Journal of Medicinal Chemistry

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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.8b00727 • Publication Date (Web): 03 Aug 2018

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Discovery of 4-Benzyloxybenzo[*d*]isoxazole-3-amine Derivatives as Highly Selective and Orally Efficacious Human Sphingomyelin Synthase 2 Inhibitors that Reduce Chronic Inflammation in *db/db* Mice

Mingguang Mo[†], Jintong Yang[†], Xian-Cheng Jiang[†], Yu Cao[‡], Jinyu Fei[†], Yang Chen[‡], Xiangyu Qi[†], Yong Chu[†], Lu Zhou*[,†], Deyong Ye*[,†]

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ABSTRACT

Sphingomyelin synthase 2 (SMS2) is a promising therapeutic target for several chronic inflammation-associated diseases, including atherosclerosis, fatty liver and insulin resistance. Herein, we report the identification of 4-benzyloxybenzo[*d*]isoxazole-3-amine derivatives as potent and highly selective SMS2 inhibitors through a conformational restriction strategy. After systematic structural modifications, several compounds with high selectivity and good potency *in vitro* were selected for further evaluation. Compound **15w** demonstrated good pharmacokinetics (oral bioavailability, $F = 56\%$) *in vivo* and has an inhibitory potency against sphingomyelin synthase activity when ICR (Institute of Cancer Research) mice are provided with an oral dose of this compound. In addition, compound **15w** attenuated chronic inflammation significantly in *db/db* mice after oral dosing for 6 weeks.

INTRODUCTION

The sphingomyelin synthase (SMS) family has three members, sphingomyelin synthase 1 (SMS1), sphingomyelin synthase 2 (SMS2) and sphingomyelin synthase-related protein (SMSr), whose genes were identified[1-2] 30 years after confirmation that sphingomyelin was produced from ceramide and phosphatidylcholine (PC) by a ^{14}C-labeled substrate approach.[3] The substrates PC and ceramide for SMS1 and SMS2, and the reaction products diacylglycerol (DAG) and sphingomyelin (SM) are important signaling molecules.[4-5] The highly conserved SMSr, with few SM synthase activity,[1] yields ethanolamine phosphorylceramide (EPC) and DAG from phosphatidylethanolamine (PE) and ceramide.[6] Thus, sphingomyelin synthase family members serve as central regulators in various physiological pathways such as cell proliferation, cell death and apoptosis, and intracellular trafficking. The levels of SM synthesized by SMSs serve as a risk factor for coronary artery disease.[7] In addition, high levels of serum SM with saturated acyl chains are associated with insulin resistance in young obese adults.[8] Results from *in vivo* studies showed that decreased atherosclerotic effects were observed in SMS2 knockout mice because the levels of inflammatory cytokines were reduced,[9] whereas SMS1 knockout mice exhibited some side effects including dysfunction and increased reactive oxygen and hearing impairment.[10-11] Interestingly, dynamic modification of SM in SMS2 knockout mice showed that these mice are protected from diet-induced obesity, fatty liver, Type 2 diabetes mellitus (T2DM) and insulin resistance with few negative side effects.[12-14] Amelioration of chronic inflammation is a new strategy for the treatment of metabolic diseases, such as obesity, fatty liver, T2DM and insulin resistance,[15-16]

and a selective SMS2 inhibitor may be a promising alternative for the treatment of metabolic diseases. [9, 12-13, 17-18] However, the lack of specific SMS2 inhibitors hampers the development of pharmacological studies of SMS2 inhibitors *in vitro* and *in vivo*.

Our research group has spent many years focusing on the discovery of SMS inhibitors. We published previously a series of aminonitrile derivatives as SMS inhibitors and a series of 2-(4-(*N*-phenethylsulfamoyl)phenoxy)acetamides (SAPAs) as SMS1 inhibitors.[19-20] Very recently, selective SMS2 inhibitors, which are oxazolopyridine derivatives, were reported by our team using purified SMS1 and SMS2, and enzyme inhibitory assays.[21] However, the potency of these inhibitors against SMS2 was found to be poor with IC_{50} values in the micromolar range.

In an effort to discover selective SMS2 inhibitors with higher potency, compound **1**, an originally synthesized and patented compound[22] used to prevent and treat atherosclerosis,[23] was selected as the lead compound. As seen in Figure 1, a scaffold hopping conformational restricted approach was used to afford a new 4-benzyloxybenzo[*d*]isoxazole-3-amine scaffold. Compound **3a** displayed good potency and good selectivity against human SMS2. Encouraged by these results, we proceeded with further structure and activity relationship (SAR) exploration of 4-benzyloxybenzo[*d*]isoxazole-3-amine.

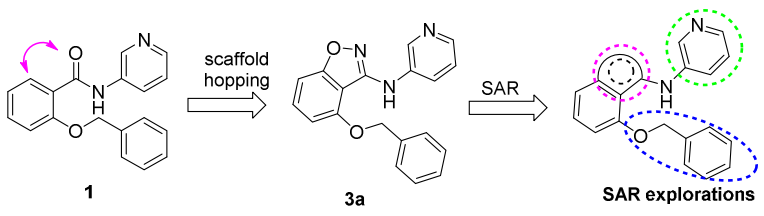


Figure 1. Design strategy of selective SMS2 inhibitors and systematic SAR

explorations of 4-benzyloxybenzo[*d*]isoxazole-3-amine derivatives.

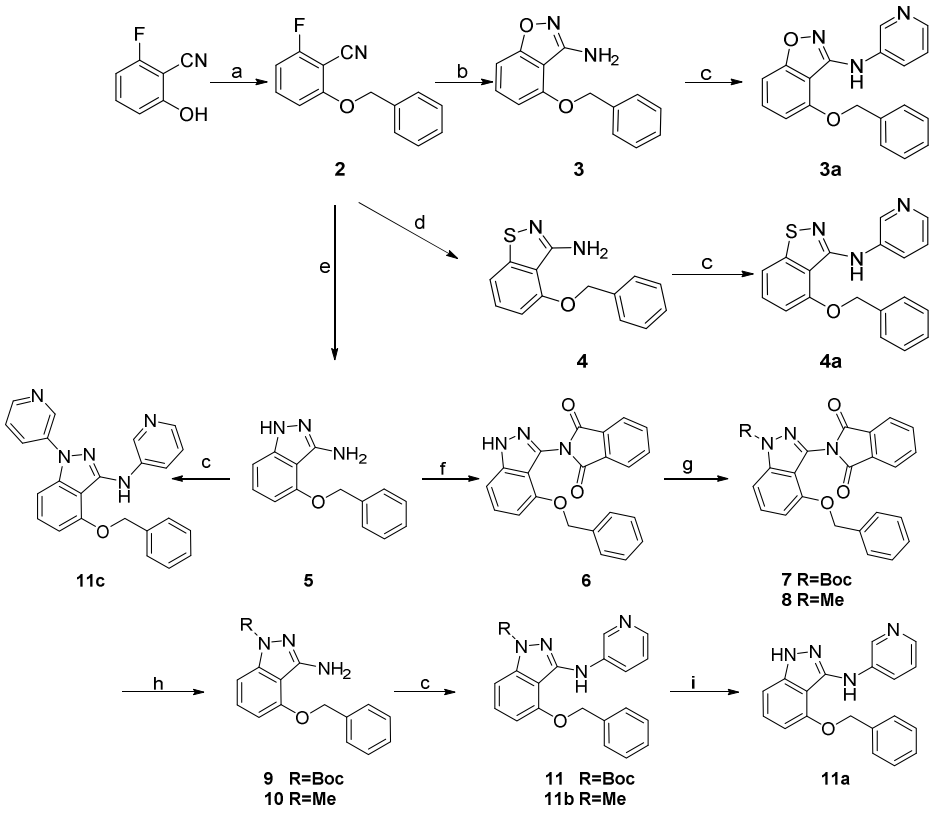
Herein, we describe the discovery of 4-benzyloxybenzo[*d*]isoxazole-3-amine derivatives as a novel class of human SMS2 inhibitors. These compounds possessed potent activity and high selectivity against pure SMS2, and exhibited good pharmacokinetic profiles. Compound **15w** is the first selective SMS2 inhibitor with high potency that reduces chronic inflammation significantly in *db/db* mice.

CHEMISTRY

A general synthetic strategy was used to prepare the target compounds with various scaffolds (Schemes 1–3). The synthetic routes for the compounds, *i.e*., scaffold hopping of **1**, are shown in Scheme 1. A simple nucleophilic substitution between 2-fluoro-6-hydroxybenzonitrile and (bromomethyl)benzene yielded intermediate **2**, which was followed by adding acetohydroxamic acid and *t*-BuOK to afford 4-(benzyloxy)benzo [*d*]isoxazol-3-amine (**3**).[24] Compound **2** was heated with sodium sulfide overnight and then treated with concentrated aqueous ammonium hydroxide and aqueous sodium hypochlorite in an ice-water bath to give compound **4**. Cyclization of **2** via hydrazine hydrate yielded **5**. The 3-pyridine moiety was introduced to **3**–**5** using the Pd(0) catalyzed Buchwald-Hartwig cross-coupling reaction to yield **3a**, **4a** and **11c**. Protection of **5** via phthalic anhydride yielded **6**, and a *tert*-butyl or methyl carbonate was introduced to afford **7** and **8**. The amino group of **7** and **8** was deprotected by hydrazine hydrate, followed with a similar Buchwald-Hartwig cross-coupling reaction yielded **11** and **11b**, respectively. Acid

hydrolysis of the *tert*-butyl carbonate of **11** yielded **11a**.

Scheme 1. **Synthesis of Analogues 3a, 4a and 11a-11c**[a]

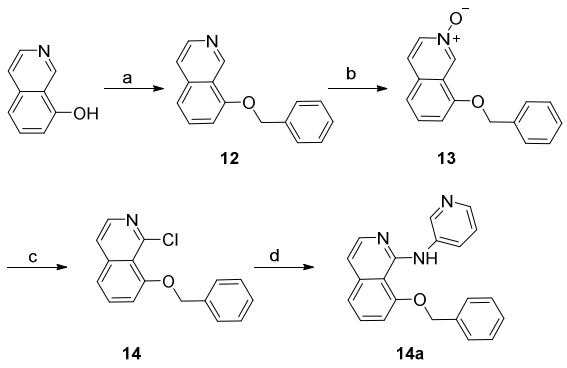


[a] Reagents and conditions: (a) K_2CO_3, KI, (bromomethyl)benzene, acetonitrile, rt; (b) *t*-BuOK, acetohydroxamic acid, DMF, rt; (c) 3-bromopyridine, $Pd_2(dba)_3$, Xantphos, K_2CO_3, dioxane, 125 °C, 15 h; (d) Na_2S, DMSO, 70 °C, 12 h; then, 0 °C, 25% NH_3 aqueous solution, 15% NaClO aqueous solution, rt, 5 h; (e) N_2H_4 H_2O, EtOH, reflux; (f) phthalic anhydride, 170 °C, 0.5 h; (g) **7**: DMAP, $(Boc)_2O$, DCM, rt, overnight; **8**: K_2CO_3, CH_3I, DMF, rt, 3 h; (h) N_2H_4 H_2O, EtOH, rt; (i) TFA, DCM, rt, 2 h.

Synthesis of **14a** is shown in Scheme 2. A simple nucleophilic substitution gave 8-benzyloxy isoquinoline (**12**). *N*-oxide (**13**) was prepared from 8-benzyloxy isoquinoline (**12**) with *m*-CPBA. 8-benzyloxy-1-chloroisoquinoline (**14**) was prepared

from **13** with phosphorus oxychloride. Finally, a Buchwald-Hartwig crossing between pyridin-3-amine and **14** gave **14a**.

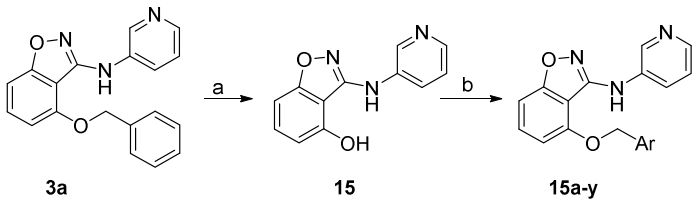
Scheme 2. **Synthesis of Analogue 14a**[a]

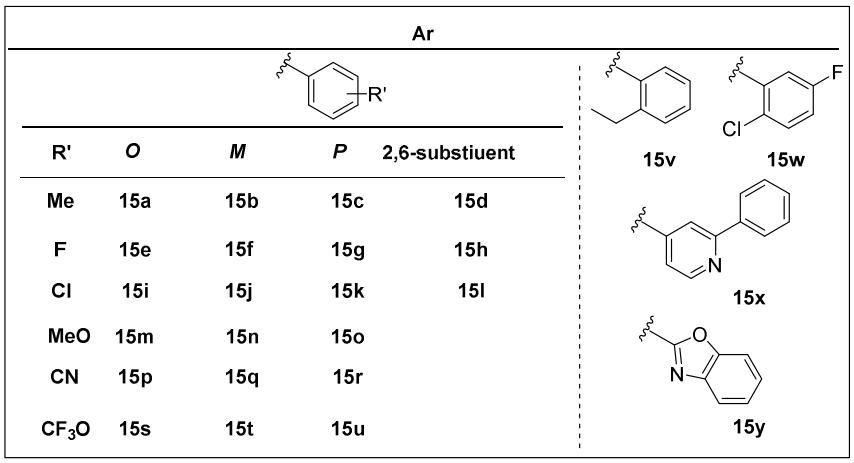


[a] Reagents and conditions: (a) K_2CO_3, KI, (bromomethyl)benzene, acetonitrile, rt; (b) *m*-CPBA, DCM, overnight; (c) $POCl_3$, 90 °C, 5 h; (d) pyridin-3-amine, $Pd_2(dba)_3$, Xantphos, K_2CO_3, dioxane, 125 °C, 15 h.

The synthesis approach for compounds **15a–y** is outlined in Scheme 3. Treating **3a** with a hydrobromic acid solution and acetic acid provided intermediate **15**. A number of commonly available benzyl bromide and 4-bromomethyl-2-phenylpyridine (details in Supporting Information) compounds were reacted with key intermediate **15** to afford the desired compounds **15a–y**.

Scheme 3. Synthesis of Analogues 15a–y[a]





[a] Reagents and conditions: (a) 40% HBr solution, HOAc, 65 °C, overnight; (b) $ArCH_2$-Br, K_2CO_3, acetone, rt.

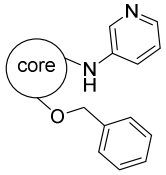
RESULTS AND DISCUSSION

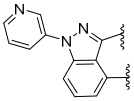
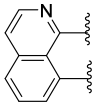
SAR In Vitro. Compounds were evaluated initially for their *in vitro* inhibitory activities against SMS2 at 10 μM and against SMS1 at 50 μM. Compounds that demonstrated acceptable inhibitory activity in these initial tests were further evaluated by determining their IC_{50} values against SMS2 and SMS1 to ascertain their precise inhibitory potency. SMS activities were measured using human SMS1 and SMS2.

SAR modification of **3a** focused mainly on the three zones of the molecule (Figure. 1): the core moiety, the substituted benzyloxy and the pyridine moiety. The new benzo[*d*]isoxazole scaffold (**3a**) (Figure. 1) showed improved potency when compared with that of **1** (0.34 μM *vs* 1.5 μM). Intriguingly, replacing the benzo[*d*]isoxazole with benzo[*d*]isothiazole (**4a**), *1H*-indazole (**11a**) or isoquinoline (**14a**) reduced inhibitory potency, and a dramatic loss in potency was observed when substituting the *1H*-indazole with either a methyl (**11b**) or 3-pyridinyl (**11c**) moiety.

Thus, we assumed that SMS2 activity is gradually lost as the size of the substituent increased (Table 1), and hypothesized that the benzo[*d*]isoxazole moiety played a key role in both hydrophobic interactions with SMS2 and that the size of the ligand binding site of SMS2 was strictly limited. The benzoisoxazole compound **3a**, which possessed the best potency in both SMS2 inhibitory activities and ligand efficiencies (*LE*) among the scaffolds **3a**, **4a**, **11a**, **14a**, was selected for further analoging.

Table 1. Scaffold Hopping of 1



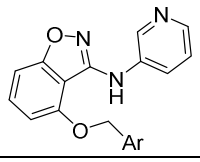
Compd.	Core	SMS2 IC_{50} ± SEM (μM)[a]	SMS1 inhibition rate (%) at 50 μM[b]	*LE*[c]
1		1.5 ± 0.3	20	0.35
3a		0.34 ± 0.15	24	0.38
4a		1.2 ± 0.2	13	0.34
11a		9.1 ± 2	–7	0.29
11b		>10	1	–
11c		Inhibition ratio –25%@ 10 μM[b]	–70	–
14a		>10	18	–

[a]IC_{50} values are the mean of at least two separate determinations and each determination represents the mean of triplicate experiments. Statistical calculation of IC_{50} values was performed using GraphPad Prism 7.0 (GraphPad Software, Inc.), which were determined at more than five concentrations of each inhibitor. [b]The inhibition rate was derived from the mean value of two independent experiments. "–", not tested. [c]$LE = -1.4 \ln IC_{50(SMS2)} / HAC$.[25]

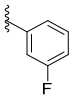
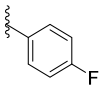
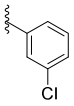
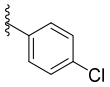
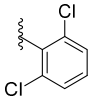
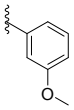
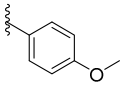
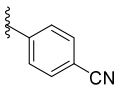
Our next objective was to investigate the influence of substituents on the benzyl aromatic ring to the activity of SMS2 and selectivity over SMS1. As shown in Table 2, a methyl (**15a**) or electron-withdrawing fluorine (**15e**) substituent introduced to the benzyl *ortho*-position resulted in no significant change in SMS2 potency. However, introduction of a bulkier substituent such as chlorine (**15i**) or methoxy (**15m**) to the *ortho*-position were well tolerated in SMS2 inhibitory activity. Encouraged by the bioactivity results of **15i** and **15m**, we then focused on some bulkier groups such as ethyl, trifluoromethoxy and cyano groups. Intriguingly, ethyl (**15v**) and trifluoromethoxy (**15s**) slightly increased SMS2 potency, whereas a cyano group (**15p**) resulted in the loss of the majority of the SMS2 potency. Further incorporation of a chloro group to the chloro-substituted derivative (**15i**) yielded **15l**, which possessed higher SMS2 inhibitory activity and high selectivity (780-fold) over SMS1. In contrast, smaller groups without electron-withdrawing properties were more tolerated in the *meta*-position for SMS2 inhibitory activity, such as a fluorine group (**15f**) *vs* chloro group (**15j**) (0.16 μM *vs* 0.10 μM). However, SMS2 inhibitory activity decreased when *para*-substituents were tested. In addition, replacement of the phenyl

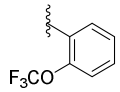
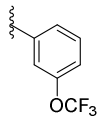
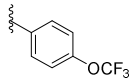
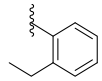
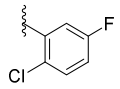
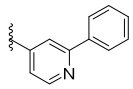
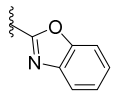
group with a larger group such as the 2-phenylpridyl group (**15x**) and the benzo[*d*]oxazole group (**15y**) resulted in a significant loss of potency towards both SMS1 and SMS2. Interestingly, incorporation of **15f** and **15i** substituents to give, **15w** yielded a compound with similar potency and selectivity (IC_{50} = 0.10 μM against SMS2, 560-fold selectivity for SMS2 over SMS1) and also exhibited unchanged *LE* values. These SARs led us to postulate that electron-donating groups with hydrophobic substituents at the *meta*-position of the tail aromatic ring improved inhibitory activity against SMS2, whereas electron-donating groups with hydrophilic substituents were better at the *ortho*- rather than *meta*-position, such as methoxy and trifluoromethoxy.

Table 2. Modification of Benzyl Aromatic Substituents



Comp	Ar	SMS2 IC_{50} ± SEM (μM)[a]	SMS1 Inhibition rate (%) at 50 μM [b]	SMS1 IC_{50} ± SEM (μM) [a]	Select-ivity ratio[c]	*LE*[d]
3a		0.33 ± 0.15	24	–	–	0.38
15a		0.47 ± 0.06	41	70 ± 7	150	0.35
15b		0.19 ± 0.03	20	–	–	0.38
15c		>10	5	–	–	–
15d		0.15 ± 0.01	53	47 ± 4	310	0.37

15e		0.67 ± 0.08	6	–	–	–
15f		0.16 ± 0.04	6	–	–	0.38
15g		1.7 ± 0.3	7	–	–	–
15h		0.74 ± 0.11	30	–	–	–
15i		0.27 ± 0.01	41	79 ± 8	290	0.37
15j		0.10 ± 0.01	44	80 ± 5	800	0.39
15k		>10	5	–	–	–
15l		0.090 ± 0.004	25	70 ± 7	780	0.38
15m		0.24 ± 0.05	49	48 ± 8	200	0.36
15n		0.52 ± 0.02	17	–	–	–
15o		>10	–5	–	–	–
15p		4.4 ± 0.5	4	–	–	–
15q		0.96 ± 0.04	8	–	–	–
15r		>10	–18	–	–	–

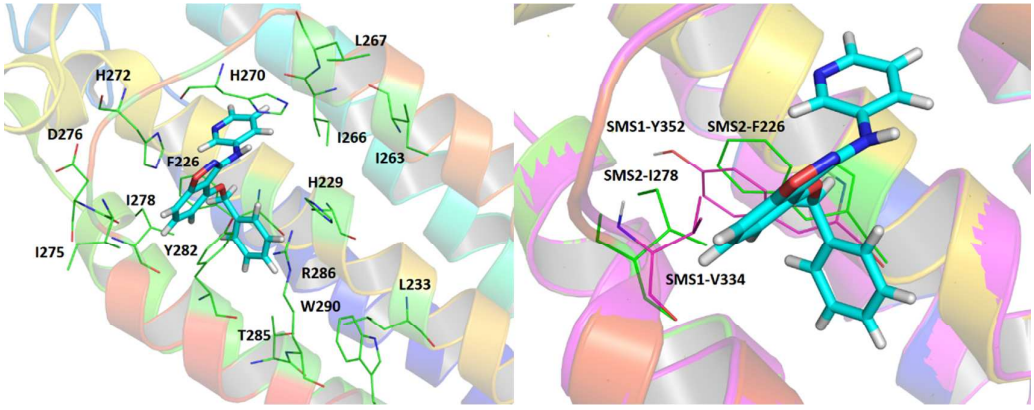
15s		0.11 ± 0.02	59	67 ± 3	610	0.34
15t		1.3 ± 0.2	30	–	–	–
15u		>10	5	–	–	–
15v		0.24 ± 0.01	65	33 ± 2	140	0.37
15w		0.10 ± 0.01	44	56 ± 7	560	0.38
15x		>10	–6	–	–	–
15y		>10	–35	–	–	–

[a]IC_{50} values are the mean of at least two separate determinations and each determination was the mean of triplicate experiments. Statistical calculation of IC_{50} values was performed using GraphPad Prism 7.0 (GraphPad Software, Inc.), which were determined at more than five concentrations of each inhibitor. "–", not tested. [b]The inhibition rate was derived from the mean value of two independent experiments. [c]Selectivity ratio = SMS1 IC_{50}/SMS2 IC_{50}. [d]$LE = -1.4 \ln IC_{50(SMS2)} / HAC$[25].

Finally, we explored the effects of the pyridine moiety on SMS2 activity and selectivity. Replacement of pyridine with pyrimidine while keeping the 2-chloro-5-fluro substituted benzyloxy tail resulted in a significant loss of potency (Table S1, details in the Supporting Information). Replacing the pyridine moiety with 2-(dimethylamino)acetamide analogues also resulted in loss of potency (Table S1,

details in the Supporting Information). Thus, we hypothesized that the terminal pyridine ring plays a key role in the interaction with SMS2.

To gain further insight into the binding interactions between these 4-benzyloxybenzo[*d*]isoxazole-3-amine derivatives and SMS1 or SMS2, docking studies were performed to investigate the docking pose of **3a** with SMS1 or SMS2 (Figure 2). The key binding forces between **3a** and SMS2 were hydrophobic interactions and π-π stacking interactions. Here, the pyridine ring formed an edge-to-face π-π stacking interaction with H272 and H270, and the benzo[*d*]isoxazole moiety formed another face-to-face π-π stacking interaction with Y282. Moreover, the central benzo[*d*]isoxazole moiety formed hydrophobic interactions with I278 and F226 (Figure 2B). In contrast, the corresponding residues V334 and Y352 of SMS1 formed a pocket with lower hydrophobicity. This difference may contribute to the inhibitory selectivity between the isoforms SMS1 and SMS2.



(A) (B)

Figure 2. Docking mode of **3a** with SMS2. Carbon, oxygen, nitrogen and hydrogen atoms of the **3a** molecule are colored cyan, red, blue and gray, respectively. (A) The binding mode of **3a** to SMS2. (B) The superposition of **3a** binding to SMS1 (magenta)

and SMS2 (same color as shown in (A)).

***Pharmacokinetic* Evaluation** Compounds with relatively good selectivity towards SMS2 over SMS1 were chosen for evaluation of their pharmacokinetic profiles in male ICR (Institute of Cancer Research) mice. The key pharmacokinetic parameters are summarized in Table 3. Compound **15l** displayed the highest exposure in plasma (C_{max} = 0.71 μg/mL, AUC = 3.0 μg·h/mL) and a moderate $T_{1/2}$ value (2.0 h). Compounds **15j** and **15w** had better half-lives than **15l** (**15j**, 2.5 h; **15w**, 2.3 h) with moderate exposure in plasma. To our delight, compounds **15l** and **15w** possessed significant oral bioavailabilities (F = 57% and 56%) and compounds **15j** (F = 41%) and **15s** (F = 53%) possess slightly lower ones. These results led us to postulate that these four compounds possess reasonable physicochemical properties for oral dosing, according to Lipinski rule[26] and Veber rule.[27] What's more, the good oral bioavailability and hight Vd indicated that these compounds maybe good permeability. SMS2 mainly distribute in membrane in the organs such as liver, and compound with higher Vd may be highly lipophilic and tend to bind to tissue. Compounds **15s** and **15w** had higher Vd, and **15w** was selected for further valuation and the results showed it had lower clearance(Cl) (6.2 *vs* 7.8 L/h/kg) and higher C_{max}(0.51 *vs* 0.36 μg/mL) comparing with **15s**.

Table 3. Pharmacokinetic Parameters of selected Compounds after Oral Administration to ICR Mice[a]

	pharmacokinetics (iv)			pharmacokinetics (po)				
Comp	AUC_{0-24} (μg·h/mL)	Cl (L/h/kg)	Vd (L/kg)	AUC_{0-24} (μg·h/mL)	$T_{1/2}$ (h)	C_{max} (μg/mL)	T_{max} (h)	F (%)

15j	2.2 ± 0.3	4.8 ± 0.5	9.9 ± 1	1.8 ± 0.4	2.5±0.5	0.49 ± 0.06	0.9 ± 0.1	41
15l	2.6 ± 0.2	3.9 ± 0.3	6.9 ± 0.5	3.0 ± 2.5	2.0 ± 0.1	0.71 ± 0.08	1.8 ± 0.2	57
15s	1.4 ± 0.3	7.8 ± 0.2	6.1 ± 0.3	1.5 ± 0.4	2.0 ± 0.3	0.36 ± 0.05	1.4 ± 0.4	53
15w	1.7 ± 0.1	6.2 ± 0.2	12.5 ± 0.7	1.9 ± 0.3	2.3 ± 0.7	0.51 ± 0.04	2.2 ± 0.2	56

[a] n = 4 animals/group. Dose iv: administered at 10 mg/kg, vehicle was 5% DMSO/5% Polyoxyl 35 Castor oil in saline. Dose po: administered at 20 mg/kg, vehicle was 0.5% carboxymethylcellulose sodium. Data are the mean ± SEM.

Assessment of the Inhibitory Activities Against SM Synthase Activity *In Vivo.* Based on the pharmacokinetic results, the inhibitory activity of compound **15w** was evaluated in male ICR mice through oral administration at single doses of 50 mg/kg. The whole livers were collected 2 h after the oral dose of **15w** and assay SMS activity following detailed measurements that have been described previously.[28] Compound **15w** was found to inhibit SMS activity significantly (Figure 3A). To further evaluate the inhibitory activities against SM synthase *in vivo*, we injected NBD-ceramide (substrate of SMS activity) (*i.v.)* to initiate SM synthase activity and collected plasma at 1 and 2 h after the oral dose of **15w** (50 or 100 mg/kg). We then measured NBD-SM (product) in the plasma. Compound **15w** was found to inhibit SM synthase activity in a dose-dependent fashion (Figure 3B). What's more, we further evaluated the plasma SM level in ICR after treating with **15w** (intraperitoneal injection, 40 mg/kg) for 7 days. The plasma SM levels were measured by the enzymatic measurement reported previously.[29] Compound **15w** reduced the levels of plasma SM in ICR significantly (Figure 3C).

The observed inhibition of SM synthase activity reflects a combination of all the activity *in vivo*. Noticeably, **15w** could not reduce total SM synthase activity below 50% even when a higher dose was administered, which may be due to **15w** SMS2 selectivity. (IC_{50}, 0.10 μM *vs* 56 μM, SMS2 *vs* SMS1). We postulate that compound **15w** has very weak inhibitory activity against SMS1, while SMS1 was responsible for about 70% of the total SM synthase activity *in vivo*,[30] which correlates with the *in vivo* effects of compound **15w**.

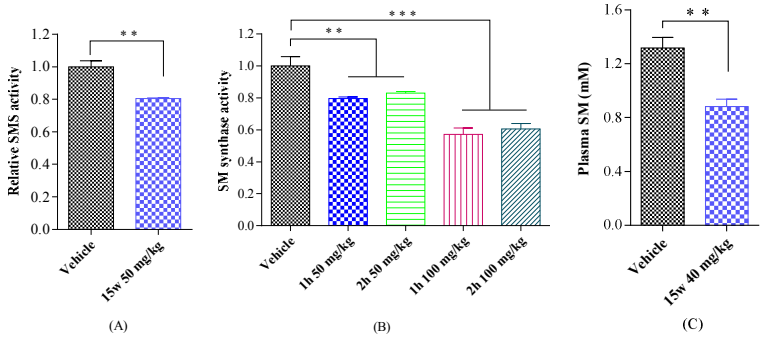


Figure 3. A) Relative SMS activities in liver tissues of ICR mice after a single oral dose of **15w** (50 mg/kg $n = 3$). B) Relative SM synthase activity in ICR mice after a single oral dose of **15w** (50 mg/kg or 100 mg/kg; $n = 5$). C) Plasma SM levels of ICR mice after intraperitoneal injection of **15w** (40 mg/kg; $n = 3$).(**) $P < 0.01$ versus vehicle. (***) $P < 0.001$ versus vehicle. Error bars indicate SEM.

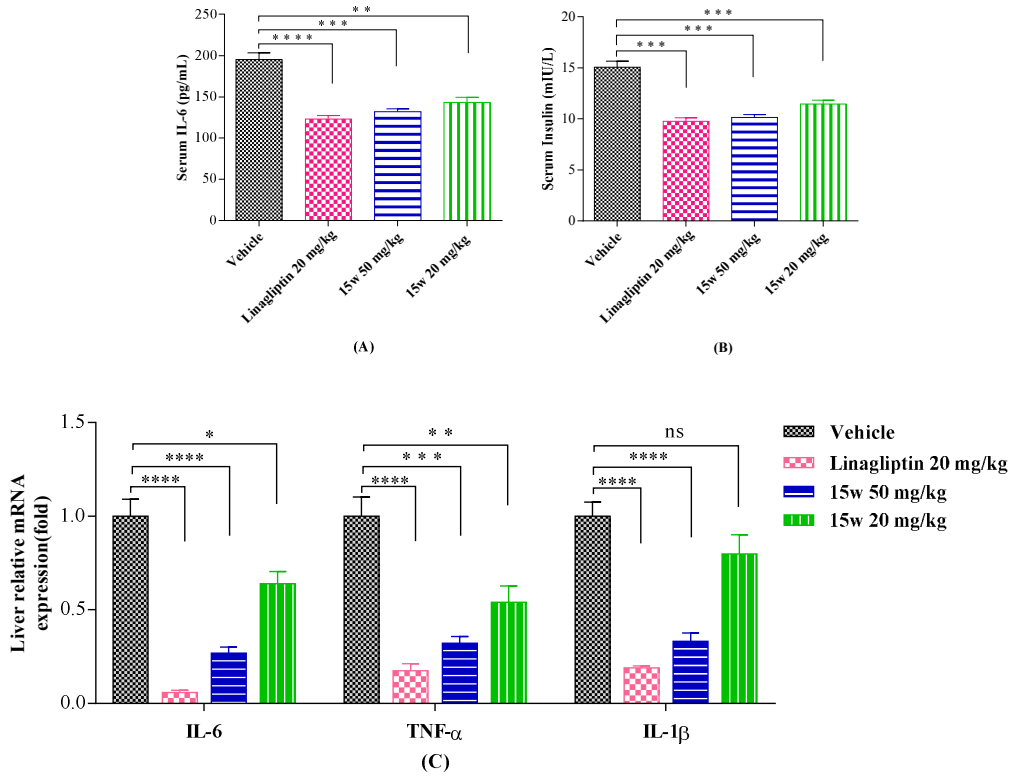
Effects of 15w on Reducing Chronic Inflammation in the *db/db* Mouse Model. T2DM is characterized by obesity, fast hyperglycemia and severe chronic inflammation.[31] The *db/db* mouse model, a T2DM model provides an ideal pathological model for evaluating whether SMS2-selective inhibition can have a potential therapeutic effect against chronic inflammatory responses. Elevated levels of

interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were observed in T2DM patients.[32-33] Pro-inflammatory cytokines IL-6, TNF-α and IL-1β were now regarded a key regulator in T2DM. IL-1β activates NF-κB pathways to generate other inflammatory mediators (TNF-α and IL-1β itself).[34] SMS2-KO macrophages were shown previously to attenuated NF-κB (downstream of TLRs) activation[35] by diminishing recruitment of the toll like receptor 4 (TLR4)-MD-2 complex on the surface of macrophages induced by LPS.[35] What's more, SMS2 was found to associated with caveolin 1 in lipid microdomains[13] and caveolin 1 induces the activation of NF-κB pathways by tumor necrosis factor receptor-associated factor-2 (TRAF-2).[36] In addition, a SMS2 inhibitor **Dy105** (SMS2 IC_{50}, 21 μM *vs* SMS1 57% @100 μM) was found to attenuate the activation of NF-κB.[21, 37] Inhibition of the NF-κB pathway and downstream inflammatory signaling pathways is a pharmacological strategy to reduce chronic inflammation in T2DM.[38] After daily oral administration of the compound (20 mg/kg/day or 50 mg/kg/day) for 6 weeks, compound **15w** reduced significantly IL-6 ($P < 0.005$) (Figure 4A) and insulin levels ($P < 0.001$) (Figure 4B). However, there was no significant change in fasting blood glucose levels during 4-6 weeks treated with 20 or 50 mg/kg/day of compound **15w** (Figure S1, details in the Supporting Information). It was in line with previous effects of SMS2-KO studies.[12, 18] To further confirm expression of pro-inflammatory cytokines, we measured the IL-6, TNF-α and IL-1β mRNA expression levels in both liver and white adipose tissues by real-time PCR. Data showed that compound **15w** decreased significantly the relative mRNA expression levels of pro-inflammatory

cytokines, such as IL-6, TNF-α and IL-1β, in both liver and adipose tissues (Figure 4C–D), except for IL-1β relative mRNA expression in liver treated with 20 mg/kg/day of compound **15w**. In addition, it was reported that knockdown of SMS2 improved TG accumulation in the liver of *ob/ob* mice by injection of SMS2 siRNA.[13] We found that compound **15w** markedly attenuated TG (Figure 4E), TC (Figure 4F), NEFA (Figure 4G) and LDL-C (Figure 4H) levels in the liver of *db/db* mice treated with 50 mg/kg/day of compound **15w** for 6 weeks. Moreover, compound **15w** also increased the HDL-C (Figure 4I) level in the liver of *db/db* mice treated with 50 mg/kg/day.

Furthermore, we found that compound **15w** reduced about 20% SM (d18:1/16:0) level but mildly changed ceramide (16:0) level (Figure S2, details in the Supporting Information) in adipose tissue of *db/db* mice treated with 20 mg/kg/day. The results for highly selective SMS2 inhibitor might be reasonable, because the SMS2 KO reduced SM level by about 20%, without significant effect on ceramide or PC levels.[13] Interestingly, both linagliptin and compound **15w** treatment improved similar effect on lipid metabolism such as TG, NEFA, LDL-C, with amelioration of inflammation. Linagliptin was reported to ameliorate inflammation via TLR2-MyD88 pathway,[39] another dipeptidyl peptidase-4 (DDP-4) inhibitor was also found to reduce the expression of TLR4,[40] and MyD88 is the adaptor protein of both TLR2 and TLR4. Those results suggested that both DDP-4 and SMS2 could regulate TLR signals to suppress inflammation. Additionally, silent of SMS1, SMSr but SMS2 were reported to induce cell apoptosis for the position of ceramide accumulation rather than the amounts.[30, 41-42] Compounds **15l** and **15w** showed to induce few apoptosis at least in

RAW264.7 cell with 1 and 5 μM (Figure S3, details in the Supporting Information), which correlates with their selectivity. Although further studies are required to understand the link between chronic inflammation, insulin, lipid metabolism and SMS2, these preliminary positive results provide a foundation to treat chronic inflammation and improve lipid metabolism disorder by a selective SMS2 inhibitor in *db/db* mice. These effects observed *in vivo* indicate that 4-benzyloxybenzo[*d*]isoxazole-3-amine derivatives should be valuable tools for further investigation of SMS2-relevant pathways.



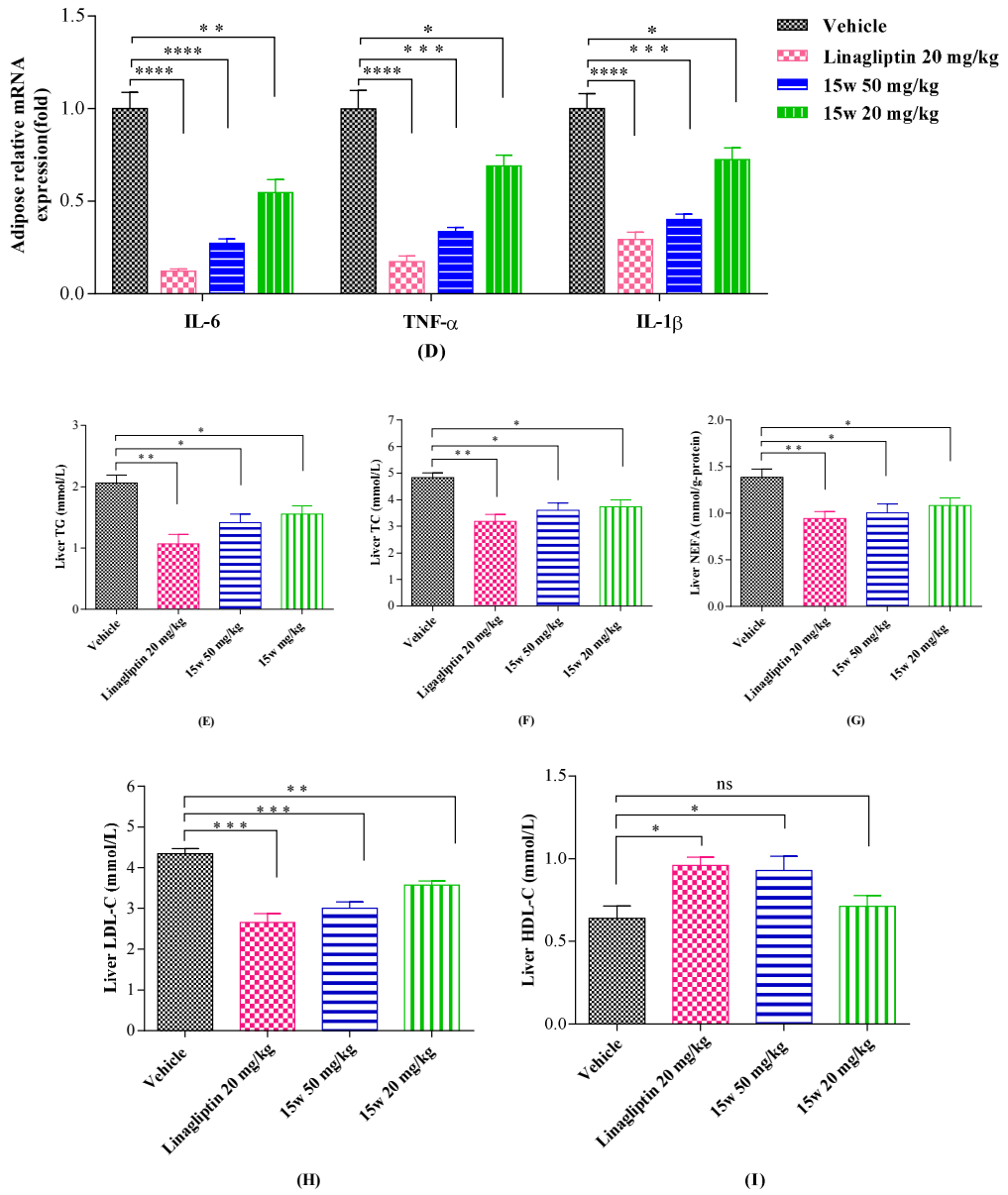


Figure 4. Treating chronic inflammation with compound **15w** (*n* = 4–5, 20 mg/kg or 50 mg/kg, po, 6 weeks) in *db/db* mice. Linagliptin and **15w** were formulated in 0.5% sodium carboxymethycellulose and the vehicle was 0.5% sodium carboxymethycellulose. (A) Serum IL-6 levels. (B) Serum insulin levels. (C) Relative mRNA expression of IL-6, TNF-α and IL-1β levels in the liver. (D) Relative mRNA expression of IL-6, TNF-α and IL-1β levels in adipose tissue. (E)–(I) Triglyceride

(TG), total cholesterol (TC), non-esterified fatty acid (NEFA), LDL-C and HDL-C levels in liver. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (****) $P < 0.0005$, ns = no significant difference versus the control. Error bars indicate SEM.

CONCLUSIONS

We have discovered 4-benzyloxybenzo[*d*]isoxazole-3-amine derivatives as potent SMS2 selective inhibitors. The IC_{50} values of particular 4-benzyloxybenzo[*d*]isoxazole-3-amine derivatives against SMS2 were < 0.10 μM and selectivity over SMS1 was > 500-fold. Among the derivatives, compound **15w** was found to be the most promising lead with an IC_{50} value of 0.10 μM against SMS2 and 56 μM against SMS1. In addition, compound **15w** inhibited SM synthase activity *in vivo*. These results suggest that 4-benzyloxybenzo[*d*]isoxazole-3-amine derivatives with reasonable physicochemical properties are potent and orally efficacious selective SMS2 inhibitors.

Previous studies have examined the effects of SMS2-KO on particular features of chronic inflammation-associated diseases, such as atherosclerosis, obesity, fatty liver and insulin resistance, but the report herein is the first to provide a study of a selective SMS2 inhibitor on chronic inflammation-associated T2DM in *db/db* mice. Compound **15w** decreased expression of pro-inflammatory cytokines significantly, such as IL-6, TNF-α and IL-1β, in both liver and adipose tissues, and improved lipid metabolism disorder after dosing 50 mg/kg/day for 6 weeks. Our study provides a foundation to further develop selective SMS2 inhibitors. Further studies on

inflammation-associated diseases are currently underway and the results will be presented in the near future.

EXPERIMENTAL SECTION

I*n vitro* Assay to Measure the Inhibitory Activity of SMS1 and SMS2. Activities against human SMS1 and SMS2 purified enzymes (unpublished work from Prof. Yu Cao, Institute of Precision Medicine, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine) were measured using C6-NBD-ceramide and 2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) as the substrates. (a) The inhibitory activity of SMS1 was measured as follows: 75 μL of the SMS1 enzyme (0.041 μg/μL) in n-dodecyl-beta-D-maltoside (DDM) (150 mM NaCl, 20 mM Hepes (pH 7.5) 1 mM DDM) and 5 μL of a DMSO solution of the compound were incubated at rt for 5 min. Then, 20 μL of the DDM solution of the two substrates, C6-NBD-ceramide and DMPC (1 μL of anhydrous ethanol solution of DMPC (40 mmol/L) and 1 μL of the DMSO solution of C6-NBD-ceramide (1.16 mmol/L) diluted with 18 μL of DDM), were added and mixed in a total volume of 100 μL. After incubating at 37 °C for 30 min, the reaction was quenched with 200 μL of anhydrous ethanol. The mixture was then centrifuged at 7178 g for 10 min to produce the supernatant collected for the HPLC test.[43] (b) The inhibitory activity of SMS2 was measured as follows: the assay was similar to the assay used for measuring SMS1 activity, except 79 μL of the SMS2 enzyme (0.0041 μg/μL) in DDM and 1 μL of a DMSO solution of the compound were used. Inhibition activity was determined by

the following equation:

$$\text{Inhibition (\%)} = \frac{A_{blank} - A_{compound}}{A_{blank}} \times 100\% \quad (1)$$

A_{blank}: AUC of C6-NBD-SM contained DMSO without test compound.

$A_{compound}$: AUC of C6-NBD-SM containing the test compound.

Docking Protocols. A published three-dimensional structure of human sphingomyelin synthase 1 (*h*SMS1)[44] and a *h*SMS2 homology model derived from *h*SMS1, which was optimized through molecular dynamics and proved rationality (unpublished work),[21] were used as the receptor structures. The active site was defined according to the HHD.[1, 44-45] Water molecules and ions were deleted. The receptor was prepared using Protein Preparation and Induced Fit Docking tools in the Schrödinger Maestro interface. The docked conformation of the compound with the lowest energy was selected for the study.

Animals. Male ICR mice (from the Shanghai SLAC Laboratory Animal Co. Ltd. Shanghai, China) and 4–5 weeks old B6.Cg-m +/+ *Lepr*^{db}/J(*db/db*) mice (Jackson Laboratory, Bar Harbor, ME, USA) were bred at Fudan University. The animals were maintained under a 12 h light-dark cycle with free access to water and food. Animal experiments were approved by the Animal Care and Use Committee, Fudan University.

Pharmacokinetic Studies. Test compounds were formulated for intravenous (10 mg/kg) in 5% DMSO/5% Polyoxyl 35 Castor oil in saline and oral administrations (20 mg/kg) in 0.5% carboxymethylcellulose sodium, which were subjected to pharmacokinetic studies on male ICR mice with four animals in each group. Serial

specimens were collected pre-dose 5, 15 and 30 min and 1, 3, 5, 7 and 24 h following intravenous administration, as well as 0.5, 1, 2, 3, 5, 7, 12 and 24 h after administration, and quantified by LC-MS/MS. Pharmacokinetic parameters were calculated from the mean serum concentration by WinNonlin Prefessional Edition Version 2.1.

Assay to Measure the Inhibitory Activity of SMSs *In Vivo.* Compound **15w** was formulated in 0.5% sodium carboxymethylcellulose and the vehicle was formulated in 0.5% sodium carboxymethylcellulose without the test compound for oral administration.The vehicle was formulated in 5% DMSO/5% Cremophor EL/0.9% NaCl aqueous solution for intraperitoneal injection. A group of three male ICR mice were fasted overnight and administered test compounds (50 mg/kg). After 2 h, the whole livers were obtained from ICR mice and homogenized, centrifugation to get supernatant as a SMS solution in buffer (0.25 M sucrose, 50 mM Tris HCl, pH 7.4, 1 mM EDTA) and SMS activity assays were conducted according to the method for sphingomyelin synthase activity using HPLC analysis of C6-NBD-Cer and C6-NBD-SM. The detailed instructions have been described previously.[28] A group of five male ICR mice were fasted overnight and administered test compounds (50 mg/kg or 100 mg/kg) by gavage. Compound **15w** was formulated in 0.5% sodium carboxymethylcellulose and the vehicle was 0.5% sodium carboxymethylcellulose without the test compound. C6-NBD-ceramide (in 5% ethanol/5% Cremophor EL/0.9% NaCl aqueous solution) was injected into the tail vein at pre-dose, and at 1 and 2 h after administration. After 5 min, blood samples were drawn to measure SM synthase

activity.[43] SM synthase activity of ICR mice was measured according to the method described for measurement of SMS1 and SMS2 inhibitory activity *in vitro*. Daily intraperitoneal injection of test compounds (40 mg/kg) into a group of three male ICR mice (20-23g) for 7 days, and plasma samples were drawn to measure SM levels by the enzymatic measurement reported previously.[29]

Effects of 15w on Reducing Chronic Inflammation in the *db/db* Mouse Model. Four-to-five week old *db/db* mice were divide randomly into four groups (n = 4–5 mice/group). Compound **15w** (20 mg/kg/day, 50 mg/kg/day), Linagliptin (20 mg/kg/day) or vehicle (0.5% sodium carboxymethylcellulose) was orally administered for 6 weeks. Mice serum was obtained from mice eyes after 6 h fasting. Mouse plasma IL-6 and insulin amounts were determined using commercially available ELISA kits (Affymetrix eBioscience, CUSABIO) and performed according to the manufacturer's instructions. Total RNA extraction from liver or white adipose tissue using the Trizol reagent and real-time quantitative PCR was performed on SYBR Green labeling (Bio-Rad, Hercules, CA, USA) following detailed instructions that have been described previously[39] and specific primers (see Supporting Information). The levels of TG, TC, NEFA, LDL-C and HDL-C in liver tissues were determined using a triglyceride kit, a total cholesterol kit, a non-esterified fatty acid kit, a LDL-C kit and a HDL-C-kit, respectively, all of which were purchased from the Nanjing Jiancheng Bioengineering Institute (China).[46]

Synthetic Materials and Methods. All reagents were purchased from commercial suppliers and used without further purification unless otherwise stated.

Yields were not optimized. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC400 or a Bruker AC600 NMR spectrometer using tetramethylsilane as an internal reference. Low-resolution mass spectra were determined on an Agilent liquid-chromatography mass spectrometer system that consisted of an Agilent 1260 infinity LC coupled to an Agilent 6120 Quadrupole mass spectrometer (electrospray positive ionization; ESI). High-resolution mass spectra were conducted on a triple TOF 5600^{+} MS/MS system (AB Sciex, Concord, Ontario, Canada) in the positive ESI mode. The purity of test compounds was determined by HPLC (Agilent ChemStation 1260, Waters XBridge Shield RP C18, 5 μm, 2.1 × 50 mm, 40 °C, UV 254 nM, flow rate = 0.25 mL/min), eluting with mixtures of water (containing 0.1% HCOOH)/methanol. The ratio of mobile methanol was increased linearly from 40 to 95% over 1 min and then maintained at 95% over the next 3 min, the methanol was decreased linearly from 95 to 40% over 3.2 min, followed by re-equilibration at 40% methanol for 1.8 min. All the assayed compounds possess ≥95% purity. Column chromatography was performed on silica gel (300−400 mesh) and preparative TLC was performed on HSGF 254 (0.4–0.5 mm thickness; Yantai Jiangyou Company, Yantai, Shangdong, China).

2-(Benzyloxy)-6-fluorobenzonitrile (2). To a mixture of K_2CO_3 (10.07 g, 73.0 mmol), KI (200 mg) and 2-fluoro-6-hydroxybenzonitrile (5.00 g, 36.5 mmol) in acetonitrile (75 mL) was added bromomethyl benzene (6.55 g, 38.3 mmol), the whole was stirred at rt overnight. After the reaction was complete, most of the solution was removed under reduced pressure, the residue was added water, and extracted with EA

(40 mL × 3), the organic layers were combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified with column chromatography (PE/EA = 10:1) to yield **2** (7.90 g, 95%) as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.73 – 7.62 (m, 1H), 7.43 (d, J = 7.0 Hz, 2H), 7.38 (t, J = 7.4 Hz, 2H), 7.32 (d, J = 7.1 Hz, 1H), 7.16 (d, J = 8.7 Hz, 1H), 7.03 (t, J = 8.8 Hz, 1H), 5.27 (s, 2H). MS (ESI): *m/z* 228 $[M+H]^+$.

4-(Benzyloxy)benzo[*d*]isoxazol-3-amine (3). To a solution of acetohydroxamic acid (0.99 g, 13.2 mmol) in dry DMF (30 mL) was added t-BuOK (1.48 g, 13.2 mmol), the whole was stirred at rt for 0.5h. Then, 2-(benzyloxy)-6-fluorobenzonitrile (2.00 g, 8.8 mmol) was added. After addition 5 h of stirring, the reaction was poured into a mixture of brine and extracted with EA (40 mL × 4), the organic layers were combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified with column chromatography (PE/EA = 4:1) to yield **3** (0.74 g, 35%) as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.50 (d, J = 6.8 Hz, 2H), 7.42 – 7.34 (m, 3H), 7.34 – 7.28 (m, 1H), 6.96 (d, J = 8.4 Hz, 1H), 6.76 (d, J = 8.0 Hz, 1H), 5.86 (s, 2H), 5.29 (s, 2H). MS (ESI): *m/z* 241 $[M+H]^+$.

4-(Benzyloxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (3a). A mixture of 3-bromopyridine (494 mg, 3.1 mmol), **6** (500 mg, 2.1 mmol), $Pd_2(dba)_3$ (191 mg, 0.21 mmol), Xantphos (241 mg, 0.42 mmol) and K_2CO_3 (574 mg, 4.2 mmol) in dioxane under N_2 was stirred at 125 °C for 15 h. After the reaction was complete, the whole was filtered, washed with EA, the filtrate was concentrated under reducing pressure, the residue was extracted with EA (40 mL × 3), the organic layers were combined,

washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified with column chromatography (PE/EA = 3:1) to yield **3a** (400 mg, 60%) as white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.73 (d, J = 2.8 Hz, 1H), 8.28 (s, 1H), 8.18 (dd, J = 4.7, 1.4 Hz, 1H), 8.05 (dd, J = 7.9, 1.8 Hz, 1H), 7.54 (d, J = 7.5 Hz, 2H), 7.47 (t, J = 8.2 Hz, 1H), 7.43 – 7.34 (m, 3H), 7.31 (t, J = 7.2 Hz, 1H), 7.11 (d, J = 8.4 Hz, 1H), 6.85 (d, J = 8.0 Hz, 1H), 5.41 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.00, 154.63, 153.23, 142.26, 139.72, 136.78, 136.34, 132.47, 128.41(2C), 127.90, 127.32(2C), 124.25, 123.49, 105.87, 105.08, 102.23, 69.70. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{19}H_{16}N_3O_2$: 318.1237, found: 318.1237. HPLC purity 99.2%.

4-(Benzyloxy)benzo[*d*]isothiazol-3-amine (4). A mixture of Na_2S (78 mg, 1.0 mmol) and **2** (228 mg, 1.0 mmol) in DMSO under N_2 was stirred at 70°C for 12h. Then, whole was cooled down to 0°C and dropped with 25% ammonium hydroxide (1.4 mL) and 15% NaClO solution (1.4 mL). Then, the whole was warmed to rt slowly and stirred for another 5h. After the reaction was complete, the whole was diluted with water and extracted with EA (30 mL × 3), the organic layers were combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified with column chromatography (PE/EA = 4:1) to yield **4** (200 mg, 78%) as light yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.50 (s, 1H), 7.48 (s, 1H), 7.42 – 7.32 (m, 4H), 7.36 – 7.27 (m, 1H), 6.89 (d, J = 7.4 Hz, 1H), 6.45 (s, 2H), 5.28 (s, 2H). MS (ESI): *m/z* 257 $[M+H]^+$.

4-(Benzyloxy)-*N*-(pyridin-3-yl)benzo[*d*]isothiazol-3-amine (4a). The title compound was obtained as a white solid from **4** according to the procedure of **3a** in

54% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 9.03 (s, 1H), 8.44 (d, J = 2.7 Hz, 1H), 8.23 (dd, J = 8.6, 1.8 Hz, 1H), 8.12 (dd, J = 4.7, 1.8 Hz, 1H), 7.62 (d, J = 7.2 Hz, 2H), 7.58 (d, J = 8.1 Hz, 1H), 7.53 – 7.38 (m, 4H), 7.30 (dd, J = 8.6, 4.7 Hz, 1H), 7.07 (d, J = 7.8 Hz, 1H), 5.42 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 154.83, 154.04, 152.38, 142.16, 138.97, 136.80, 135.95, 130.28, 128.64(2C), 128.44, 128.19(2C), 123.47, 123.21, 116.47, 113.03, 106.73, 70.40. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{19}H_{16}N_3OS$: 334.1009, found: 334.1014. HPLC purity 98.3%.

4-(Benzyloxy)-*1H*-indazol-3-amine (5). A mixture of **2** (1.0 g, 4.4 mmol) in 85% hydrazine hydrate (4 mL) and EtOH (10 mL) was stirred at 100°C overnight. After the reaction was complete, the whole was concentrated. The residue was added 5 mL water and stirred at rt for 15 minutes; then, the suspension was filtered washed with water to obtain **5** (840 mg, 80%) as a white solid, which was dry under infrared light. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 11.39 (s, 1H), 7.47 (d, J = 7.5 Hz, 2H), 7.37 (t, J = 7.5 Hz, 2H), 7.29 (t, J = 7.3 Hz, 1H), 7.04 (t, J = 8.0 Hz, 1H), 6.73 (d, J = 8.3 Hz, 1H), 6.35 (d, J = 7.7 Hz, 1H), 5.17 (s, 2H), 4.90 (s, 2H). MS (ESI): *m/z* 240 $[M+H]^+$.

2-(4-(Benzyloxy)-*1H*-indazol-3-yl)isoindoline-1,3-dione (6). A mixture of phthalic anhydride (148 mg, 1.0 mmol) and **5** (240 mg, 1.0 mmol) was heated to 170°C for 0.5 h. Then, the whole cooled down to rt and added EA to the residue. The resulted suspension was filtered to obtain **6** (185 mg, 50%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 13.46 (s, 1H), 7.93 – 7.83 (m, 4H), 7.32 (t, J = 8.0 Hz, 1H), 7.15 (d, J = 8.4 Hz, 1H), 7.08 – 6.97 (m, 3H), 6.92 (t, J = 7.6 Hz, 2H), 6.65 (d, J

= 7.7 Hz, 1H), 4.96 (s, 2H). MS (ESI): *m/z* 370 $[M+H]^+$.

***Tert*-butyl-4-(benzyloxy)-3-(1,3-dioxoisoindolin-2-yl)-*1H*-indazole-1-carboxyl ate (7).** A mixture of **6** (200 mg, 0.5 mmol), DMAP (198 mg, 1.6 mmol) and $(Boc)_2O$ (142 mg, 0.6 mmol) in DCM was stirred at rt overnight. The whole was diluted with water and extracted with DCM (20 mL × 3), the organic layers were combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified with column chromatography (PE/EA = 6:1) to yield **7** (80 mg, 31%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.85 (s, 4H), 7.71 (d, J = 8.5 Hz, 1H), 7.61 (t, J = 8.2 Hz, 1H), 7.04 (t, J = 7.2 Hz, 1H), 7.02 – 6.96 (m, 3H), 6.94 (t, J = 7.5 Hz, 2H), 4.99 (s, 2H), 1.63 (s, 9H). MS (ESI): *m/z* 470 $[M+H]^+$.

2-(4-(Benzyloxy)-1-methyl-*1H*-indazol-3-yl)isoindoline-1,3-dione (8). To a mixture of **6** (150 mg, 0.4 mmol) and K_2CO_3 (112 mg, 0.8 mmol) in DMF (5 mL) was added CH_3I (75 mg, 0.5 mmol), the whole was stirred at rt for 3 h. The whole was diluted with water and extracted with DCM (20 mL × 3), the organic layers were combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified with column chromatography (PE/EA = 4:1) to yield **8** (100 mg, 64%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.77 (t, J = 1.8 Hz, 4H), 7.31 – 7.23 (m, 1H), 7.16 (dd, J = 8.6, 1.8 Hz, 1H), 6.95 – 6.86 (m, 3H), 6.86 – 6.77 (m, 2H), 6.58 (dd, J = 7.6, 1.7 Hz, 1H), 4.86 (s, 2H), 3.94 (s, 3H). MS (ESI): *m/z* 384$[M+H]^+$.

***Tert*-butyl 3-amino-4-(benzyloxy)-*1H*-indazole-1-carboxylate (9).** A mixture of

7 (50 mg, 0.1 mmol) and 85% hydrazine hydrate (7 mg, 0.2 mmol) in EtOH (3 mL) was stirred at rt for 5 h. The whole was diluted with water and extracted with DCM (20 mL × 3), the organic layers were combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified with column chromatography (PE/EA = 4:1) to yield **9** (20 mg, 56%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.51 – 7.42 (m, 3H), 7.40 – 7.32 (m, 3H), 7.33 – 7.27 (m, 1H), 6.78 (d, J = 7.9 Hz, 1H), 5.81 (s, 2H), 5.26 (s, 2H), 1.52 (s, 9H). MS (ESI): *m/z* 340$[M+H]^+$.

4-(Benzyloxy)-1-methyl-*1H*-indazol-3-amine (10). The title compound was obtained as a white solid from **8** according to the procedure of **9** in 70% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 7.47 (d, J = 7.6 Hz, 2H), 7.37 (dd, J = 8.4, 6.7 Hz, 2H), 7.29 (t, J = 7.2 Hz, 1H), 7.08 (t, J = 8.0 Hz, 1H), 6.82 (d, J = 8.4 Hz, 1H), 6.36 (d, J = 7.6 Hz, 1H), 5.18 (s, 2H), 4.97 (s, 2H), 3.63 (s, 3H). MS (ESI): *m/z* 254$[M+H]^+$.

***Tert*-butyl-4-(benzyloxy)-3-(pyridin-3-ylamino)-*1H*-indazole-1-carboxylate (11).** The title compound was obtained as a white solid from **9** according to the procedure of **3a** in 54% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.74 (s, 1H), 8.18 (s, 1H), 8.15 (d, J = 2.4 Hz, 1H), 8.14 – 8.10 (m, 1H), 7.55 (d, J = 7.5 Hz, 2H), 7.52 – 7.43 (m, 2H), 7.40 (t, J = 7.5 Hz, 2H), 7.37 – 7.29 (m, 2H), 6.89 (d, J = 7.5 Hz, 1H), 5.40 (s, 2H), 1.59 (s, 9H). MS (ESI): *m/z* 417$[M+H]^+$.

4-(Benzyloxy)-*N*-(pyridin-3-yl)-*1H*-indazol-3-amine (11a). A mixture of **11** (50

mg, 0.1 mmol) in DCM (5 mL) and TFA (0.3 mL) was stirred at rt for 2h. The whole was diluted with water and extracted with DCM (10 mL × 3), the organic layers were combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified with column chromatography (PE/EA = 3:1) to yield **11a** (20 mg, 53%) as a light yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.16 (s, 1H), 8.54 (d, J = 2.7 Hz, 1H), 7.98 (d, J = 4.6 Hz, 1H), 7.96 – 7.91 (m, 1H), 7.86 (s, 1H), 7.48 (d, J = 7.4 Hz, 2H), 7.36 (t, J = 7.4 Hz, 2H), 7.30 (d, J = 7.1 Hz, 1H), 7.23 (dd, J = 8.4, 4.7 Hz, 1H), 7.16 (t, J = 8.0 Hz, 1H), 6.89 (d, J = 8.3 Hz, 1H), 6.49 (d, J = 7.7 Hz, 1H), 5.27 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 152.58, 143.57, 142.33, 139.82, 139.08, 137.71, 136.80, 128.36, 128.33(2C), 127.75, 127.26(2C), 123.38, 121.42, 105.24, 102.65, 99.78, 69.20. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{19}H_{17}N_4O$: 317.1397, found: 317.1397. HPLC purity 97.4%.

4-(Benzyloxy)-1-methyl-*N*-(pyridin-3-yl)-*1H*-indazol-3-amine (11b). The title compound was obtained as a white solid from **10** according to the procedure of **3a** in 57% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.56 (d, J = 2.7 Hz, 1H), 8.00 (d, J = 4.7 Hz, 1H), 7.95 (dt, J = 8.5, 2.0 Hz, 1H), 7.90 (s, 1H), 7.47 (d, J = 7.4 Hz, 2H), 7.36 (t, J = 7.4 Hz, 2H), 7.30 (d, J = 7.2 Hz, 1H), 7.27 – 7.17 (m, 2H), 7.00 (d, J = 8.4 Hz, 1H), 6.51 (d, J = 7.7 Hz, 1H), 5.29 (s, 2H), 3.84 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 152.64, 142.61, 142.02, 140.03, 138.86, 137.86, 136.76, 128.46, 128.34(2C), 127.76, 127.25(2C), 123.37, 121.43, 105.48, 101.98, 99.83, 69.25, 35.05. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{19}N_4O$: 331.1553, found: 331.1555. HPLC purity 96.8%.

4-(Benzyloxy)-N,1-di(pyridin-3-yl)-*1H*-indazol-3-amine (11c). The title compound was obtained as a white solid from **5** according to the procedure of **3a** in 80% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 9.02 (d, J = 2.6 Hz, 1H), 8.70 (d, J = 2.7 Hz, 1H), 8.45 (dd, J = 4.7, 1.4 Hz, 1H), 8.19 (dd, J = 8.4, 2.8, 1.4 Hz, 1H), 8.16 (s, 1H), 8.14 (dd, J = 2.8, 1.4 Hz, 1H), 8.09 (dd, J = 4.7, 1.4 Hz, 1H), 7.56 (d, J = 6.9 Hz, 2H), 7.54 – 7.51 (m, 1H), 7.42 – 7.36 (m, 4H), 7.35 – 7.30 (m, 2H), 6.74 (dd, J = 5.5, 2.8 Hz, 1H), 5.39 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 152.99, 146.04, 145.67, 141.44, 141.03, 140.03, 138.61, 137.72, 136.64, 136.53, 130.60, 128.45(2C), 127.95, 127.44, 127.35(2C), 124.14, 123.54, 122.64, 107.60, 103.00, 102.63, 69.60. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{24}H_{20}N_5O$: 394.1662, found: 394.1659. HPLC purity 98.3%.

8-Benzyloxy isoquinoline (12). The title compound was obtained as a white solid from isoquinolin-8-ol according to the procedure of 2-(benzyloxy)-6-fluorobenzonitrile (**2**) in 90% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 10.70 (s, 1H), 7.48 (d, J = 7.6 Hz, 2H), 7.39 (t, J = 8.0 Hz, 1H), 7.24 (t, J = 7.5 Hz, 2H), 7.14 (t, J = 7.3 Hz, 1H), 6.99 (d, J = 7.9 Hz, 1H), 6.94 (t, J = 6.5 Hz, 1H), 6.87 (d, J = 8.1 Hz, 1H), 6.25 (d, J = 7.0 Hz, 1H), 5.07 (s, 2H). MS (ESI): *m/z* 236$[M+H]^+$.

8-Benzyloxy isoquinoline 2-oxide (13). A mixture of **12** (290 mg, 1.2 mmol) and *m*-CPBA (255 mg, 1.5 mmol) in DCM (5 mL) was stirred at rt overnight. Then the reaction was quenched with Sat.Na_2CO_3 solution, diluted with water and extracted with EA (20 mL × 3), the organic layers were combined, washed with brine, dried

over Na_2SO_4, filtered, and concentrated. The residue was purified with column chromatography (PE/EA = 1:1) to yield **13** (260 mg, 84%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.61 (t, J = 1.2 Hz, 1H), 8.02 (dd, J = 7.1, 1.8 Hz, 1H), 7.76 (d, J = 7.1 Hz, 1H), 7.43 – 7.34 (m, 4H), 7.28 (t, J = 7.4 Hz, 2H), 7.21 (t, J = 7.2 Hz, 1H), 7.12 – 7.06 (m, 1H), 5.18 (s, 2H). MS (ESI): *m/z* 252$[M+H]^+$.

8-Benzyloxy-1-chloroisoquinoline (14). A mixture of **13** (200 mg, 0.8 mmol) in $POCl_3$ (1.5 mL) was heated to 90°C for 5 h. After the reaction was complete, most of the solution was removed under reduced pressure, the residue was added water, added Sat.Na_2CO_3 to pH=8-9, and then extracted with DCM (20 mL × 3), the organic layers were combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated to give crude product. The product was then taken on to the next reaction immediately without further purification.

8-Benzyloxy-1-chloroisoquinoline (14a). The title compound was obtained as a white solid from **14** and pyridin-3-amine according to the procedure of **3a** in 30% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 10.01 (s, 1H), 8.19 (d, J = 2.6 Hz, 1H), 8.14 (dd, J = 8.6, 2.4 Hz, 1H), 8.08 (d, J = 4.0 Hz, 1H), 7.97 (d, J = 5.7 Hz, 1H), 7.72 – 7.64 (m, 2H), 7.63 (t, J = 8.0 Hz, 1H), 7.54 – 7.45 (m, 3H), 7.40 (d, J = 8.0 Hz, 1H), 7.27 (d, J = 7.8 Hz, 1H), 7.23 (dd, J = 8.3, 4.7 Hz, 1H), 7.13 (d, J = 5.7 Hz, 1H), 5.40 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 154.94, 151.84, 142.09, 140.70, 140.45, 139.33, 136.95, 135.52, 130.65, 128.94(2C), 128.80(3C), 125.23, 123.14, 119.66, 112.41, 109.36, 108.28, 71.31. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{21}H_{18}N_3O$: 328.1444, found: 328.1447. HPLC purity 95.3%.

3-(Pyridin-3-ylamino)benzo[*d*]isoxazol-4-ol (15). A mixture of **3a** (3.10 g, 9.8 mmol) in 40% HBr aqueous solution (10 mL) and HOAc (10 mL) was heated to 65°C overnight. After the reaction was complete, most of the solution was removed under reduced pressure, the residue was added water, added Sat.Na_2CO_3 to pH=8-9, and then extracted with DCM (50 mL × 3), the organic layers were combined, washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue was purified with column chromatography (DCM/MeOH = 25:1) to yield **16** (1.55 g, 70%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 11.20 (s, 1H), 8.90 (d, J = 2.7 Hz, 1H), 8.35 (s, 1H), 8.20 – 8.13 (m, 2H), 7.40 – 7.32 (m, 2H), 6.97 (d, J = 8.3 Hz, 1H), 6.65 (d, J = 7.8 Hz, 1H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.19, 154.75, 153.05, 142.04, 140.00, 136.99, 132.24, 124.41, 123.32, 107.25, 105.08, 100.03. MS (ESI): *m/z* 228[M+H]$^+$.

General Synthetic Procedure for 15a-y. To a solution of **15** (0.3 mmol) and corresponding benzyl bromide/benzyl chloride (0.4 mmol) in acetone (4 mL) was added K_2CO_3 (0.6 mmol) at rt for 5 h. Then diluted with water, and extracted with ethyl acetate (15 mL × 3), washed with brine, dried over Na_2SO_4, filtered, and concentrated. The residue obtained was purified with column chromatography (PE/EA = 5:1 to 1:1) to yield the desired compound.

4-((2-Methylbenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15a). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-2-methylbenzene according to the general procedure in 60% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.54 (s, 1H), 8.17 – 7.98 (m, 2H), 7.89 (d, J

= 8.4 Hz, 1H), 7.34 (d, *J* = 8.0 Hz, 2H), 7.29 – 7.17 (m, 1H), 7.09 (s, 3H), 6.99 (d, *J* = 8.4 Hz, 1H), 6.75 (d, *J* = 8.0 Hz, 1H), 5.28 (s, 2H), 2.24 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 162.99, 154.53, 153.23, 142.18, 139.38, 136.72, 135.98, 133.98, 132.47, 130.16, 127.99, 127.92, 125.66, 124.06, 123.51, 105.81, 105.00, 102.21, 68.37, 18.33. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{18}N_3O_2$: 332.1394, found: 332.1393. HPLC purity 96.2%.

4-((3-Methylbenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15b). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-3-methylbenzene according to the general procedure in 67% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.71 (d, *J* = 2.7 Hz, 1H), 8.28 (s, 1H), 8.16 (d, *J* = 4.6 Hz, 1H), 8.03 (d, *J* = 8.4 Hz, 1H), 7.46 (t, *J* = 8.2 Hz, 1H), 7.35 (q, *J* = 5.3 Hz, 2H), 7.30 (d, *J* = 7.7 Hz, 1H), 7.25 (t, *J* = 7.5 Hz, 1H), 7.10 (d, *J* = 8.1 Hz, 2H), 6.82 (d, *J* = 8.0 Hz, 1H), 5.35 (s, 2H), 2.26 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 162.97, 154.61, 153.31, 142.23, 139.63, 137.58, 136.76, 136.28, 132.49, 128.53, 128.31, 127.75, 124.33, 124.14, 123.48, 105.89, 105.08, 102.22, 69.84, 20.85. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{18}N_3O_2$: 332.1394, found: 332.1396. HPLC purity 97.6%.

4-((4-Methylbenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15c). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-4-methylbenzene according to the general procedure in 65% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.73 (s, 1H), 8.27 (s, 1H), 8.17 (s, 1H), 8.05 (d, *J* = 8.3 Hz, 1H), 7.44 (t, *J* = 8.2 Hz, 3H), 7.37 (d, *J* = 7.5 Hz, 1H), 7.17 (d, *J* = 7.6

Hz, 2H), 7.10 (d, J = 8.4 Hz, 1H), 6.83 (d, J = 7.9 Hz, 1H), 5.36 (s, 2H), 2.25 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 162.96, 154.62, 153.24, 142.25, 139.71, 137.18, 136.75, 133.27, 132.43, 128.94(2C), 127.41(2C), 124.25, 123.47, 105.87,105.14, 102.14, 69.66, 20.61. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{18}N_3O_2$: 332.1394, found: 332.1394. HPLC purity 96.2%.

4-((2,6-Dimethylbenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15d). The title compound was obtained as a white solid from **15** and 2-(bromomethyl)-1,3-dimethylbenzene according to the general procedure in 45% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.27 (d, J = 2.7 Hz, 1H), 8.11 (d, J = 4.6 Hz, 1H), 7.79 (d, J = 5.5 Hz, 2H), 7.56 (t, J = 8.2 Hz, 1H), 7.29 (dd, J = 8.5, 4.7 Hz, 1H), 7.16 (dd, J = 7.8, 5.8 Hz, 2H), 7.08 (d, J = 7.5 Hz, 2H), 7.01 (d, J = 8.0 Hz, 1H), 5.34 (s, 2H), 2.36 (s, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 162.91, 154.44, 153.75, 142.22, 138.84, 137.55, 136.39, 132.68, 131.94, 128.56, 128.11(2C), 123.63, 123.58(2C), 105.75, 105.00, 102.44, 65.72, 19.14(2C). HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{21}H_{20}N_3O_2$: 346.1550, found: 346.1551. HPLC purity 98.2%.

4-((2-Fluorobenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15e). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-2-fluorobenzene according to the general procedure in 50% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.67 (d, J = 2.7 Hz, 1H), 8.20 (s, 1H), 8.15 (d, J = 4.6 Hz, 1H), 8.00 (d, J = 8.5 Hz, 1H), 7.60 (t, J = 7.6 Hz, 1H), 7.48 (t, J = 8.2 Hz, 1H), 7.35 (td, J = 9.2, 8.7, 5.6 Hz, 2H), 7.21 (dt, J = 15.3, 8.6 Hz, 2H), 7.12 (d, J = 8.6 Hz, 1H), 6.89 (d, J = 8.1 Hz, 1H), 5.44 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6)

δ (ppm) 162.99, 160.04 (d, J = 245.8 Hz), 154.52, 152.92, 142.25, 139.53, 136.69, 132.53, 130.38, 130.10, 124.49, 124.13, 123.50, 123.00(d, J = 14.1 Hz), 115.35(d, J = 20.9 Hz), 105.93, 104.92, 102.57, 64.23. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{19}H_{15}FN_3O_2$: 336.1143, found: 336.1145. HPLC purity 98.6%.

4-((3-Fluorobenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15f). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-3-fluorobenzene according to the general procedure in 55% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.75 (s, 1H), 8.34 (s, 1H), 8.16 (dd, J = 4.7, 1.5 Hz, 1H), 8.06 (dd, J = 8.4, 1.5 Hz, 1H), 7.48 (d, J = 8.2 Hz, 1H), 7.46 – 7.39 (m, 2H), 7.36 (dd, J = 8.7, 3.9 Hz, 2H), 7.12 (t, J = 8.4 Hz, 2H), 6.82 (d, J = 8.0 Hz, 1H), 5.41 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.06, 162.11 (d, J = 243.6 Hz), 154.62, 153.01, 142.24, 139.79, 139.29, 139.24, 136.86, 132.47, 130.47, 130.41, 124.35, 123.47, 123.24, δ (ppm) 114.65 (d, J = 20.9 Hz), 114.05 (d, J = 22.0 Hz),105.91, 105.01, 102.40, 68.89. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{19}H_{15}FN_3O_2$: 336.1143, found: 336.1145. HPLC purity 96.1%.

4-((4-Fluorobenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15g). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-4-fluorobenzene according to the general procedure in 63% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.71 (s, 1H), 8.25 (s, 1H), 8.14 (d, J = 4.6 Hz, 1H), 8.02 (d, J = 8.4 Hz, 1H), 7.65 – 7.51 (m, 2H), 7.43 (t, J = 8.2 Hz, 1H), 7.33 (dd, J = 8.3, 4.7 Hz, 1H), 7.18 (t, J = 8.7 Hz, 2H), 7.08 (d, J = 8.4 Hz, 1H), 6.81 (d, J = 8.1 Hz, 1H), 5.35 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.02, 162.68 (d, J =

244.0Hz),154.59, 153.06, 142.18, 139.67, 136.80, 132.50, 132.43, 129.70, 129.65, 124.39, 123.48, 115.28, 115.14, 105.87, 105.04, 102.27, 68.97. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{19}H_{15}FN_3O_2$: 336.1143, found: 336.1143. HPLC purity 95.5%.

4-((2,6-Difluorobenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15h). The title compound was obtained as a white solid from **15** and 2-(bromomethyl)-1,3-difluorobenzene according to the general procedure in 63% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.60 (s, 1H), 8.19 (d, J = 4.3 Hz, 1H), 8.11 (s, 1H), 7.97 (d, J = 8.2 Hz, 1H), 7.66 – 7.48 (m, 2H), 7.37 (dd, J = 8.3, 4.4 Hz, 1H), 7.26 – 7.14 (m, 3H), 7.07 (d, J = 7.9 Hz, 1H), 5.49 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 162.98,160.99(d, J = 248.0Hz, 2C), 154.38, 152.73, 142.17, 139.28, 136.64, 132.62, 131.75, 124.08, 123.57, 111.82, 111.66, 111.41,106.03, 105.05, 102.93, 58.98. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{19}H_{14}F_2N_3O_2$: 354.1049, found: 354.1047. HPLC purity 97.2%.

4-((2-Chlorobenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15i). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-2-chlorobenzene according to the general procedure in 40% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.67 (s, 1H), 8.23 (s, 1H), 8.15 (s, 1H), 8.01 (d, J = 8.4 Hz, 1H), 7.60 (s, 1H), 7.49 (q, J = 8.9, 7.5 Hz, 2H), 7.34 (s, 3H), 7.14 (d, J = 8.4 Hz, 1H), 6.85 (d, J = 7.9 Hz, 1H), 5.46 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.03, 154.50, 152.88, 142.25, 139.51, 136.70, 133.39, 132.55, 132.10, 129.92, 129.81, 129.39, 127.29, 124.11, 123.51, 105.90, 104.94, 102.62, 67.40. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{19}H_{15}ClN_3O_2$: 352.0847, found: 352.0858.

HPLC purity 97.4%.

4-((3-Chlorobenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15j). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-3-chlorobenzene according to the general procedure in 58% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.77 (d, J = 2.6 Hz, 1H), 8.33 (s, 1H), 8.21 – 8.14 (m, 1H), 8.11 – 8.03 (m, 1H), 7.67 (s, 1H), 7.48 (dd, J = 9.4, 7.2 Hz, 2H), 7.37 (td, J = 10.2, 8.7, 5.8 Hz, 3H), 7.13 (d, J = 8.4 Hz, 1H), 6.83 (d, J = 8.0 Hz, 1H), 5.41 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.07, 154.61, 153.00, 147.79, 142.26, 139.85, 136.82, 135.82, 132.46, 129.26(2C), 124.38, 123.42, 120.96(2C), 105.88, 104.97, 102.37, 68.72. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{19}H_{15}ClN_3O_2$: 352.0847, found: 352.0848. HPLC purity 98.3%.

4-((4-Chlorobenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15k). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-4-chlorobenzene according to the general procedure in 65% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.77 (d, J = 2.7 Hz, 1H), 8.30 (s, 1H), 8.19 (d, J = 4.6 Hz, 1H), 8.11 – 8.02 (m, 1H), 7.58 (d, J = 8.1 Hz, 2H), 7.45 (dd, J = 8.1, 6.2 Hz, 3H), 7.37 (dd, J = 8.4, 4.7 Hz, 1H), 7.12 (d, J = 8.4 Hz, 1H), 6.83 (d, J = 8.0 Hz, 1H), 5.41 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.04, 154.60, 152.98, 142.26, 139.83, 136.81, 135.35, 132.43(2C), 129.20(2C), 128.37(2C), 124.40, 123.45, 105.87, 105.03, 102.32, 68.83. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{19}H_{15}ClN_3O_2$: 352.0847, found: 352.0850. HPLC purity 95.8%.

4-((2,6-Dichlorobenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15l). The title compound was obtained as a white solid from **15** and 2-(bromomethyl)-1,3-dichlorobenzene according to the general procedure in 53% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.46 (d, J = 2.7 Hz, 1H), 8.15 (d, J = 4.7 Hz, 1H), 7.94 (s, 1H), 7.89 (d, J = 9.5 Hz, 1H), 7.60 (d, J = 7.6 Hz, 3H), 7.50 (dd, J = 8.9, 7.2 Hz, 1H), 7.34 (dd, J = 8.4, 4.6 Hz, 1H), 7.23 (d, J = 8.4 Hz, 1H), 7.13 (d, J = 8.0 Hz, 1H), 5.54 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.00, 154.34, 153.05, 142.22, 139.23, 136.49, 135.76(2C), 132.67, 131.73, 130.75, 128.82(2C), 123.86, 123.54, 105.94, 105.29, 103.03, 65.84. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{19}H_{14}Cl_2N_3O_2$: 386.0458, found: 386.0461. HPLC purity 98.0%.

4-((2-Methoxybenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15m). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-2-methoxybenzene according to the general procedure in 40% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.66 (d, J = 2.7 Hz, 1H), 8.18 (d, J = 7.1 Hz, 2H), 8.01 (d, J = 8.0 Hz, 1H), 7.53 – 7.43 (m, 2H), 7.39 – 7.30 (m, 2H), 7.10 (dd, J = 19.3, 8.3 Hz, 2H), 6.94 (t, J = 7.3 Hz, 1H), 6.85 (d, J = 8.0 Hz, 1H), 5.38 (s, 2H), 3.81 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 162.92, 156.75, 154.58, 153.35, 142.25, 139.39, 136.65, 132.57, 129.55, 128.80, 124.05, 123.69, 123.57, 120.31, 111.00, 105.92, 105.04, 102.26, 65.57, 55.51. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{18}N_3O_3$: 348.1343, found: 348.1345. HPLC purity 95.9%.

4-((3-Methoxybenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15n).

The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-3-methoxybenzene according to the general procedure in 48% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.74 (d, J = 2.7 Hz, 1H), 8.31 (s, 1H), 8.17 (d, J = 4.7 Hz, 1H), 8.06 (d, J = 8.1 Hz, 1H), 7.46 (t, J = 8.2 Hz, 1H), 7.40 – 7.32 (m, 1H), 7.28 (t, J = 7.9 Hz, 1H), 7.13 (s, 1H), 7.10 (t, J = 7.7 Hz, 2H), 6.85 (t, J = 8.9 Hz, 2H), 5.37 (s, 2H), 3.69 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 162.98, 159.27, 154.64, 153.22, 142.26, 139.74, 137.92, 136.77, 132.46, 129.55, 124.27, 123.45, 119.34, 113.19, 112.99,105.89, 105.09, 102.24, 69.61, 54.91. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{18}N_3O_3$: 348.1343, found: 348.1346. HPLC purity 98.1%.

4-((4-Methoxybenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15o). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-4-methoxybenzene according to the general procedure in 56% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.71 (d, J = 2.7 Hz, 1H), 8.22 (s, 1H), 8.16 (d, J = 4.6 Hz, 1H), 8.03 (d, J = 8.0 Hz, 1H), 7.47 (s, 1H), 7.36 (dd, J = 8.3, 4.8 Hz, 1H), 7.29 (d, J = 5.3 Hz, 1H), 7.22 (d, J = 6.6 Hz, 1H), 7.14 (d, J = 7.7 Hz, 1H), 7.09 (d, J = 8.4 Hz, 1H), 6.91 (s, 1H), 6.85 (s, 1H), 5.32 (s, 2H), 3.69 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 162.94, 158.97, 154.63, 153.25, 142.26, 139.69, 136.70, 132.43, 129.19(2C), 128.12, 124.24, 123.48, 113.78(2C), 105.89, 105.19, 102.10, 69.52, 54.94. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{18}N_3O_3$: 348.1343, found: 348.1341. HPLC purity 97.0%.

4-((2-Cyanobenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15p). The

title compound was obtained as a white solid from **15** and 2-(bromomethyl)benzonitrile according to the general procedure in 40% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.71 (d, J = 2.6 Hz, 1H), 8.17 (d, J = 4.7 Hz, 2H), 8.04 (dd, J = 8.3, 2.8, Hz, 1H), 7.96 – 7.88 (m, 1H), 7.83 (d, J = 7.8 Hz, 1H), 7.79 – 7.71 (m, 1H), 7.61 – 7.47 (m, 2H), 7.36 (dd, J = 8.4, 4.7 Hz, 1H), 7.17 (d, J = 8.4 Hz, 1H), 6.95 (d, J = 8.0 Hz, 1H), 5.60 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.08, 154.43, 152.55, 142.21, 139.60, 139.29, 136.67, 133.38, 133.34, 132.55, 129.54, 129.14, 124.24, 123.51, 117.45, 110.74, 105.80, 104.85, 102.81, 67.83. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{15}N_4O_2$: 343.1190, found: 343.1193. HPLC purity 98.1%.

4-((3-Cyanobenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15q). The title compound was obtained as a white solid from **16** and 3-(bromomethyl)benzonitrile according to the general procedure in 58% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.74 (s, 1H), 8.33 (s, 1H), 8.14 (d, J = 4.6 Hz, 1H), 8.04 (d, J = 5.3 Hz, 2H), 7.85 (d, J = 7.9 Hz, 1H), 7.76 (d, J = 7.6 Hz, 1H), 7.58 (d, J = 7.7 Hz, 1H), 7.45 (t, J = 8.2 Hz, 1H), 7.39 – 7.29 (m, 1H), 7.11 (d, J = 8.4 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 5.42 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.11, 154.59, 152.85, 142.21, 139.81, 138.01, 136.88, 132.45, 132.09, 131.63, 130.90, 129.62, 124.38, 123.41, 118.48, 111.35, 105.92, 104.94, 102.51, 68.54. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{15}N_4O_2$: 343.1190, found: 343.1190. HPLC purity 96.3%.

4-((4-Cyanobenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15r). The

title compound was obtained as a white solid from **15** and 4-(bromomethyl)benzonitrile according to the general procedure in 63% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.78 (s, 1H), 8.37 (s, 1H), 8.21 – 8.10 (m, 1H), 8.06 (d, J = 8.4 Hz, 1H), 7.83 (d, J = 7.8 Hz, 2H), 7.69 (d, J = 7.9 Hz, 2H), 7.44 (t, J = 8.2 Hz, 1H), 7.35 (t, J = 6.7 Hz, 1H), 7.11 (d, J = 8.4 Hz, 1H), 6.76 (d, J = 8.0 Hz, 1H), 5.49 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.12, 154.60, 152.81, 142.26, 142.10, 139.92, 136.89, 132.43, 132.32(2C), 127.85(2C), 124.51, 123.43, 118.53,110.493, 105.88, 104.93, 102.51, 68.71. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{15}N_4O_2$: 343.1190, found: 343.1192. HPLC purity 95.7%.

4-((2-Trifluoromethoxybenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15s). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-2-(trifluoromethoxy)benzene according to the general procedure in 43% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.68 (d, J = 2.7 Hz, 1H), 8.21 (s, 1H), 8.17 (d, J = 4.7 Hz, 1H), 8.02 (d, J = 7.9 Hz, 1H), 7.73 (d, J = 7.6 Hz, 1H), 7.55 – 7.47 (m, 2H), 7.44 (d, J = 7.6 Hz, 2H), 7.36 (dd, J = 8.4, 4.8 Hz, 1H), 7.16 (d, J = 8.4 Hz, 1H), 6.85 (d, J = 8.0 Hz, 1H), 5.48 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.04, 154.50, 152.85, 146.22, 142.20, 139.49, 136.72, 132.57, 130.19(2C), 128.49, 127.56, 124.17, 123.53, 120.66, 120.04(q, J = 256.7 Hz), 105.81, 104.75, 102.63, 64.82. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{15}F_3N_3O_3$: 402.1060, found: 402.1063. HPLC purity 96.1%.

4-((3-Trifluoromethoxybenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15t). The title compound was obtained as a white solid from **15** and

1-(bromomethyl)-3-(trifluoromethoxy)benzene according to the general procedure in 63% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.75 (d, J = 2.8 Hz, 1H), 8.33 (s, 1H), 8.17 (d, J = 4.6 Hz, 1H), 8.09 – 8.01 (m, 1H), 7.57 (d, J = 10.4 Hz, 2H), 7.50 (dt, J = 12.3, 8.1 Hz, 2H), 7.39 – 7.27 (m, 2H), 7.13 (d, J = 8.4 Hz, 1H), 6.86 (d, J = 8.0 Hz, 1H), 5.45 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.08, 154.62, 152.96, 148.38, 142.24, 139.80, 139.18, 136.85, 132.46, 130.41, 126.32, 124.36, 123.41, 120.27, 119.89(q, J = 256.7 Hz), 119.79, 105.93, 105.04, 102.47, 68.76. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{15}F_3N_3O_3$: 402.1060, found: 402.1063. HPLC purity 98.2%.

4-((4-Trifluoromethoxybenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15u). The title compound was obtained as a white solid from **15** and 1-(bromomethyl)-4-(trifluoromethoxy)benzene according to the general procedure in 66% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.76 (d, J = 2.7 Hz, 1H), 8.31 (s, 1H), 8.18 (d, J = 4.6 Hz, 1H), 8.05 (d, J = 8.5 Hz, 1H), 7.67 (d, J = 8.3 Hz, 2H), 7.48 (t, J = 8.2 Hz, 1H), 7.38 (d, J = 8.1 Hz, 3H), 7.12 (d, J = 8.4 Hz, 1H), 6.84 (d, J = 7.9 Hz, 1H), 5.44 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.07, 154.61, 152.99, 142.25, 139.82, 138.92, 136.84, 133.10, 132.47, 130.30, 128.33(q, J = 257.1Hz), 127.80, 127.08, 125.86, 124.31, 123.44, 105.92, 105.00, 102.43, 68.80. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{15}F_3N_3O_3$: 402.1060, found: 402.1056. HPLC purity 97.7%.

4-((2-Ethylbenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15v). The title compound was obtained as a white solid from **15** and

2-(bromomethyl)-1-ethylbenzene according to the general procedure in 60% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.64 (d, J = 2.7 Hz, 1H), 8.22 (s, 1H), 8.15 (d, J = 4.6 Hz, 1H), 8.00 (dt, J = 8.6, 2.0 Hz, 1H), 7.48 (t, J = 7.8 Hz, 2H), 7.35 (dd, J = 8.4, 4.7 Hz, 1H), 7.28 – 7.21 (m, 2H), 7.22 – 7.15 (m, 1H), 7.11 (d, J = 8.4 Hz, 1H), 6.89 (d, J = 8.0 Hz, 1H), 5.44 (s, 2H), 2.73 (t, J = 7.6 Hz, 2H), 1.11 (t, J = 7.5 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.00, 154.54, 153.25, 142.20, 142.09, 139.39, 136.70, 133.28, 132.53, 128.48, 128.44, 128.31, 125.68, 124.01, 123.53, 105.80, 105.03, 102.24, 68.09, 24.51, 14.97. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{21}H_{20}N_3O_2$: 346.1550, found: 346.1551. HPLC purity 95.8%.

4-((2-Chloro-5-fluorobenzyl)oxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15w). The title compound was obtained as a white solid from **15** and 2-(bromomethyl)-1-chloro-4-fluorobenzene according to the general procedure in 58% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.72 (d, J = 2.7 Hz, 1H), 8.32 (s, 1H), 8.18 (dd, J = 4.7, 1.5 Hz, 1H), 8.05 (d, J = 8.4 Hz, 1H), 7.64 – 7.51 (m, 3H), 7.38 (dd, J = 8.4, 4.7 Hz, 1H), 7.33 – 7.24 (m, 1H), 7.20 (d, J = 8.4 Hz, 1H), 6.90 (d, J = 8.0 Hz, 1H), 5.46 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.11, 160.72(d, J = 244.6 Hz)154.51, 152.69, 142.21, 139.61, 136.82, 135.90 (d, J = 7.8 Hz), 132.57, 131.14 (d, J = 8.3 Hz), 127.08, 124.13, 123.49, 116.72 (d, J = 23.0 Hz), 116.49 (d, J = 24.5 Hz), 105.97, 104.90, 102.82, 66.97. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{19}H_{14}ClFN_3O_2$: 370.0753, found: 370.0756. HPLC purity 98.0%.

4-((2-Phenylpyridin-4-yl)methoxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15x). The title compound was obtained as a white solid from **15** and

4-bromomethyl-2-phenylpyridine (detail in support information) according to the general procedure in 28% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.85 (d, J = 2.8 Hz, 1H), 8.66 (d, J = 5.0 Hz, 1H), 8.53 (s, 1H), 8.19 (dd, J = 4.7, 1.4 Hz, 1H), 8.16 (s, 1H), 8.10 (dd, J = 8.4, 2.8 Hz, 1H), 8.08 – 8.03 (m, 2H), 7.52 (d, J = 8.2 Hz, 1H), 7.50 – 7.41 (m, 4H), 7.38 – 7.33 (m, 1H), 7.17 (d, J = 8.4 Hz, 1H), 6.87 (d, J = 8.0 Hz, 1H), 5.55 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 163.19, 156.10, 154.67, 152.95, 149.63, 146.57, 142.27, 139.97, 138.29, 136.98, 132.51, 129.07, 128.62(2C), 126.35(2C), 124.55, 123.42, 120.36, 118.04, 105.96, 104.99, 102.62, 68.36. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{24}H_{19}N_4O_2$: 395.1503, found: 395.1506. HPLC purity 97.1%.

4-(Benzo[d]oxazol-2-ylmethoxy)-*N*-(pyridin-3-yl)benzo[*d*]isoxazol-3-amine (15y). The title compound was obtained as a white solid from **15** and 2-(chloromethyl)benzo[d]oxazole according to the general procedure in 38% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.90 (d, J = 2.6 Hz, 1H), 8.62 (s, 1H), 8.20 (dd, J = 4.7, 1.6 Hz, 1H), 8.17 – 8.12 (m, 1H), 7.79 – 7.70 (m, 2H), 7.55 (t, J = 8.3 Hz, 1H), 7.45 – 7.36 (m, 3H), 7.21 (d, J = 8.4 Hz, 1H), 7.03 (d, J = 8.0 Hz, 1H), 5.76 (s, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm) 162.87, 161.58, 154.47, 152.64, 150.14, 142.29, 140.04, 139.70, 136.80, 132.58, 125.55, 124.73, 124.25, 123.54, 119.61, 110.92, 106.07, 105.31, 103.22, 63.31. HRMS (ESI): *m/z* $[M+H]^+$ calculated for $C_{20}H_{15}N_4O_3$: 359.1139, found: 359.1141. HPLC purity 96.4%.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Supplemental figures, Experimental procedures for 4-bromomethyl-2-phenylpyridine and replacement of pyridine with pyrimidine series compounds. The spectral data of compound **3a**, **15**, **15j**, **15l**, **15s**, **15w**. (PDF)

Molecular formula strings and associated biochemical and biological data (CSV).

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Notes

The authors declare no conflicts of interest.

■ ACKNOWLEDGMENT

This work was financially supported by grant from the Chinese National Natural Science Foundation (Grant No. 30973641), the Shanghai Municipal Committee of Science and Technology (14XD1400300 & 17431902300), the program for Shanghai Rising Star (15QA1400300), and the open grant of the State Key Laboratory of

Bio-organic and Natural Products Chemistry, CAS. We also owe great thanks to the generous grant of Prof Yu Cao from Institute of Precision Medicine, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, with whose help did we succeed to have materials to evaluate our inhibitors. We thank Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

■ ABBREVIATIONS USED

BOP, benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphates; cLogP, calculated logP; DMPC, 2-dimyristoyl-sn-glycero-3-phosphocholine; DDM, n-dodecyl-beta-*D*-maltoside; EA, ethyl acetate; HAC, heavy atom count; *m*-CPBA, 3-chloroperbenzoic acid; PE, petroleum ether (boiling range: 60 – 90℃); rt, room temperature;SAR, structure and activity relationship; SM, sphingomyelin; SMS, sphingomyelin synthase; T2DM, type 2 diabetes mellitus; TEA, triethylamine; TMSOTf, trimethylsilyl trifluoromethanesulfonate;

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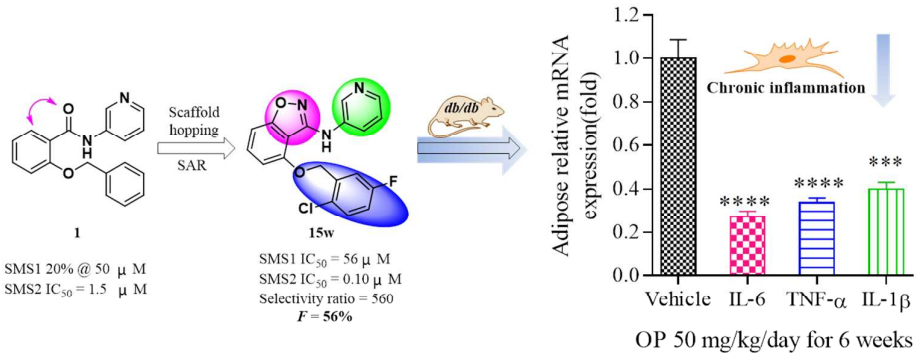
Table of Contents graphic



1

SMS1 20% @ 50 μ M
SMS2 IC_{50} = 1.5 μ M

15w

SMS1 IC_{50} = 56 μ M
SMS2 IC_{50} = 0.10 μ M
Selectivity ratio = 560
***F* = 56%**