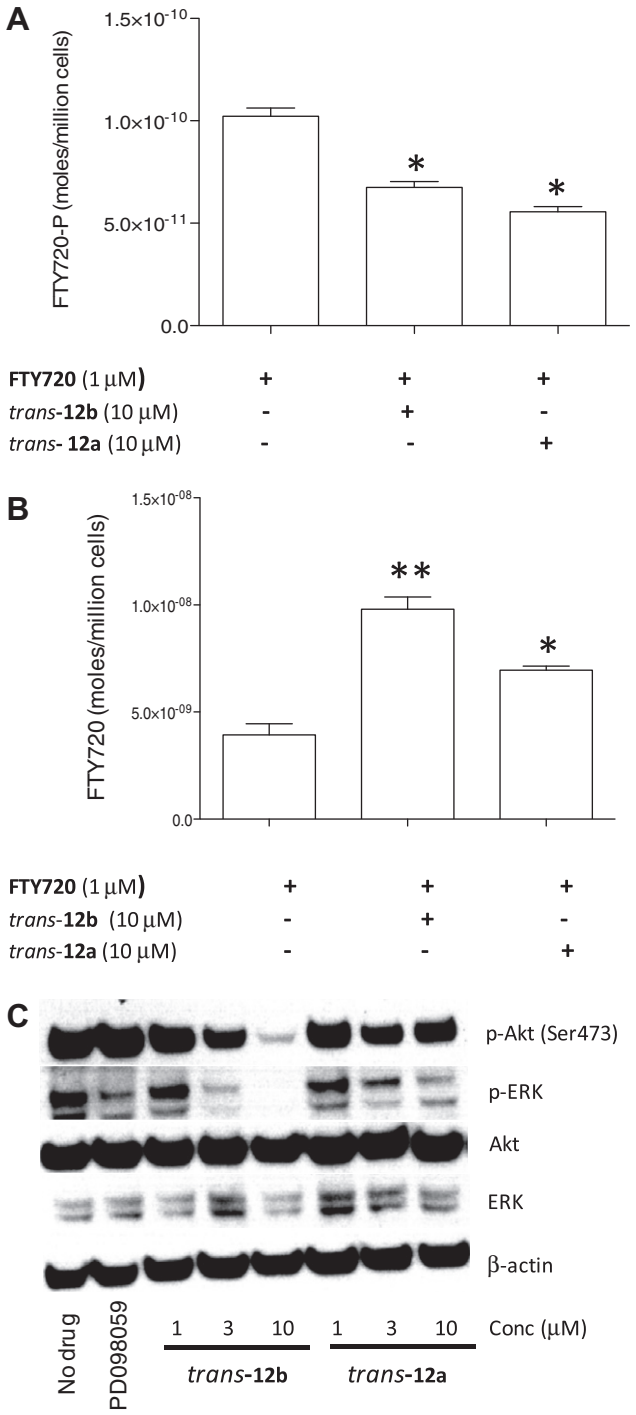


Figure 3. SphK2 inhibition in U937 cells. (A) and (B) Cultured U937 cells were exposed to FTY720 and 10 μM inhibitors as indicated in the figure. After 2 h, cells were harvested by centrifugation and the amounts of FTY720-P and FTY720 associated with the cell pellet were measured by LC/MS. (A): FTY720-P; (B): FTY720. Amounts are expressed as the number of moles *per* million cells. Data are means ± SD of three independent experiments. $^{*}p$ <0.05; $^{**}p$ <0.005 (Unpaired two-tailed *T*-test). (C) *trans*-**12b** and *trans*-**12a** inhibit Akt and ERK phosphorylation. U937 cells were pretreated with indicated concentration of inhibitor for 24 h. Cells were lysed and equal amounts of proteins were analyzed by Western blotting with the indicated antibodies. PD098059 (10 μM) was used as a positive control for inhibition of ERK phosphorylation.

($CDCl_3$: 7.26 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), and integration. ^{13}C NMR spectra were recorded on an EclipsePlus-500 (126 MHz) spectrometer or a Varian Inova-400 (101 MHz) spectrometer with complete proton decoupling. Chemical shifts are reported in ppm with the solvent resonance as the internal standard ($CDCl_3$: 77.16 ppm). Low resolution mass spectrometry (ESI-MS) was performed on a Thermo Instrument TSQ triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA, USA), equipped with an ESI source, which was used in the positive ion mode. High resolution mass spectroscopy (HRMS) was performed on an Agilent 6220 LC/MS time-of-flight mass spectrometer using either electrospray ionization (ESI) or fast atom bombardment (FAB). Column chromatography was performed either on a CombiFlash® Rf automated chromatography or by either using flash grade silica gel (SiO_2, 32–63 μm) or neutral, activated, Brockmann I aluminum oxide (Al_2O_3, ~150 mesh, 58 Å). Thin layer chromatography (TLC) was performed either on EMD silica gel 60 F_{254} plates or EMD aluminum oxide 60 F_{254} neutral plates. All reactions were conducted in oven or flame dried glassware under an inert atmosphere of nitrogen using magnetic stirring. Solvents were dried using the PureSolv™ solvent purification system. All other chemical reagents were purchased from commercial sources and were used without further purification.

4.1.2. Synthesis and characterization of compounds

4.1.2.1. 8-(4-Octylphenyl)-1,4-dioxaspiro[4.5]decan-8-ol (2). To a solution of 1-bromo-4-octylbenzene (1 g, 3.60 mmol) in 25 mL THF at −78 °C, *n*-butyllithium (2.5 M in hexanes, 1.965 mL, 4.32 mmol) was added and the solution stirred for 10 min. Then, a solution of 1,4-dioxaspiro[4.5]decan-8-one (0.696 g, 4.32 mmol) in 10 mL THF was added dropwise at −78 °C. The reaction was stirred for 2 h at −78 °C, warmed to 0 °C and quenched with dropwise addition of a saturated solution of NH_4Cl. The reaction mixture was partitioned between water and EtOAc. The aqueous phase was extracted with EtOAc and the combined organic phases were washed with brine, dried over anhydrous sodium sulfate and concentrated on a rotary evaporator. The resulting residue was purified by column chromatography over silica gel (95/5 dichloromethane/acetone) to give the title compound (0.96 g, 75%) as a white solid, mp 52 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.44–7.40 (m, 2H), 7.18–7.13 (m, 2H), 4.03–3.93 (m, 4H), 2.67–2.54 (m, 2H), 2.22–2.04 (m, 4H), 1.85–1.77 (m, 2H), 1.72–1.55 (m, 5H), 1.40–1.20 (m, 10H), 0.87 (t, J = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 145.7, 141.6, 128.2, 124.4, 108.5, 72.2, 64.3, 64.2, 36.6, 35.5, 31.9, 31.5, 30.8, 29.5, 29.4, 29.3, 22.7, 14.1; HRMS (FAB+) *m/z* calcd for $C_{22}H_{33}O_2$ $[M-OH]^+$ 329.2481, found 329.24603.

4.1.2.2. 4-(4-Octylphenyl)cyclohexanone (3). *p*-Toluenesulfonic acid monohydrate (0.106 g, 0.556 mmol) was added to a solution of **2** (1.96 g, 5.66 mmol) in toluene (40 mL) and the reaction mixture was heated at 65 °C for 1 h. Toluene was evaporated under reduced pressure to give a pink oil which was dissolved in EtOAc. It was washed with $NaHCO_3$, brine, dried under sodium sulfate, and the solution concentrated on a rotary evaporator. The resulting oil was dissolved in ethanol and transferred to a 2-neck flask equipped with a magnetic stirrer. 10% Pd on activated carbon (10 mol %) was added and the reaction run under H_2 gas for 20 h. Pd/C was filtered through a plug of celite and the filtrate concentrated on a rotary evaporator. Acetic acid (45 mL) and water (15 mL) was added to the resulting oil and the solution heated at 65 °C for 2 h. The reaction mixture was cooled to rt and partitioned between hexanes and water. The aqueous layer was extracted with hexanes and the combined organic extracts washed with $NaHCO_3$, brine, and dried with sodium sulfate. The organic solvents were evaporated under reduced pressure and the resulting oil purified by column chromatography over silica gel (90/10 hexanes/EtOAc) to give the title compound (1.24 g, 76%) as a white solid, mp 36.0–37.0 °C; ^1H NMR (500 MHz, $CDCl_3$) δ 7.18–7.10 (m, 4H),

2.99 (tt, J = 3.0 Hz, 12.0 Hz, 1H), 2.62–2.43 (m, 6H), 2.26–2.16 (m, 2H), 1.99–1.86 (m, 2H), 1.65–1.53 (m, 2H), 1.36–1.20 (m, 10H), 0.87 (t, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 211.3, 141.9, 141.2, 128.6, 126.5, 42.4, 41.4, 35.6, 34.1, 31.9, 31.5, 29.5, 29.4, 29.3, 22.7, 14.1; HRMS (FAB+) m/z calcd for $C_{20}H_{30}O$ $[M+H]^+$ 287.2375, found 287.23669.

4.1.2.3. 1-Amino-4-(4-octylphenyl)cyclohexanecarbonitrile (4). A solution of potassium cyanide (0.091 g, 1.396 mmol) and ammonium chloride (0.021 g, 0.394 mmol) in water (2.5 mL) was added to a solution of **3** (0.1 g, 0.349 mmol) in methanol (2.5 ml). The mixture was stirred overnight at 60 °C. After cooling to rt, the mixture was diluted with water and extracted with EtOAc. The organic layer was washed with brine, dried with sodium sulfate and evaporated under reduced pressure. The residue obtained was purified by column chromatography on silica gel (90/10 EtOAc/hexanes) to give the title compound as a colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.16–7.09 (m, 4H), 2.58–2.54 (m, 2H), 2.48 (tt, J = 3.6 Hz, 12.3 Hz, 1H), 2.18–2.12 (m, 2H), 1.98–1.78 (m, 6H), 1.67–1.61 (m, 2H), 1.61–1.56 (m, 2H), 1.35–1.21 (m, 10H), 0.87 (t, J = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 142.5, 141.1, 128.4, 126.6, 123.7, 51.5, 42.6, 38.3, 35.5, 31.9, 31.5, 30.7, 29.4, 29.4, 29.2, 22.6, 13.9.

4.1.2.4. Methyl 2-(4-(4-octylphenyl)cyclohexylidene)acetate (5). To a solution of methyl diethylphosphonoacetate (0.329 mL, 1.745 mmol) in CH_2Cl_2 (5 mL) at −78 °C was added sodium *tert*-butoxide (0.138 g, 1.396 mmol) over a period of 15 min. The reaction mixture was stirred for 1 h at −78 °C. Then, a solution of **3** (0.2 g, 0.698 mmol) in CH_2Cl_2 (2 mL) was added dropwise. The solution was allowed to warm to rt and then stirred overnight. The reaction was quenched by the addition of saturated NH_4Cl. The reaction mixture was partitioned between CH_2Cl_2 and water and the aqueous layer extracted with CH_2Cl_2. The combined organic layers were washed with saturated $NaHCO_3$, brine, dried over sodium sulfate and filtered. The filtrate was concentrated on a rotary evaporator and the residue was purified by column chromatography over silica gel (95/5 hexanes/EtOAc, R_f = 0.38) to give the title compound (0.195 g, 82%) as a white solid, mp 39.1–40.0 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.11 (s, 4H), 5.69 (s, 1H), 4.02–3.93 (m, 1H), 3.71 (s, 3H), 2.77 (tt, J = 3.8 Hz, 11.9 Hz, 1H), 2.57 (t, J = 7.9 Hz, 2H), 2.44–2.31 (m, 2H), 2.11–2.00 (m, 3H), 1.69–1.55 (m, 4H), 1.37–1.22 (m, 10H), 0.89 (t, J = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.4, 162.6, 143.3, 141.0, 128.6, 126.8, 113.4, 51.1, 43.9, 38.0, 35.9, 35.8, 35.1, 32.1, 31.8, 29.8, 29.7, 29.6, 29.5, 22.9, 14.3; ESI-MS m/z calcd for $C_{23}H_{34}O_2$ $[M+H]^+$ 343.26, found 343.30.

4.1.2.5. 2-(4-(4-Octylphenyl)cyclohexylidene)ethanol (6). Diisobutylaluminum hydride (1 M in toluene, 2.2 mL, 2.2 mmol) was added dropwise to a solution of **5** (0.247 g, 0.721 mmol) in CH_2Cl_2 (7.2 mL) at 0 °C. The solution was allowed to warm to rt and stirred for 1 h. The solution was diluted with careful dropwise addition of 0.09 mL water, 0.14 mL 10% NaOH and then 0.22 mL water and stirred for 30 min. The resulting precipitate was filtered through a plug of celite and the filtrate was washed with brine solution and dried with sodium sulfate. Evaporation of the organic solvent gave a residue which was purified by column chromatography on silica gel (75/25 hexanes/EtOAc, R_f = 0.32) to give the title compound (0.179 g, 79%) as a white solid, mp 36.7–37.4 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.10 (s, 4H), 5.44 (t, J = 7.1 Hz, 1H), 4.18 (d, J = 7.1 Hz, 2H), 2.79–2.73 (m, 1H), 2.68 (tt, J = 3.5 Hz, 12.2 Hz, 1H), 2.58–2.53 (m, 2H), 2.39–2.29 (m, 1H), 2.27–2.18 (m, 1H), 2.03–1.88 (m, 3H), 1.63–1.41 (m, 4H), 1.36–1.21 (m, 11H), 0.87 (t, J = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.8, 143.2, 140.6, 128.3, 126.6, 120.9, 58.7, 44.1, 36.8, 35.6, 35.5, 35.1, 31.9, 31.5, 29.5, 29.4, 29.3, 28.6, 22.7, 14.1; HRMS (FAB+) m/z calcd for $C_{22}H_{33}$ $[M-OH]^+$ 297.25823, found 297.25844.

4.1.2.6. 1-(Nitromethyl)-4-(4-octylphenyl)cyclohexanol (7). Nitromethane (0.37 mL, 6.98 mmol) was added to a solution of **3** (0.4 g, 1.396 mmol) in ethanol (8 mL). The mixture cooled to 0 °C and sodium ethoxide (0.114 g, 1.676 mmol) dissolved in ethanol (4 mL) was added dropwise. The mixture was warmed to rt and stirred for 4 h. It was quenched by the addition of saturated NH_4Cl. It was then partitioned between EtOAc and water. The aqueous layer was extracted with EtOAc and the combined organic extracts were washed with brine and dried with sodium sulfate. The organic solvents were evaporated under reduced pressure and the resulting residue purified by column chromatography over silica gel (75/25 hexanes/EtOAc, R_f = 0.37) to give the title compound (0.3 g, 62%) as a white solid, mp 45.8–46.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12 (s, 4H), 4.68 (s, 2H), 3.09 (s, 1H), 2.62 (tt, J = 3.0 Hz, 12.5 Hz, 1H), 2.57 (t, J = 7.5 Hz, 2H), 2.01–1.90 (m, 4H), 1.77 (td, J = 3.7 Hz, 13.4 Hz, 2H), 1.63–1.49 (m, 4H), 1.37–1.21 (m, 10H), 0.87 (t, J = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 142.2, 141.1, 128.5, 126.5, 81.3, 71.2, 42.2, 35.9, 35.5, 31.9, 31.5, 30.5, 29.4, 29.4, 29.2, 22.6, 14.1.

4.1.2.7. 4-(4-Octylphenyl)cyclohexanone oxime (8). Hydroxylamine hydrochloride (72.8 mg, 1.047 mmol) was added to a solution of **3** (100 mg, 0.349 mmol) in pyridine (1 mL) and the resulting solution was heated at 80 °C for 24 h. Pyridine was evaporated under reduced pressure and the resulting residue was dissolved in EtOAc. It was washed with $NaHCO_3$, brine, dried over sodium sulfate and filtered. The resulting solution was concentrated on a rotary evaporator to give the title compound (87 mg, 83%) as a white solid, mp 69.2–70.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (br s, 1H), 7.04 (s, 4H), 3.44–3.36 (m, 1H), 2.68 (tt, J = 3.3 Hz, 12.1 Hz, 1H), 2.53–2.42 (m, 3H), 2.19 (td, J = 4.7 Hz, 13.6 Hz, 1H), 2.05–1.93 (m, 2H), 1.81 (td, J = 5.2 Hz, 13.9 Hz, 1H), 1.68–1.47 (m, 4H), 1.30–1.14 (m, 10H), 0.80 (t, J = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.9, 142.8, 140.9, 128.5, 126.5, 43.3, 35.6, 34.1, 33.0, 32.0, 31.9, 31.5, 29.5, 29.4, 29.3, 24.2, 22.7, 14.2; HRMS (FAB+) m/z calcd for $C_{20}H_{32}NO$ $[M+H]^+$ 302.2484, found 302.2457.

4.1.2.8. (1r,4r)-4-(4-Octylphenyl)cyclohexanol (9). Sodium borohydride (0.031 g, 0.826 mmol) was added to a solution of **3** (0.1 g, 0.349 mmol) in methanol (2 mL) at 0 °C. The reaction was warmed to rt and stirred overnight. The solvent was removed by evaporation under reduced pressure and water was added to the resulting residue. The aqueous phase was extracted 3 times with EtOAc and the combined organic extracts were washed with brine and dried over sodium sulfate. The solvent was evaporated under reduced pressure and the resulting residue was purified by column chromatography on silica gel (50/50 hexanes/EtOAc) to give the title compound (0.073 mg, 72% yield) as a white solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.10 (s, 4H), 3.72-3.65 (m, 1H), 2.58–2.53 (m, 2H), 2.46 (tt, J = 3.5 Hz, 12.2 Hz, 1H), 2.12–2.06 (m, 2H), 1.95–1.89 (m, 2H), 1.62–1.22 (m, 16H), 0.87 (t, J = 7.5 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 143.8, 140.8, 128.4, 126.7, 70.8, 43.1, 36.1, 35.6, 32.6, 32.0, 31.6, 29.6, 29.5, 29.4, 22.8, 14.2; HRMS (FAB+) m/z calcd for $C_{20}H_{32}O$ 288.2453, found 288.2457.

4.1.3. General procedure for reductive amination with sodium triacetoxyborohydride

Primary amine (1.1 equiv) was added to a solution of **3** (1 equiv) in CH_2Cl_2 and the solution stirred for 5 min. Sodium triacetoxyborohydride (1.4 equiv) was added and the mixture was stirred for 1 h. The reaction mixture was diluted by the addition of saturated $NaHCO_3$ and stirred for an additional 10 min. It was then partitioned between water and CH_2Cl_2. The aqueous layer was

extracted 3 times with CH_2Cl_2 and the combined organic layers were washed with brine, dried with sodium sulfate and filtered. The organic solvent was removed by evaporation under reduced pressure and the crude product was purified by column chromatography on neutral alumina.

4.1.4. General procedure for reductive amination with sodium cyanoborohydride

Primary amine (1.1 equiv) was added to a solution of **3** (1 equiv) in MeOH at 0 °C. After 15 min., sodium cyanoborohydride (0.7 equiv) and acetic acid (1.2 equiv) were added and the mixture was warmed to rt. After 12 h, the solution was evaporated under reduced pressure, saturated $NaHCO_3$ was added and the mixture extracted with EtOAc. The organic phase was washed with brine, dried with sodium sulfate, and concentrated on a rotary evaporator. The crude product was purified by column chromatography on neutral alumina.

4.1.5. General procedure for reductive amination with lithium borohydride

Primary amine (3 equiv) was added to a solution of **3 (1** equiv) in methanol. The mixture was stirred for 1 h at rt, cooled to −78 °C, and treated with a 2 M solution of lithium borohydride in THF (1.1 equiv). After stirring at −78 °C for 1 h, the mixture was slowly warmed to rt and stirred for 16 h. It was quenched by slow addition of a saturated solution of $NaHCO_3$. The resulting mixture was partitioned between EtOAc and water. The aqueous layer was extracted with EtOAc and the combined organic phases washed with brine, dried over sodium sulfate, filtered, and concentrated. The crude product was purified by column chromatography on neutral alumina.

4.1.5.1. (1*s*,4*s*)-*N*-Methyl-4-(4-octylphenyl)cyclohexanamine (*cis*-10a). Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18–7.13 (m, 2H), 7.13–7.07 (m, 2H), 2.79–2.74 (m, 1H), 2.59–2.48 (m, 3H), 2.43 (s, 3H), 1.89–1.71 (m, 4H), 1.68–1.55 (m, 6H), 1.37–1.20 (m, 11H), 0.88 (t, J = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.5, 140.3, 128.2, 126.7, 53.8, 43.4, 35.6, 34.2, 31.9, 31.6, 30.1, 29.5, 29.4, 29.3, 28.3, 22.7, 14.1; HRMS (FAB+) m/z calcd for $C_{21}H_{36}N$ $[M+H]^+$ 302.2848, found 302.2838.

4.1.5.2. (1*r*,4*r*)-*N*-Methyl-4-(4-octylphenyl)cyclohexanamine (*trans*-10a). Yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.16–7.05 (m, 4H), 2.58–2.52 (m, 2H), 2.51–2.36 (m, 5H), 2.10–2.02 (m, 2H), 1.96–1.88 (m, 2H), 1.65–1.54 (m, 3H), 1.54–1.44 (m, 2H), 1.34–1.17 (m, 12H), 0.87 (t, J = 7.0, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.4, 140.6, 128.4, 126.7, 58.6, 43.8, 35.7, 33.8, 33.5, 33.1, 32.0, 31.6, 29.6, 29.5, 29.4, 22.8, 14.2; HRMS (FAB+) m/z calcd for $C_{21}H_{36}N$ $[M+H]^+$ 302.2848, found 302.2838.

4.1.5.3. (1*s*,4*s*)-4-(4-Octylphenyl)-N-propylcyclohexanamine (*cis*-10b). Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17–7.12 (m, 2H), 7.12–7.07 (m, 2H), 2.88–2.82 (m, 1H), 2.59–2.49 (m, 5H), 1.85–1.72 (m, 4H), 1.69–1.46 (m, 8H), 1.37–1.19 (m, 10H), 0.94 (t, J = 7.3 Hz, 3H), 0.88 (t, J = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.5, 140.3, 128.2, 126.7, 51.8, 49.3, 43.1, 35.6, 31. 9, 31.6, 30.5, 29.5, 29.4, 29.3, 23.6, 22.7, 14.1, 11.9; HRMS (ESI+) m/z calcd for $C_{23}H_{39}N$ $[M+H]^+$ 330.3155, found 330.3126.

4.1.5.4. (1*r*,4*r*)-4-(4-Octylphenyl)-N-propylcyclohexanamine (*trans*-10b). Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.14–7.07 (m, 4H), 2.67–2.41 (m, 6H), 2.09–2.00 (m, 2H), 1.96–1.87 (m, 2H), 1.65–1.42 (m, 6H), 1.38–1.17 (m, 13H), 0.93 (t, J = 7.4 Hz, 3H), 0.88 (t, J = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.3, 140.5, 128.3, 126.6, 56.8, 49.2, 43.7, 35.6, 33.9, 33.1, 31.9, 31.5, 29.5, 29.4, 29.3, 23.6, 22.7, 14.1, 11.9; HRMS (ESI+) m/z calcd for $C_{23}H_{39}N$ $[M+H]^+$ 330.3155, found 330.3153.

4.1.5.5. (1*s*,4*s*)-*N*-Isopropyl-4-(4-octylphenyl)cyclohexanamine (*cis*-10c). Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23–7.04 (m, 4H), 3.01–2.95 (m, 1H), 2.95–2.87 (m, 1H), 2.65–2.50 (m, 3H), 1.86–1.55 (m, 10H), 1.46–1.21 (m, 11H), 1.08 (d, J = 7.5 Hz, 6H), 0.89 (t, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.5, 140.6, 128.5, 126.9, 48.6, 45.1, 43.1, 35.8, 32.2, 31.8, 30.7, 29.8, 29.7, 29.5, 28.5, 23.6, 22.9, 14.4; ESI-MS m/z calcd for $C_{23}H_{39}N$ $[M+H]^+$ 330.31, found 330.30.

4.1.5.6. (1*r*,4*r*)-*N*-Isopropyl-4-(4-octylphenyl)cyclohexanamine (*trans*-10c). White solid, mp 41.5–42.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.13–7.07 (m, 4H), 3.14–3.04 (m, 1H), 2.69 (tt, J = 3.9 Hz, 11.3 Hz, 1H), 2.55 (t, J = 8.0 Hz, 2H), 2.48 (tt, J = 3.5 Hz, 12.5 Hz, 1H), 2.12–2.03 (m, 2H), 1.98 (br s, 1H), 1.96–1.88 (m, 2H), 1.62–1.55 (m, 2H), 1.49 (qd, J = 3.0 Hz, 12.5 Hz, 2H), 1.37–1.20 (m, 12H), 1.14 (d, J = 6.3 Hz, 6H), 0.87 (t, J = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.1, 140.7, 128.4, 126.7, 53.5, 45.3, 43.5, 35.6, 33.5, 33.2, 32.0, 31.6, 29.6, 29.5, 29.3, 22.8, 14.2; ESI-MS m/z calcd for $C_{23}H_{39}N$ $[M+H]^+$ 330.31, found 330.28; HRMS (ESI+) m/z calcd for $C_{23}H_{39}N$ $[M+H]^+$ 330.3155, found 330.3168.

4.1.5.7. (1*s*,4*s*)-*N*-Butyl-4-(4-octylphenyl)cyclohexanamine (*cis*-10d). Colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.22–7.05 (m, 4H), 2.91–2.85 (m, 1H), 2.69–2.47 (m, 5H), 1.90–1.73 (m, 4H), 1.73–1.57 (m, 6H), 1.56–1.21 (m, 15H), 1.04–0.81 (m, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.7, 140.5, 128.5, 127.0, 52.1, 47.3, 43.3, 35.8, 32.8, 32.2, 31.8, 30.7, 29.8, 29.7, 29.5, 28.5, 22.9, 20.9, 14.4, 14.3; ESI-MS m/z calcd for $C_{24}H_{41}N$ $[M+H]^+$ 344.32, found 344.30.

4.1.5.8. (1*r*,4*r*)-*N*-Butyl-4-(4-octylphenyl)cyclohexanamine (*trans*-10d). Yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18–7.05 (m, 4H), 2.69–2.63 (m, 2H), 2.60–2.43 (m, 4H), 2.09–2.00 (m, 2H), 1.95–1.88 (m, 2H), 1.64–1.44 (m, 6H), 1.42–1.18 (m, 14H), 0.93 (t, J = 7.3 Hz, 3H), 0.88 (t, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.3, 140.5, 128.3, 126.6, 56.9, 47.0, 43.7, 35.6, 34.0, 33.1, 32.7, 31.9, 31.6, 29.5, 29.4, 29.3, 22.7, 20.6, 14.1, 14.0; ESI-MS m/z calcd for $C_{24}H_{41}N$ $[M+H]^+$ 344.32, found 344.30.

4.1.5.9. (1*s*,4*s*)-*N*-Benzyl-4-(4-octylphenyl)cyclohexanamine (*cis*-10e). Yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39–7.31 (m, 4H), 7.27–7.23 (m, 1H), 7.17–7.14 (m, 2H), 7.12–7.08 (m, 2H), 3.80 (s, 2H), 2.96–2.91 (m, 1H), 2.59–2.50 (m, 3H), 1.92–1.81 (m, 4H), 1.68–1.56 (m, 6H), 1.48 (br s, 1H), 1.37–1.22 (m, 10H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.7, 141.3, 140.4, 128.5, 128.3, 128.2, 126.9, 126.8, 51.4, 51.2, 43.4, 35.7, 32.0, 31.7, 30.6, 29.6, 29.5, 29.4, 28.3, 22.8, 14.2; ESI-MS m/z calcd for $C_{27}H_{39}N$ $[M+H]^+$ 378.31, found 378.25; HRMS (ESI+) m/z calcd for $C_{27}H_{40}N$ $[M+H]^+$ 378.3155, found 378.3168.

4.1.5.10. (1*r*,4*r*)-*N*-Benzyl-4-(4-octylphenyl)cyclohexanamine (*trans*-10e). White solid, mp 41.5–42.5 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36–7.31 (m, 4H), 7.27–7.23 (m, 1H), 7.13–7.06 (m, 4H), 3.85 (s, 2H), 2.60–2.52 (m, 3H), 2.48 (tt, J = 3.0, 12.0, 1H), 2.11–2.04 (m, 2H), 1.94–1.87 (m, 2H), 1.62–1.41 (m, 6H), 1.33–1.23 (m, 11H), 0.87 (t, J = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.3, 140.9, 140.5, 128.4, 128.3, 128.1, 126.6, 56.1, 51.2, 43.7, 35.6, 33.8, 33.1, 31.9, 31.5, 29.5, 29.4, 29.3, 22.7, 14.1; ESI-MS m/z calcd for $C_{27}H_{39}N$ $[M+H]^+$ 378.31, found 378.25; HRMS (ESI+) m/z calcd for $C_{27}H_{40}N$ $[M+H]^+$ 378.3155, found 378.3169.

4.1.5.11. (1*s*,4*s*)-*N*-Cyclopropyl-4-(4-octylphenyl)cyclohexanamine (*cis*-10f). Colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.16–7.07 (m, 4H), 3.02–2.97 (m, 1H), 2.58–2.49 (m, 3H), 2.14–2.08 (m, 1H), 1.92–1.84 (m, 2H), 1.80–1.70 (m, 2H), 1.67–1.55 (m, 7H), 1.35–1.21 (m, 10H), 0.87 (t, J = 6.8 Hz, 3H), 0.46–0.40 (m, 2H), 0.40–0.32 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.7, 140.4, 128.3, 126.8, 52.3, 43.3, 35.6, 32.0, 31.6, 30.8, 29.6, 29.5, 29.4, 28.7, 28.5, 22.8, 14.2, 6.4; ESI-MS m/z calcd for $C_{23}H_{37}N$ $[M+H]^+$ 328.29, found 328.32.

4.1.5.12. (1*r*,4*r*)-*N*-Cyclopropyl-4-(4-octylphenyl)cyclohexanamine (*trans*-10f). Yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.15–7.08 (m, 4H), 2.66 (tt, J = 4.0 Hz, 11.5 Hz, 1H), 2.57 (t, J = 7.5 Hz, 2H), 2.47 (tt, J = 3.5 Hz, 12.0 Hz, 1H), 2.19–2.10 (m, 3H), 1.95–1.89 (m, 2H), 1.68–1.47 (m, 5H), 1.38–1.19 (m, 12H), 0.89 (t, J = 7.0 Hz, 3H), 0.49–0.42 (m, 2H), 0.41–0.34 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.4, 140.6, 128.4, 126.8, 57.6, 43.9, 35.7, 34.3, 33.3, 32.0, 31.6, 29.6, 29.5, 29.4, 28.5, 22.8, 14.2, 6.6; ESI-MS m/z calcd for $C_{23}H_{37}N$ $[M+H]^+$ 328.29, found 328.33.

4.1.5.13. (1*s*,4*s*)-*N*-(Prop-2-ynyl)-4-(4-octylphenyl)cyclohexanamine (*cis*-10g). Colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.19–7.15 (m, 2H), 7.14–7.09 (m, 2H), 3.47 (d, J = 2.4 Hz, 2H), 3.16–3.10 (m, 1H), 2.61–2.50 (m, 3H), 2.22 (t, J = 2.4 Hz, 1H), 1.90–1.77 (m, 4H), 1.70–1.58 (m, 6H), 1.38–1.22 (m, 10H), 1.12 (br s, 1H), 0.90 (t, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.6, 140.4, 128.3, 126.8, 82.9, 71.0, 50.2, 43.6, 35.8, 35.7, 32.0, 31.7, 30.4, 29.6, 29.5, 29.4, 28.3, 22.7, 14.2; ESI-MS m/z calcd for $C_{23}H_{35}N$ $[M+H]^+$ 326.28, found 326.27.

4.1.5.14. (1*r*,4*r*)-*N*-(Prop-2-ynyl)-4-(4-octylphenyl)cyclohexanamine (*trans*-10g). White solid, mp 48.1–49.1 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.16–7.09 (m, 4H), 3.50 (d, J = 2.4 Hz, 2H), 2.75 (tt, J = 3.4 Hz, 11.6 Hz, 1H), 2.56 (t, J = 8.0 Hz, 2H), 2.48 (tt, J = 3.0 Hz, 12.0 Hz, 1H), 2.22 (t, J = 2.4 Hz, 1H), 2.05–1.98 (m, 2H), 1.96–1.89 (m, 2H), 1.63–1.48 (m, 4H), 1.37–1.20 (m, 13H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.3, 140.7, 128.3, 126.7, 82.6, 71.4, 55.0, 43.6, 35.7, 35.4, 33.4, 32.9, 32.0, 31.6, 29.6, 29.5, 29.4, 22.7, 14.1; ESI-MS m/z calcd for $C_{23}H_{35}N$ $[M+H]^+$ 326.28, found 326.27.

4.1.5.15. (1*s*,4*s*)-*N*-Allyl-4-(4-octylphenyl)cyclohexanamine (*cis*-10h). Colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.17–7.14 (m, 2H), 7.12–7.09 (m, 2H), 6.02–5.90 (m, 1H), 5.20 (dq, J = 1.7 Hz, 17.2 Hz, 1H), 5.09 (dq, J = 1.6 Hz, 10.0 Hz, 1H), 3.27 (dt, J = 1.4 Hz, 6.0 Hz, 2H), 2.95–2.88 (m, 1H), 2.59–2.50 (m, 3H), 1.86–1.75 (m, 4H), 1.70–1.56 (m, 6H), 1.38–1.21 (m, 11H), 0.88 (t, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.5, 140.4, 137.7, 128.4, 126.7, 115.5, 51.3, 50.0, 43.2, 35.5, 32.0, 31.7, 30.5, 29.6, 29.5, 29.4, 28.3, 22.7, 14.2; ESI-MS m/z calcd for $C_{23}H_{37}N$ $[M+H]^+$ 328.29, found 328.33.

4.1.5.16. (1*r*,4*r*)-*N*-Allyl-4-(4-octylphenyl)cyclohexanamine (*trans*-10h). Colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17–7.05 (m, 4H), 6.01–5.87 (m, 1H), 5.19 (dq, J = 1.6 Hz, 17.2 Hz, 1H), 5.12–5.07 (m, 1H), 3.32 (dt, J = 1.3 Hz, 6.0 Hz, 2H), 2.61–2.42 (m, 4H), 2.10–2.00 (m, 2H), 1.96–1.86 (m, 2H), 1.63–1.44 (m, 4H), 1.38–1.17 (m, 13H), 0.87 (t, J = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.4, 140.6, 137.4, 128.4, 126.7, 115.7, 56.2, 49.8, 43.8, 35.7, 34.0, 33.2, 32.0, 31.6, 29.6, 29.5, 29.4, 22.8, 14.2; HRMS (FAB+) m/z calcd for $C_{23}H_{37}N$ $[M+H]^+$ 328.2999, found 328.3001.

4.1.5.17. 2-(((1*s*,4*s*)-4-(4-Octylphenyl)cyclohexyl)amino)ethanol (*cis*-10i). 76% Yield ($NaBH(OAc)_3$), colorless oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16–7.12 (m, 2H), 7.12–7.08 (m, 2H), 3.69–3.59 (m, 2H), 2.93–2.85 (m, 1H), 2.83–2.73 (m, 2H), 2.59–2.48 (m, 3H), 2.12 (br s, 2H), 1.84–1.53 (m, 10H), 1.39–1.18 (m, 10H), 0.87 (t, J = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 144.3, 140.4, 128.3, 126.6, 61.3, 51.5, 48.5, 43.1, 35.5, 31.9, 31.6, 30.6, 29.5, 29.4, 29.3, 28.2, 22.7, 14.1; ESI-MS m/z calcd for $C_{22}H_{38}NO$ $[M+H]^+$ 332.29, found 332.30.

4.1.5.18. 2-((1*r*,4*r*)-4-(4-Octylphenyl)cyclohexylamino)ethanol (*trans*-10i). 53% Yield ($NaBH_3CN$), white solid, mp 79.3–80.0 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12–7.07 (m, 4H), 3.65 (t, J = 5.0 Hz, 2H), 2.83 (t, J = 5.0 Hz, 2H), 2.57–2.47 (m, 4H), 2.08–2.04 (m, 2H), 1.94–1.90 (m, 2H), 1.61–1.43 (m, 4H), 1.35–1.18 (m, 12H), 0.87 (t, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.2, 140.7, 128.4, 126.7, 61.5, 56.5, 48.3, 43.7, 35.6, 34.2, 33.2, 32.0, 31.6, 29.6, 29.5, 29.3, 22.8, 14.2; HRMS (FAB+) m/z calcd for $C_{22}H_{38}NO$ $[M+H]^+$ 332.2953, found 332.2972.

4.1.5.19. (1*r*,4*r*)-4-(4-Octylphenyl)-N-(pyridin-4-ylmethyl) cyclohexanamine (*trans*-10j). 20% Yield ($LiBH_4$); white solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.58–8.53 (m, 2H), 7.31–7.27 (m, 2H), 7.10 (s, 4H), 3.88 (s, 2H), 2.59–2.43 (m, 4H), 2.12–2.04 (m, 2H), 1.96–1.88 (m, 2H), 1.64–1.55 (m, 2H), 1.55–1.42 (m, 2H), 1.39–1.21 (m, 13H), 0.88 (t, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 150.1, 149.8, 144.1, 140.6, 128.3, 126.6, 122.9, 56.2, 49.9, 43.6, 35.6, 33.9, 33.0, 31.9, 31.5, 29.5, 29.4, 29.3, 22.7, 14.1.

4.1.5.20. (1*r*,4*r*)-4-(4-Octylphenyl)cyclohexanamine (*trans*-10k). White solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.10 (s, 4H), 2.77–2.69 (m, 1H), 2.55 (t, J = 7.5 Hz, 2H), 2.44 (tt, J = 3.4 Hz, 11.6 Hz, 1H), 2.0–1.86 (m, 4H), 1.75 (br s, 2H), 1.63–1.45 (m, 4H), 1.39–1.20 (m, 12H), 0.88 (t, J = 7.5 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 143.3, 140.6, 128.7, 128.4, 50.5, 43.3, 37.1, 35.7, 33.3, 32.0, 31.6, 30.8, 29.6, 29.5, 29.4, 22.8, 14.2; HRMS (FAB+) m/z calcd for $C_{20}H_{34}N^+$ $[M+H]^+$ 288.2691, found 288.2680.

4.1.6. General procedure for the Eschweiler–Clarke methylation of secondary amines

Formic acid (4 equiv) was added to a solution of secondary amine (1 equiv) and paraformaldehyde (4 equiv) in methanol at rt. The reaction mixture was refluxed for 6 h. After cooling to rt, it was partitioned between ether and water. 10% NaOH was added to the aqueous layer until the pH is ~12. The basic aqueous layer was then extracted 3 times with ether. The combined organic extracts were washed with brine, dried with sodium sulfate and the solvent removed by evaporation under reduced pressure. The product was purified by column chromatography on neutral alumina.

4.1.6.1. (1*s*,4*s*)-*N*,*N*-Dimethyl-4-(4-octylphenyl)cyclohexanamine (*cis*-11a). 91% Yield, colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.19–7.16 (m, 2H), 7.10–7.07 (m, 2H), 2.65–2.51 (m, 3H), 2.24 (s, 6H), 2.10–2.06 (m, 1H), 1.99–1.86 (m, 4H), 1.65–1.56 (m, 4H), 1.56–1.47 (m, 2H), 1.35–1.22 (m, 10H), 0.87 (t, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.4, 140.3, 128.2, 127.0, 61.1, 43.6, 43.0, 35.6, 32.0, 31.7, 29.6, 29.5, 29.4, 28.9, 28.6, 22.8, 14.2; ESI-MS m/z calcd for $C_{22}H_{37}N$ $[M+H]^+$ 316.29, found 316.29.

4.1.6.2. (1*r*,4*r*)-*N*,*N*-Dimethyl-4-(4-octylphenyl)cyclohexanamine (*trans*-11a). 85% Yield, colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.13–7.09 (m, 4H), 2.58–2.54 (t, J = 7.5 Hz, 2H), 2.44 (tt, J = 3.0 Hz, 11.5 Hz, 1H), 2.32 (s, 6H), 2.25 (tt, J = 3.0 Hz, 11.5 Hz, 1H), 2.05–1.94 (m, 4H), 1.63–1.56 (m, 2H), 1.57–1.44 (m, 2H), 1.41–1.22 (m, 12H), 0.88 (t, J = 7.5 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.3, 140.6, 128.4, 126.7, 63.5, 43.8, 41.8, 35.7, 33.6, 32.0, 31.6, 29.6, 29.5, 29.4, 29.1, 22.8, 14.2;

HRMS (FAB+) m/z calcd for $C_{22}H_{37}N$ $[M+H]^+$ 316.3004, found 316.3009.

4.1.6.3. (1*s*,4*s*)-*N*-Methyl-4-(4-octylphenyl)-N-propylcyclohexanamine (*cis*-11b). 93% Yield, colorless oil; ^1H NMR (400 MHz, $CDCl_3$) δ 7.22–7.16 (m, 2H), 7.13–7.07 (m, 2H), 2.72–2.62 (m, 1H), 2.60–2.52 (m, 2H), 2.45–2.34 (m, 3H), 2.22 (s, 3H), 2.05–1.93 (m, 2H), 1.92–1.81 (m, 2H), 1.66–1.40 (m, 8H), 1.36–1.21 (m, 10H), 0.88 (t, J = 7.3 Hz, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.2, 140.2, 128.3, 127.1, 58.7, 56.0, 41.9, 38.9, 35.6, 32.0, 31.7, 29.6, 29.5, 29.4, 28.8, 28.1, 22.8, 19.4, 14.2, 12.1; ESI-MS m/z calcd for $C_{24}H_{41}N$ $[M+H]^+$ 344.32, found 344.35.

4.1.6.4. (1*r*,4*r*)-*N*-Methyl-4-(4-octylphenyl)-N-propylcyclohexanamine (*trans*-11b). 66% Yield, colorless oil; ^1H NMR (500 MHz, $CDCl_3$) δ 7.13–7.07 (m, 4H), 2.58–2.53 (m, 2H), 2.50–2.38 (m, 4H), 2.28 (s, 3H), 1.99–1.90 (m, 4H), 1.62–1.54 (m, 2H), 1.54–1.36 (m, 6H), 1.35–1.21 (m, 10H), 0.92–0.84 (m, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.4, 140.6, 128.4, 126.7, 62.3, 56.0, 44.0, 38.1, 35.7, 33.9, 32.0, 31.6, 29.6, 29.5, 29.4, 28.7, 22.8, 21.2, 14.2, 12.1; HRMS (FAB+) m/z calcd for $C_{24}H_{41}N$ $[M+H]^+$ 344.3312, found 344.3314.

4.1.6.5. (1*s*,4*s*)-*N*-Isopropyl-N-methyl-4-(4-octylphenyl)cyclohexanamine (*cis*-11c). 86% Yield, colorless oil; ^1H NMR (400 MHz, $CDCl_3$) δ 7.21–7.15 (m, 2H), 7.14–7.07 (m, 2H), 3.27–3.10 (m, 1H), 2.73–2.52 (m, 4H), 2.14 (s, 3H), 2.05–1.86 (m, 4H), 1.67–1.48 (m, 6H), 1.39–1.19 (m, 10H), 1.01 (d, J = 6.6 Hz, 6H), 0.89 (t, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 140.9, 139.0, 129.0, 127.2, 71.7, 63.9, 44.7, 35.6, 34.2, 32.1, 31.7, 29.7, 29.6, 29.5, 29.1, 22.9, 22.4, 17.8, 14.3; ESI-MS m/z calcd for $C_{24}H_{41}N$ $[M+H]^+$ 344.32, found 344.32.

4.1.7. General procedure for the synthesis of quaternary ammonium salts from tertiary amines

Methyl iodide (10 equiv) was added to a solution of the tertiary amine (1 equiv) in acetonitrile. The reaction mixture was refluxed for 2 h. The organic solvent was evaporated under reduced pressure and the residue dissolved in diethyl ether. The precipitate was collected by filtration and washed 3 times with diethyl ether to give the pure quaternary ammonium salt.

4.1.7.1. (1*s*,4*s*)-*N*,*N*,*N*-Trimethyl-4-(4-octylphenyl)cyclohexanaminium iodide (*cis*-12a). 69% Yield, pink solid, mp 238.4–239.8 °C; ^1H NMR (500 MHz, $CDCl_3$) δ 7.22–7.17 (m, 2H), 7.17–7.12 (m, 2H), 4.04 (tt, J = 3.3 Hz, 12.1 Hz, 1H), 3.31 (s, 9H), 3.10 (s, 1H), 2.62–2.52 (m, 2H), 2.51–2.43 (m, 2H), 2.16–2.00 (m, 4H), 1.69–1.50 (m, 4H), 1.37–1.18 (m, 10H), 0.86 (t, J = 7.0 Hz, 3H); ^{13}C NMR (126 Hz, $CDCl_3$) δ 140.9, 138.7, 128.9, 127.0, 74.6, 51.6, 35.5, 33.8, 32.0, 31.5, 29.6, 29.5, 29.3, 28.5, 22.7, 22.1, 14.2; ESI-MS m/z calcd for $C_{25}H_{44}N^+$ 330.32, found 330.31.

4.1.7.2. (1*r*,4*r*)-*N*,*N*,*N*-trimethyl-4-(4-octylphenyl)cyclohexanaminium iodide (*trans*-12a). 72% Yield, white solid; ^1H NMR (500 MHz, CD_3OD) δ 7.17–7.06 (m, 4H), 3.53 (m, 1H), 3.14 (s, 9H), 2.61–2.51 (m, 3H), 2.40–2.30 (m, 2H), 2.14–2.05 (m, 2H), 1.81–1.52 (m, 6H), 1.37–1.21 (m, 10H), 0.88 (t, J = 7.5 Hz, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 144.5, 140.8, 128.2, 126.3, 74.1, 50.4, 42.2, 35.2, 32.5, 31.7, 31.4, 29.2, 29.1, 29.0, 26.1, 22.4, 13.1; HRMS (FAB+) m/z calcd for $C_{23}H_{40}N^+$ 330.3161, found 330.3168.

4.1.7.3. (1*s*,4*s*)-*N*,*N*-Dimethyl-4-(4-octylphenyl)-N-propylcyclohexanaminium iodide (*cis*-12b). 68% Yield, yellow solid; ^1H NMR (400 MHz, $CDCl_3$) δ 7.23–7.18 (m, 2H), 7.17–7.13 (m, 2H), 3.98–3.84 (m, 1H), 3.48–3.39 (m, 2H), 3.21 (s, 6H), 3.12 (m, 1H), 2.61–2.54 (m, 2H), 2.54–2.45 (m, 2H), 2.12–2.00 (m, 4H), 1.90–1.77 (m, 2H), 1.68–1.56 (m, 4H), 1.38–1.19 (m, 10H), 1.04 (t, J = 7.3 Hz, 3H), 0.87 (t, J = 6.9 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 140.9, 138.6, 128.9, 127.0, 72.7, 64.1, 49.0, 35.5, 33.9, 32.0, 31.5, 29.6, 29.5, 29.4, 28.8, 22.8, 21.9, 16.5, 14.2, 10.9; ESI-MS m/z calcd for $C_{25}H_{44}N^+$ 358.35, found 358.34.

4.1.7.4. (1*r*,4*r*)-*N*,*N*-Dimethyl-4-(4-octylphenyl)-N-propylcyclohexanaminium iodide (*trans*-12b). 71% Yield, white solid, mp 162.6–163.7 °C; ^1H NMR (400 MHz, $CDCl_3$) δ 7.12–7.05 (m, 4H), 3.77–3.68 (m, 1H), 3.60–3.51 (m, 2H), 3.34 (s, 6H), 2.60–2.50 (m, 3H), 2.37–2.29 (m, 2H), 2.17–2.10 (m, 2H), 1.92–1.80 (m, 2H), 1.78–1.65 (m, 4H), 1.61–1.52 (m, 2H), 1.35–1.20 (m, 10H), 1.07 (t, J = 7.3 Hz, 3H), 0.85 (t, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 141.7, 141.4, 128.6, 126.6, 72.0, 64.5, 49.5, 42.4, 35.6, 32.6, 32.0, 31.6, 29.6, 29.5, 29.3, 26.6, 22.7, 16.5, 14.2, 10.9; ESI-MS m/z calcd for $C_{25}H_{44}N^+$ 358.35, found 358.34; HRMS (FAB+) m/z calcd for $C_{25}H_{44}N^+$ 358.3463, found 358.3470.

4.1.7.5. (1*s*,4*s*)-*N*-Isopropyl-N,N-dimethyl-4-(4-octylphenyl)cyclohexanaminium iodide (*cis*-12c). 70% Yield, white solid; ^1H NMR (500 MHz, $CDCl_3$) δ 7.20-7.07 (m, 4H), 4.06–3.98 (m, 1H), 3.84 (tt, J = 3.2 Hz, 11.8 Hz, 1H), 3.14–3.05 (m, 1H), 2.96 (s, 6H), 2.54 (t, J = 7.9 Hz, 2H), 2.49–2.42 (m, 2H), 2.17–2.00 (m, 4H), 1.68–1.52 (m, 4H), 1.48 (d, J = 6.5 Hz, 6H), 1.34–1.20 (m, 10H), 0.85 (t, J = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 140.9, 139.0, 129.0, 127.2, 71.7, 63.9, 44.7, 35.6, 34.2, 32.1, 31.7, 29.7, 29.6, 29.5, 29.1, 22.9, 22.4, 17.8, 14.3; ESI-MS m/z calcd for $C_{25}H_{44}N^+$ 358.35, found 358.32.

4.1.8. General procedure for the synthesis of quaternary ammonium salts from secondary amines

Methyl iodide (10 equiv) was added to a solution of the secondary amine (1 equiv) and K_2CO_3 (3 equiv) in acetonitrile. The reaction mixture was refluxed for 2 h. The organic solvent was evaporated under reduced pressure and the residue dissolved in diethyl ether. The precipitate was collected by filtration and washed three times with diethyl ether. The precipitate was dissolved in $CHCl_3$, the inorganic precipitates filtered and the filtrate concentrated under reduced pressure to give the pure quaternary ammonium salt.

4.1.8.1. (1*r*,4*r*)-*N*-Isopropyl-*N*,*N*-dimethyl-4-(4-octylphenyl)cyclohexanaminium iodide (*trans*-12c). 68% Yield, white solid; ^1H NMR (400 MHz, $CDCl_3$) δ 7.10 (s, 4H), 4.20–4.08 (m, 1H), 3.72 (tt, J = 2.8 Hz, 11.7 Hz, 1H), 3.13 (s, 6H), 2.64–2.52 (m, 3H), 2.45–2.35 (m, 2H), 2.20–2.11 (m, 2H), 1.91–1.66 (m, 4H), 1.65–1.49 (m, 8H), 1.39–1.19 (m, 10H), 0.88 (t, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 141.6, 141.2, 128.5, 126.5, 71.2, 63.7, 44.7, 42.4, 35.5, 32.7, 31.9, 31.5, 29.5, 29.4, 29.2, 26.8, 22.7, 17.4, 14.1; ESI-MS m/z calcd for $C_{25}H_{44}N^+$ 358.35, found 358.32.

4.1.8.2. (1*r*,4*r*)-*N*-Benzyl-*N*,*N*-dimethyl-4-(4-octylphenyl)cyclohexanaminium iodide (*trans*-12e). 73% Yield, white solid, mp 183.9–185.0 °C; ^1H NMR (500 MHz, $CDCl_3$) δ 7.69–7.65 (m, 2H), 7.46–7.38 (m, 3H), 7.08–7.01 (m, 4H), 4.98 (s, 2H), 3.83 (tt, J = 2.8 Hz, 12.0 Hz, 1H), 3.20 (s, 6H), 2.59–2.41 (m, 5H), 2.17–2.05 (m, 2H), 1.91–1.76 (m, 2H), 1.67–1.52 (m, 4H), 1.35–1.16 (m, 10H), 0.85 (t, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 141.7, 141.3, 133.4, 130.9, 129.4, 128.6, 127.3, 126.6, 72.1, 65.1, 47.8, 42.2, 35.6, 32.5, 32.0, 31.6, 29.6, 29.5, 29.3, 27.1, 22.7, 14.2; ESI-MS m/z calcd for $C_{29}H_{44}N^+$ 406.35, found 406.31.

4.1.8.3. (1*r*,4*r*)-*N*-Allyl-*N*,*N*-dimethyl-4-(4-octylphenyl)cyclohexanaminium iodide (*trans*-12h). 72% Yield, white solid; ^1H NMR (400 MHz, $CDCl_3$) δ 7.10–7.02 (m, 4H), 6.14–5.99 (m, 1H), 5.94–5.86 (m, 1H), 5.78–5.70 (m, 1H), 4.37 (d, J = 7.2 Hz, 2H), 3.65 (tt, J = 3.1 Hz, 12.0 Hz, 1H), 3.30 (s, 6H), 2.61–2.48 (m,

3H), 2.43–2.32 (m, 2H), 2.18–2.08 (m, 2H), 1.87–1.50 (m, 6H), 1.36–1.16 (m, 10H), 0.85 (t, J = 6.9 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 141.6, 141.2, 130.0, 128.5, 126.5, 124.3, 71.8, 64.7, 48.6, 42.2, 35.5, 32.4, 31.9, 31.5, 29.5, 29.4, 29.2, 26.6, 22.7, 14.1; ESI-MS m/z calcd for $C_{25}H_{42}N^+$ 356.35, found 356.32.

4.1.8.4. (1*s*,4*s*)-*N*-(2-Hydroxyethyl)-*N*,*N*-dimethyl-4-(4-octylphenyl)cyclohexanaminium iodide (*cis*-12i). 52% Yield, white solid; ^{1}H NMR (400 MHz, CD_3OD) δ 7.38–7.24 (m, 2H), 7.22–7.07 (m, 2H), 4.09–3.91 (m, 2H), 3.91–3.68 (m, 1H), 3.56–3.43 (m, 2H), 3.15–2.95 (m, 7H), 2.56 (t, J = 7.7 Hz, 2H), 2.53–2.43 (m, 2H), 2.25–2.14 (m, 1H), 2.14–1.91 (m, 3H), 1.80–1.38 (m, 4H), 1.38–1.13 (m, 11H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 140.3, 138.7, 128.4, 126.7, 74.0, 63.4, 55.4, 35.0, 33.7, 31.5, 31.2, 29.1, 29.0, 28.9, 28.4, 22.3, 21.4, 13.4; HRMS (FAB+) m/z calcd for $C_{24}H_{42}NO^+$ 360.3266, found 360.3273.

4.1.8.5. (1*r*,4*r*)-*N*-(2-Hydroxyethyl)-N,N-dimethyl-4-(4-octylphenyl)cyclohexanaminium iodide (*trans*-12i). 39% Yield, white solid, mp 153.3–154.2 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.11–7.04 (m, 4H), 4.34–4.28 (m, 1H), 4.22–4.16 (m, 2H), 3.95–3.87 (m, 1H), 3.81–3.76 (m, 2H), 3.30 (s, 6H), 2.52 (t, J = 7.5 Hz, 2H), 2.42–2.35 (m, 2H), 2.13–2.02 (m, 2H), 1.79–1.65 (m, 4H), 1.60–1.50 (m, 2H), 1.34–1.18 (m, 10H), 0.86 (t, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 141.8, 141.3, 128.6, 126.7, 73.4, 64.1, 55.9, 50.1, 42.5, 35.6, 32.5, 32.0, 31.6, 29.6, 29.5, 29.4, 26.7, 22.8, 14.2; HRMS (FAB+) m/z calcd for $C_{24}H_{42}NO^+$ 360.3266, found 360.3259.

4.1.8.6. 5-(4-Octylphenyl)pyrazin-2-aminium chloride (13). $Pd(OAc)_2$ (0.232 mg, 1.032 μmol), and S-Phos (2.89 mg, 7.03 μmol) were added to a suspension of K_2CO_3 (243 mg, 1.758 mmol), 5-bromopyrazin-2-amine (61.2 mg, 0.352 mmol), and (4-octylphenyl)boronic acid (107 mg, 0.457 mmol) (prepared according to Ishi-I, T. et al.[49]) in acetonitrile/water (1.5:1). The suspension was degassed for 5 min and then refluxed for 6 h. The reaction mixture was extracted with EtOAc, washed with brine, dried with $MgSO_4$ and filtered. After evaporation of the organic solvent under reduced pressure, the resulting residue was purified by column chromatography over silica gel (100% hexanes to 50/50 hexanes/EtOAc) to provide an orange solid (95% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (s, 1H), 8.04 (s, 1H), 7.78 (d, J = 8.1 Hz, 2H), 7.26 (d, J = 8.1 Hz, 2H), 4.82 (br s, 1H), 2.64 (t, J = 7.7 Hz, 2H), 1.63 (m, 2H), 1.38–1.20 (m, 8H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.8, 143.2, 143.0, 138.4, 134.3, 131.8, 128.9, 125.5, 35.7, 31.9, 31.4, 29.5, 29.3, 29.2, 22.7, 14.1; HRMS (ESI+) m/z calcd for $C_{18}H_{26}N_3$ $[M+H]^+$ 284.2121, found 284.2136.

The above amine was dissolved in 10 mL methanol and HCl (g) was bubbled through the solution for one minute. After evaporation of the methanol, diethyl ether was added and the solid filtered. It was washed with cold diethyl ether to yield the title compound as a pale yellow solid (95% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.44(s, 1H), 8.29 (s, 1H), 7.82 (d, J = 7.9 Hz, 2H), 7.26 (d, J = 7.8 Hz, 2H), 2.59 (t, J = 7.3 Hz, 2H), 1.65–1.49 (m, 2H), 1.40–1.10 (m, 8H), 0.84 (t, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 151.6, 143.1, 139.5, 136.2, 133.8, 131.8, 129.3, 125.4, 35.4, 31.8, 31.4, 29.4, 29.3, 29.2, 22.6, 14.5; HRMS (ESI+) m/z calcd for $C_{18}H_{26}N_3$ $[M+H]^+$ 284.2121, found 284.2119.

4.1.8.7. 4-(6-(4-Octylphenyl)pyridin-2-yl)piperazin-1-ium chloride (14). $Pd(OAc)_2$ (0.115 mg, 0.514 μmol), and S-Phos (0.211 mg, 0.514 μmol) were added to a suspension of K_2CO_3 (17.75 mg, 0.128 mmol), 1-bromo-4-octylbenzene (6.12 μl, 0.026 mmol), *tert*-butyl 4-(6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-yl)piperazine-1-carboxylate (10 mg, 0.026 mmol), in acetonitrile/water (1.5:1). The suspension was degassed for 5 min and then refluxed for 6 h. The reaction mixture was extracted with EtOAc, washed with brine, dried with $MgSO_4$ and filtered. After evaporation of the organic solvent under reduced pressure, the resulting residue was purified by column chromatography over silica gel (100% hexanes to 70/30 hexanes/EtOAc) to provide a yellow oil (93% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.90 (d, J = 8.3 Hz, 2H), 7.58–7.50 (m, 1H), 7.23 (d, J = 7.6 Hz, 2H), 7.10 (d, J = 7.6 Hz, 1H), 6.57 (d, J = 8.5 Hz, 1H), 3.64–3.55 (m, 8H), 2.67–2.60 (m, 2H), 1.67–1.57 (m, 2H), 1.49 (s, 9H), 1.36–1.24 (m, 8H), 0.87 (t, J = 6.8 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 158.9, 155.5, 155.0, 143.8, 138.3, 137.3, 128.7, 126.7, 109.9, 105.4, 80.0, 45.2, 35.8, 32.0, 31.5, 29.6, 29.4, 29.3, 28.5, 22.8, 14.2; HRMS (ESI+) m/z calcd for $C_{28}H_{41}N_3O_2$ $[M+H]^+$ 452.3199, found 452.3605.

The Boc-protected amine was dissolved in 10 mL methanol and HCl (g) was bubbled through the solution for one minute. After evaporation of the methanol, diethyl ether was added and the solid filtered. It was washed with cold diethyl ether to yield the title compound as orange oil (92% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 8.09-8.03 (m, 1H), 7.80 (d, J = 7.3 Hz, 2H), 7.39 (d, J = 7.0 Hz, 2H), 7.33 (d, J = 7.0 Hz, 1H), 7.29–7.20 (m, 1H), 4.05–3.96 (m, 4H), 3.50–3.39 (m, 4H), 2.70 (t, J = 7.6 Hz, 2H), 1.71–1.59 (m, 2H), 1.40–1.20 (m, 8H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR (126 MHz, CD_3OD) δ 155.1, 151.8, 146.2, 143.6, 129.0, 127.7, 114.6, 113.0, 109.8, 44.0, 42.9, 35.4, 31.7, 31.2, 29.2, 29.1, 29.0, 22.4, 13.1; HRMS (ESI+) m/z calcd for $C_{23}H_{34}N_3$ $[M+H]^+$ 352.2747, found 352.2742.

4.1.8.8. 5′-(4-Octylphenyl)-2*H*-[1,2′-bipyridin]-2-one (15). $Pd(OAc)_2$ (0.232 mg, 1.032 μmol), and S-Phos (0.424 mg, 1.032 μmol) were added to a suspension of K_2CO_3 (35.7 mg, 0.258 mmol), 1-bromo-4-octylbenzene (0.012 ml, 0.052 mmol), 5′-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-2H-[1,2′-bipyridin]-2-one (20 mg, 0.067 mmol) in acetonitrile/water (1.5:1). The suspension was degassed for 5 minutes and refluxed for 6 h. The reaction mixture was extracted with EtOAc, washed with brine, dried with $MgSO_4$ and filtered. After evaporation of the organic solvent under reduced pressure, the resulting residue was purified by column chromatography over silica gel (100% hexanes to 40/60 hexanes/EtOAc) to provide the title compound as an off-white solid (81% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.75 (t, J = 1.7 Hz, 1H), 8.00 (d, J = 1.6 Hz, 3H), 7.92 (dd, J = 2.1 Hz, 7.1 Hz, 1H), 7.52 (d, J = 8.3 Hz, 2H), 7.41 (m, 1H), 7.31 (d, J = 8.3 Hz, 2H), 6.71–6.63 (m, 1H), 6.36–6.27 (m, 1H), 2.66 (t, J = 7.8 Hz, 2H), 1.71–1.59 (m, 2H), 1.37–1.22 (m, 8H), 0.88 (t, J = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.3, 150.5, 147.0, 143.5, 140.2, 136.3, 136.0, 135.9, 134.1, 129.3, 127.0, 122.1, 121.1, 106.3, 35.7, 31.9, 31.5, 29.5, 29.4, 29.3, 29.2, 22.7, 14.1; HRMS (ESI+) m/z calcd for $C_{24}H_{28}N_2O$ $[M+Na]^+$ 383.2094, found 383.2113.

4.2. Biology

4.2.1. Sphingosine kinase assay

Human SphK1 and mouse SphK2 cDNAs were used to generate recombinant baculoviruses that encoded the respective proteins. Infection of Sf9 insect cells with the viruses for 72 h resulted in >1000-fold increases in SphK activity in 10,000×g supernatant fluid from homogenized cell pellets. The enzyme assay conditions were exactly as described,[43] except infected Sf9 cell extract containing 2-3 μg protein was used as a source of enzyme.

4.2.2. LC/MS protocol

Analyses were performed by Liquid Chromatography/ESI Mass Spectrometry (LC/MS) using a triple quadrupole mass spectrometer (Sciex 4000 Q-Trap) coupled to a Shimadzu LC-20AD LC system. A binary solvent gradient with a flow rate of 1 mL/min was used to separate FTY720 and FTY720-P by reverse phase chromatography using a Supelco Discovery C18 column (50 mm × 2.1 mm, 5 μm

bead size). Mobile phase A consisted of water: methanol: formic acid (79:20:1) while mobile phase B was methanol: formic acid (99:1). The run started with 100% A for 0.5 minutes. Solvent B was then increased linearly to 100% B in 5.1 min and held at 100% for 4.3 min. The column was finally re-equilibrated to 100% A for 1 min. Natural sphingolipids were detected using multiple reaction monitoring (MRM) methods previously described[50] as follows: C17S1P (366.4→250.4); FTY720 (308.4→255.1); FTY720-P (388.4→255.1); C17sphingosine (286.4→250.3); C17S1P and C17sphingosine were used as the internal standards. All analytes were analyzed simultaneously using the aforementioned MRMs. Voltages (DP, EP, CE and CXP) for C17S1P and C17sphingosine were: 35, 10, 25, 6; and 156, 10, 25, 14 volts, respectively. Retention times for all analytes under our experimental conditions were between 5.1 and 5.6 min. Quantification was carried out by measuring peak areas using commercial software (Analyst 1.5.1).

4.2.3. Western blot analysis

Cells were incubated with various concentrations of inhibitor for the times indicated (usually 2 h). After incubation, cells were washed with phosphate-buffered saline and lysed using a Dounce homogenizer. Equal amounts of protein were resolved by SDS–PAGE analysis using 10% polyacrylamide gels and resolved proteins transferred to a nitrocellulose membrane. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS, pH 7.4) containing 0.1% Tween 20 for 1 h at room temperature. After rinsing, membranes were incubated with antibodies (diluted 1:1000 in TBS) against ERK, p-ERK, Akt, p-Akt, and β-actin for 1 h. After washing three times in TBS buffer, the nitrocellulose membrane was incubated with a 1:2000 dilution (in TBS) of HRP-conjugated anti-IgG antibody. Detection was accomplished by chemiluminesence using a commercial kit (Perkin Elmer Western Lightning).

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Supplementary data

Supplementary data (explanation of stereoselectivity, GC analysis of reductive amination reactions, and ^{1}H and ^{13}C NMR spectral data for all compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.11.011.

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