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Discovery of novel ketoxime ether derivatives with potent FXR agonistic activity, oral effectiveness and high liver/blood ratio

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ABSTRACT

The farnesoid X receptor (FXR) is a promising therapeutic target for nonalcoholic steatohepatitis (NASH) and other bile acid related diseases because it plays a critical role in fibrosis, inflammation and bile acid homeostasis. Obeticholic acid (OCA), a FXR agonist which was synthesized from chenodeoxycholic acid, showed desirable curative effects in clinical trials. However, the pruritus which was the main side effect of OCA limited its further applications in NASH. Although pruritus was also observed in the clinical trials of non-steroidal FXR agonists, the proportion of patients with pruritus was much smaller than that of OCA. Thus, we decided to develop non-steroidal FXR agonists and discovered a series of novel FXR agonists which were synthesized from GW4064 by replacing the stilbene group with ketoxime ether. Encouragingly, in the following biological tests, our target compounds **13j** and **13z** not only showed potent FXR agonistic activities in vitro, but also effectively promoted the expression of target genes in vivo. More importantly, in the pharmacokinetic experiments, compounds **13j** and **13z** displayed high liver/blood ratio characteristics which were helpful to reduce the potential side effects which were caused by prolonged systemic activation of FXR. In summary, our compounds were good choices for the development of non-steroidal FXR agonists and were deserved further investigation.

1. Introduction

The farnesoid X receptor (FXR) was identified as an orphan nuclear receptor in 1999. It is expressed in the liver, intestines, kidney, adipose tissue and cardiovascular system. It acts as a key regulator which maintains the balance of bile acid by regulating the synthesis, transport and metabolism of bile acid, protecting the liver from the detrimental effects of bile acid accumulation.[1–3] In the liver, activated FXR inhibits the synthesis of bile acid by inducing the small-heterodimer partner (SHP) which down-regulates cholesterol 7a-hydroxylase (CYP7A1).[4] In the intestines, activation of FXR promotes the secretion of fibroblast growth factor 15 (FGF15) in mice or the secretion of fibroblast growth factor 19 (FGF19) in humans. Then they return to the liver via the enterohepatic circulation and down-regulate CYP7A1, thereby inhibiting the synthesis of bile acid.[5] In addition, FXR regulates the bile salt export pump (BSEP) gene which is one of the downstream target genes of FXR to control the secretion of bile acid from hepatocytes to gall.[6] Moreover, Activated FXR will protect liver from inflammation and fibrosis by inhibiting the activation of the hepatic stellate cells (HSC).[7] Due to its multifunctional activities in fibrosis, inflammation and bile

Abbreviations: LDL, low density lipoprotein; SAR, structure activity relationships; EC_{50}, half maximal effective concentration; NCS, N-Chlorosuccinimide; NBS, N-Bromosuccinimide; AIBN, 2,2′-Azobis(2-methylpropionitrile); DCM, dichloromethane; MeOH, methanol; THF, tetrahydrofuran; DMF, N, N-dimethyl formamide; DIPEA, N, N-diisopropylethylamine; TEA, triethylamine; NMR, nuclear magnetic resonance; ESI, electron spray ionization; hERG, human Ether-a-go-go Related Gene; hTGR5, human Taketa G-protein receptor 5; MTBE, methyl *tert*-butyl ether; DMEM, dulbecco's modified eagle medium; ACN, acetonitrile.

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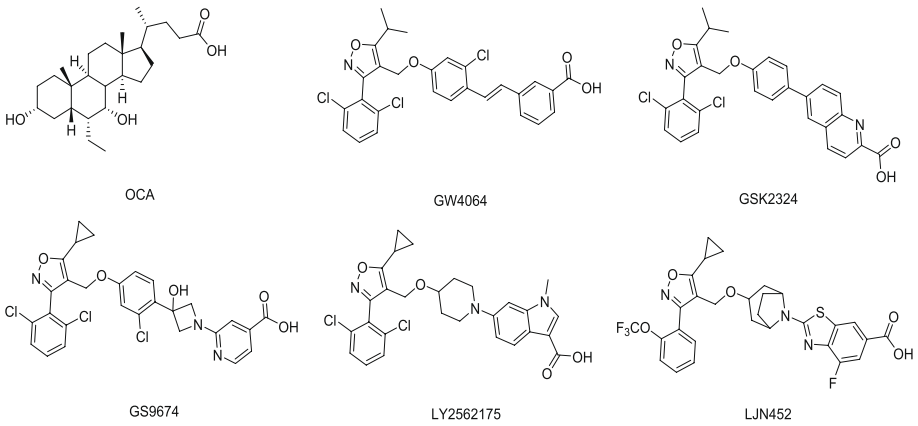


Fig. 1. Representative FXR agonists.

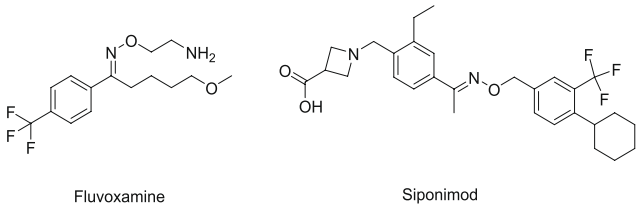


Fig. 2. The clinical drugs which contain ketoxime ether moiety.

acid metabolism, FXR presents as a promising therapeutic target for NASH.

There is no doubt that Obeticholic acid (OCA, Fig. 1) is the front-runner among various FXR agonists. However, its untoward effects are quite disturbing. In the latest phase III trial, patients in the 25 mg OCA group showed significant improvement in fibrosis compared to those in the placebo group, but unfortunately, 51% patients suffered severe pruritus and LDL cholesterol increases was observed in 17% patients.[8] Because the predicted benefits of OCA remained uncertain and they did not outweigh the potential risks sufficiently in the previous clinical trials, Intercept company needs to submit more additional evidence to prove the effectiveness and safety of OCA and the long-term clinical study of OCA is still needed. In the previous studies, it was reported that the activation of TGR5 by OCA may be responsible for the pruritus which was observed in clinical trials of OCA.[9–12] Therefore, many researchers paid more attention to the discovery of novel selective non-steroidal FXR agonists (Fig. 1) which would not activate TGR5.

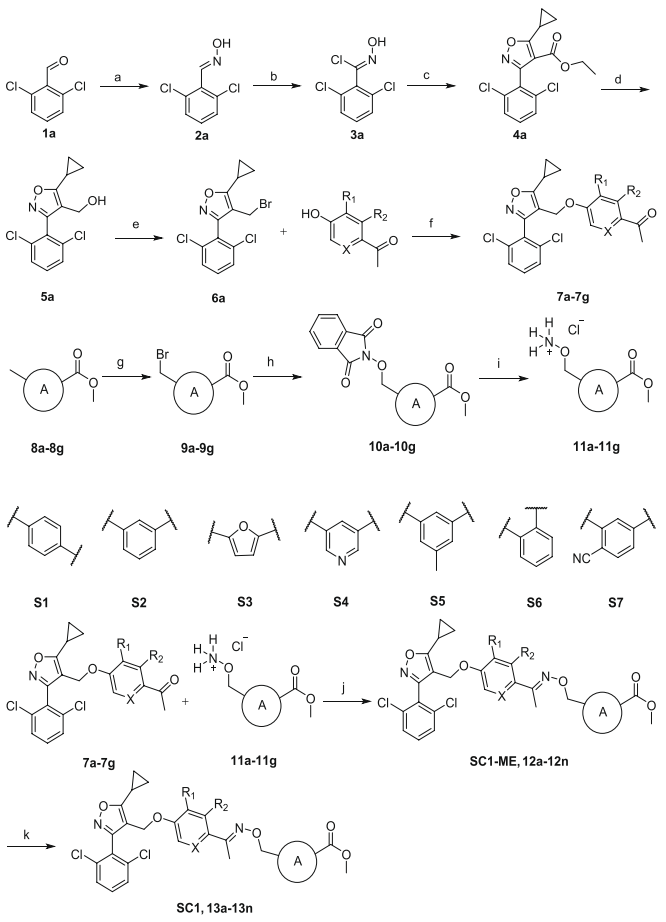
GW4064 was discovered as a classical non-steroidal FXR agonist through an iterative combinatorial library synthesis and screening approach.[13] Although GW4064 was a highly potent FXR agonist, it showed poor pharmacokinetic properties and low metabolic stability due to its stilbene group which was also a potentially toxic pharmacophore.[14–16] In order to address these issues, researchers attempted to replace the stilbene group with a stable functional group.[17] After three rounds of optimization, GSK2324 which was synthesized by replacing the stilbene group of GW4064 with heterocyclic ring showed considerable improvement in pharmacokinetic properties, but its FXR agonistic activity had not been greatly improved.[18] In order to improve the druggability, researchers from Gilead replaced the stilbene group of GW4064 with acridine ring and synthesized GS9674 (also known as Cilofexor) for the treatment of NASH and primary biliary cholangitis (PBC).[19–21] Meanwhile, the researchers in Eli Lily replaced the benzene ring of stilbene group with piperidine ring and obtained LY2562175 (as known as TERN-101). Although LY2562175 was a partial FXR agonist (41% relative to GW4064), it represented better druggability than GW4064 in the biological evaluations.[22] On the basis of LY2562175, researchers in Novartis replaced the piperidine ring with bicyclic [3.2.1] tropane linker to increase the conformational restraint and synthesized the full FXR agonist LJN452 which showed highly potent in vitro and vivo.[23] Although great progress was made in the development of FXR agonists, none of FXR agonists was approved for the treatment of NASH.

Ketoxime ether which was appeared in clinical drugs such as Fluvoxamine and Siponimod (Fig. 2),[24,25] was generally considered as the isostere of aromatic ring. Its geometric configuration and electrical arrangement were similar to that of benzene ring to some extent.[26] The C—N double bond of ketoxime ether showed considerable conformational rigidity because of its planar configuration and lone pair electrons.[27,28] Previous research proved that the agonistic activity of GW4064 would decrease when the double bond was simply reduced because the conformational rigidity was indispensable to GW4064.[29] From this perspective, we decided to replace the stilbene group of GW4064 with ketoxime ether and synthesized the start compound 1 (**SC1**) which showed considerable activating ability towards FXR. In the following studies, we continued to synthesize a series of derivatives of **SC1** and discovered **13j** and **13z** which showed some favorable properties including potent activating activity, oral effectiveness and high liver/blood ratio. High liver/blood ratio was propitious to improve the efficacy of the FXR agonists in vivo and avoid the potential side effects which were caused by the prolonged systemic activation of FXR theoretically because the main target organs of FXR agonists were liver and intestines.[23,30]

2. Result and discussion

2.1. Chemistry

The designed target compounds were synthesized from commercially available 2,6-Dichlorobenzaldehyde or 2-(Trifluoromethoxy)benzaldehyde according to the reported routes in Schemes 1 and 2.[31–34] Oxime **2a** was produced from aldehyde **1a** by the condensation with hydroxylamine hydrochloride. Then oxime **2a** was chlorinated by NCS to generate compound **3a**. Cycloaddition of compound **3a** with ethyl 3-cyclopropyl-3-oxopropanoate provided ethyl ester **4a** with a moderate yield. Then the ethyl ester **4a** was reduced by $LiAlH_4$ to give the primary alcohol **5a**. Compound **5a** was bromized via Apple reaction to generate compound **6a**. Compound **6b** was synthesized via the same route. Compounds **7a-7g** were produced from compound **6a** under nucleophilic substitution reactions. Compounds **7h-7l** were synthesized from compound **6b** in the same condition. In the meantime, compounds **9a-9g** were prepared from compounds **8a-8g** via the bromination with NBS. Then **9a-9g** were added to DMF with N-Hydroxyphthalimide and DIPEA to generate compounds **10a-10g**. Next, compounds **10a-10g** were treated with butan-1-amine and ethanol solution of hydrogen chloride to give compounds **11a-11g**. Finally, by base-mediated hydrolysis, compound **SC1** and compounds **13a-13n** were generated from the



Ring A was represented by S1, S2, S3, S4, S5, S6 and S7

SC1: R_1 = H, R_2 = H, X = C, A = S2 **13a**: R_1 = H, R_2 = H, X = C, A = S1

13b: R_1 = H, R_2 = Cl, X = C, A = S1 **13c**: R_1 = H, R_2 = Cl, X = C, A = S2

13d: R_1 = H, R_2 = Cl, X = C, A = S3 **13e**: R_1 = H, R_2 = Cl, X = C, A = S4

13f: R_1 = H, R_2 = H, X = N, A = S2 **13g**: R_1 = H, R_2 = H, X = N, A = S3

13h: R_1 = H, R_2 = Cl, X = C, A = S5 **13i**: R_1 = Cl, R_2 = H, X = C, A = S2

13j: R_1 = H, R_2 = Cl, X = C, A = S7 **13k**: R_1 = H, R_2 = Br, X = C, A = S2

13l: R_1 = H, R_2 = CF_3, X = C, A = S2 **13m**: R_1 = H, R_2 = F, X = C, A = S2

13n: R_1 = H, R_2 = Cl, X = C, A = S6

Scheme 1. Reagents and conditions. (a) $NH_2OH{\cdot}HCl$, NaOH, EtOH, H_2O, 0 °C to rt, 8 h, 89%; (b) NCS, DMF, 25 °C, 1 h, 94%; (c) TEA, ethyl 3-cyclopropyl-3-oxopropanoate, THF, 25 °C, 12 h, 59%; (d) LiAlH4, THF, 0 °C, 1 h, 75%; (e) CBr_4, PPh_3, DCM, 0 °C, 0.5 h, 86%; (f) K_2CO_3, DMF, 25 °C, 8 h, 78–90%; (g) NBS, AIBN, CCl_4, 70 °C, 6 h, 58–72%; (h) N-Hydroxyphthalimide, DIPEA, DMF, N_2, 70 °C, 8 h, 58–85%; (i) Butan-1-amine, HCl (EtOH), MeOH, 25 °C, 12 h, 78–85%; (j) MeOH, 25 °C, 12 h, 60–85%; (k) $LiOH{\cdot}H_2O$, THF, MeOH, H_2O, 40 °C, 3 h, 81–92%.

compound **SC1-ME** or compounds **12a-12n** which were produced from compounds **7a-7g** and compounds **11a-11g** in a mild condition. Compounds **13o-13z** were synthesized via the similar route. The specific structures of intermediates were shown in the supporting information.

2.2. Biological evaluation

2.2.1. FXR response element driven luciferase assay and SAR analysis

A cellular transactivation assay using the FXR response element driven luciferase method was performed according to the methods which were described in the experimental section. OCA and GW4064 were used as control compounds. All of the final compounds were evaluated and the results were shown in Tables 1 and 2.

Compound **SC1** which was the first compound that we synthesized exhibited moderate FXR activation ability with EC_{50} 179.1 nM and 147.5% maximum efficacy relative to OCA. We initially investigated the middle benzene ring of compound **SC1** and discovered that when chlorine, bromine or fluorine was introduced into the *ortho*-position of ketoxime ether in the middle benzene ring, the FXR agonistic activity of compounds would be improved. Compounds **13c**, **13k** and **13m** showed stronger activation to FXR than **SC1** with EC_{50} values of 47.4 nM, 37.2 nM and 55.8 nM respectively. However, when the middle benzene ring was replaced with heterocyclic rings such as pyridyl ring, the agonistic activity would be reduced. Compound **13f** displayed weaker agonistic activity than compound **SC1** with EC_{50} value of 233.8 nM. In the further investigations, compounds **13d** and **13e** which were synthesized by replacing the terminal benzene ring of compound **13c** with furan or pyridyl ring, showed no improvement in potency. Then we continued to introduce new substituents into the terminal benzene ring of compound **13c** and discovered that compound **13j** which was the cyanogen derivative of compound **13c** could maintain the agonistic ability with an EC_{50} of 45.2 nM. It has been reported that the pharmacokinetic properties of the isoxazole FXR agonists would be improved when the 2,6-dichlorophenyl ring was replaced with the trifluoromethoxy benzene ring,[23] so we decided to design and synthesize a series of compounds which contained trifluoromethoxy benzene ring. Surprisingly, among those compounds, compound **13z** which was optimized from compound **13j** displayed strong FXR agonistic ability with an EC_{50} of 36.1 nM. Due to their similarities in structure, both compounds **13j** and **13z** were selected for the further evaluation.

2.2.2. hTGR5 activation examination

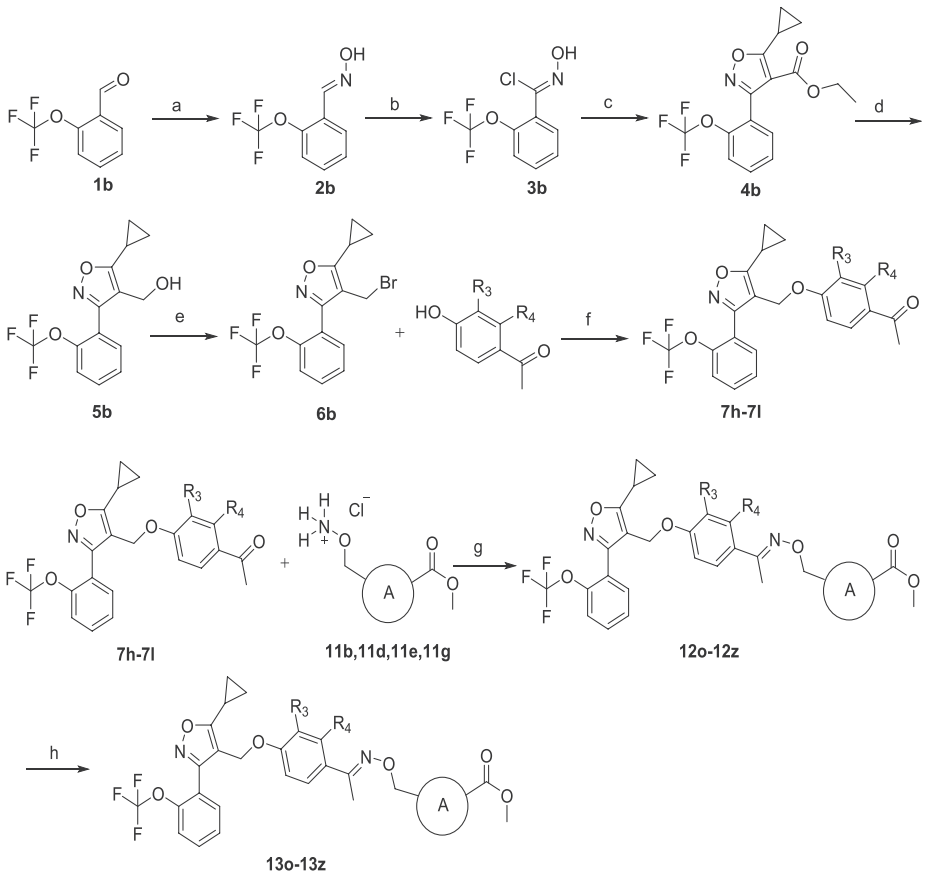
Activation of hTGR5 was supposed to be responsible for the pruritus which was observed in the clinical trials of OCA. Thus, compounds **13j**, **13z** and OCA were evaluated for their hTGR5 activation effects in a cell based reporter assay as described in the methods. As is shown in Table 3, OCA could effectively activate hTGR5 with an EC_{50} of 3.4 ± 0.6 μM and a max effect of 95.3 ± 3.6%. However, compound **13j** and compound **13z** displayed no measurable activity on TGR5, which indicated that the side effects of activating hTGR5 would probably be avoided.

2.2.3. FXR target gene expression in primary C57 mouse hepatocytes

In order to evaluate the potential of compounds **13j** and **13z** on the induction of FXR downstream genes, the mRNA levels of SHP and BSEP which are the FXR target genes were quantified in the primary C57 mouse hepatocytes. The cells were treated with vehicle (0.5% DMSO) or different compounds (**13j**, **13z** or OCA) in different concentrations (0.4 μM, 2 μM and 10 μM). After an incubation period of 24 h, the mRNA expressions of SHP and BSEP were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). As is shown in Fig. 3A, compounds **13j**, **13z** and OCA exhibited robust induction in relation to the mRNA expression of SHP in a dose-dependent manner. The maximal fold values of compounds **13j** and **13z** were greater than 40 while the maximal fold value of OCA was less than 20. As is shown in Figure 3B, compounds **13j**, **13z** and OCA also displayed strong induction in relation to BSEP mRNA expression. The results demonstrated that compounds **13j** and **13z** could effectively induce the expression of FXR target genes in the primary C57 mouse hepatocytes.

2.2.4. FXR target gene expression in C57BL/6J mouse after a single oral dose

In order to evaluate the FXR agonistic effects of compounds **13j** and **13z** in vivo, the induction effects on FXR target genes in mice were examined after a single oral dose. Compound **13j** (10 mg/kg), compound **13z** (10 mg/kg), OCA (30 mg/kg) or vehicle (0.25% CMC-Na, wt/vol) was orally given to C57BL/6J mice. Liver and ileum tissues were



Ring A was represented by S2, S4, S5 and S7

13o: R_1 = H, R_2 = Cl, A = S4 **13p**: R_1 = H, R_2 = Cl, A = S5

13q: R_1 = H, R_2 = Cl, A = S2 **13r**: R_1 = F, R_2 = H, A = S4

13s: R_1 = F, R_2 = H, A = S5 **13t**: R_1 = OCH_3, R_2 = H, A = S5

13u: R_1 = CH_3, R_2 = H, A = S5 **13v**: R_1 = CH_3, R_2 = H, A = S4

13w: R_1 = F, R_2 = H, A = S7 **13x**: R_1 = OCH_3, R_2 = H, A = S7

13y: R_1 = CH_3, R_2 = H, A = S7 **13z**: R_1 = H, R_2 = Cl, A = S7

Scheme 2. Reagents and conditions. (a) $NH_2OH{\cdot}HCl$, NaOH, EtOH, H_2O, 0 °C to rt, 8 h, 77%; (b) NCS, DMF, 25 °C, 1 h, 86%; (c) TEA, ethyl 3-cyclopropyl-3-oxopropanoate, THF, 25 °C, 12 h, 57%; (d) LiAlH4, THF, 0 °C, 1 h, 82%; (e) CBr_4, PPh_3, DCM, 0 °C, 0.5 h, 84%; (f) K_2CO_3, DMF, 25 °C, 8 h, 68–87%; (g) MeOH, 25 °C, 12 h, 58–75%; (h) $LiOH{\cdot}H_2O$, THF, MeOH, H_2O, 40 °C, 3 h, 80–91%.

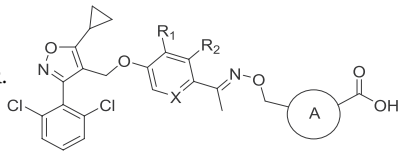
harvested 6 h post-dose, and RNA was extracted for analyzing mRNA levels of relevant genes.

As is shown in Figs. 4 and 5, OCA, compounds **13j** and **13z** all displayed strong induction or repression to FXR target genes in vivo. In the liver, OCA, compounds **13j** and **13z** all demonstrated potent induction to SHP with mRNA levels 2–3 fold above the vehicle treated animals. In the BSEP gene induction results, compounds **13j** and **13z** represented stronger inducement to the target gene than OCA. Meanwhile, the expressions of CYP7A1 and CYP8B1 were potently repressed by OCA, compounds **13j** and compound **13z**. But OCA showed less repression to CYP7A1 and CYP8B1 compared to compounds **13j** and **13z** at the mRNA expression levels. In the ileum, OCA, compounds **13j** and **13z** also displayed robust induction to SHP with mRNA levels 20 ~ 30 fold above the vehicle treated animals. Meanwhile, mRNA expressions of FGF15 were potently induced by OCA, compounds **13j** and **13z** (5–8 fold above vehicle). All the above results indicated that compounds **13j** and **13z** could effectively induce or repress the expression of FXR target genes in C57BL/6J mice at a single oral dose.

2.2.5. *Mean plasma and liver concentrations of compounds **13j** and **13z** in ICR mouse (15 mg/kg)*

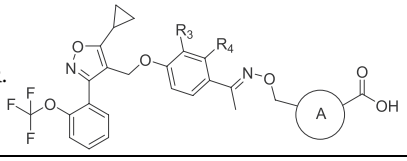
In order to clarify the distribution of compounds **13j** and **13z** in vivo, ICR mice were treated with compounds **13j** or **13z** at a single oral dose (15 mg/kg). The blood and liver samples were collected at 1.5 h, 4.0 h and 8.0 h post-dose and the concentrations of the compounds in each sample were analyzed separately. As is shown in Table 4, the concentrations of compound **13j** in the livers were about 37 ~ 57 fold higher than those in plasma at the same time point. The liver/blood ratios of compound **13z** ranged from 77 to 111. According to a previous article, the liver/blood ratio of OCA was about 5–10.[30] LJN452 which is a FXR agonist in phase II clinical trial is an analogue of GW4064 and its highest liver/ blood ratio is 20.[23] Moreover, the absolute exposures of compounds **13j** and **13z** in plasma were quite low. Although we need to conduct more experiments to analyze the distribution of compounds **13j** and **13z** in other organs like kidney and skin, we can preliminarily conclude that the high liver/ blood ratio characteristic of our compounds will help to reduce the potential side effects which were caused by the prolonged systemic activation of FXR.

Table 1
Agonist effects of the final compounds **SC1** and **13a-13n** on FXR.



Compound	R_1	R_2	X	Ring A	FXR transactivation[a]	
					EC_{50} (nM)	Efficacy (%)[b]
SC1	H	H	C	S2	179.1 ± 70.4	147.5 ± 4.7
13a	H	H	C	S1	127.9 ± 20.1	123.6 ± 21.2
13b	H	Cl	C	S1	133.7 ± 72.7	125.7 ± 16.9
13c	H	Cl	C	S2	47.4 ± 27.0	136.5 ± 5.7
13d	H	Cl	C	S3	49.7 ± 7.6	149.1 ± 4.2
13e	H	Cl	C	S4	65.0 ± 27.8	141.6 ± 17.5
13f	H	H	N	S2	233.8 ± 31.5	126.5 ± 26.4
13g	H	H	N	S3	1387.2 ± 226.8	111.7 ± 19.5
13h	H	Cl	C	S5	59.2 ± 10.6	135.5 ± 4.6
13i	Cl	H	C	S2	259.1 ± 45.3	117.9 ± 12.5
13j	H	Cl	C	S7	45.2 ± 19.5	125.2 ± 9.7
13k	H	Br	C	S2	37.2 ± 18.1	121.3 ± 23.1
13l	H	CF_3	C	S2	193.7 ± 46.4	98.5 ± 33.4
13m	H	F	C	S2	55.8 ± 4.9	128.8 ± 9.9
13n	H	Cl	C	S6	75.4 ± 12.5	120.3 ± 5.7

Table 2
Agonist effects of the final compounds **13o-13z** on FXR.



Compound	R_3	R_4	Ring A	FXR transactivation[a]	
				EC_{50} (nM)	Efficacy (%)[b]
13o	H	Cl	S4	54.8 ± 4.3	141.1 ± 2.6
13p	H	Cl	S5	158.3 ± 71.3	156.4 ± 15.8
13q	H	Cl	S2	107.5 ± 46.2	134.2 ± 26.7
13r	F	H	S4	727.8 ± 171.5	125.9 ± 11.8
13s	F	H	S5	1343.7 ± 36.5	87.9 ± 6.3
13t	OCH_3	H	S5	>1851.9	55.9 ± 21.7
13u	CH_3	H	S5	523.1 ± 181.9	106.1 ± 3.7
13v	CH_3	H	S4	1832.0 ± 978.5	82.3 ± 19.4
13w	F	H	S7	346.8 ± 100.5	134.9 ± 19.6
13x	OCH_3	H	S7	969.9 ± 363.0	126.4 ± 3.2
13y	CH_3	H	S7	440.2 ± 104.7	114.3 ± 4.5
13z	H	Cl	S7	36.1 ± 4.7	159.3 ± 8.2
OCA				324.1 ± 76.9	102.9 ± 5.0
GW4064				22.3 ± 2.7	133.7 ± 2.7

[a] Data represent means ± SD of at least three independent experiments.
[b] Efficacy: maximum efficacy of the test compound relative to 10 μM OCA (100%).

Table 3
The hTGR5 activation ability of compounds **13j**, **13z** and OCA.

Compound	EC_{50} (μM)[a]	Efficacy (%)[b]
13j	>100	1.1 ± 0.7
13z	>100	0.6 ± 0.8
OCA	3.4 ± 0.6	95.3 ± 3.6

[a] EC_{50} values represent the mean ± SD of three independent experiments.
[b] Efficacy: maximum efficacy of the test compound relative to positive control OCA (20 μM).

3. Conclusion

We discovered a series of non-steroidal FXR agonists which contained a ketoxime ether group and their agonistic activities were evaluated on FXR response element driven luciferase assay. Among them, compounds **13j** and **13z** displayed robust agonistic activities on FXR without TGR5 activation. Meanwhile, in the primary C57 mouse hepatocytes, both compounds effectively induced the expression of target genes SHP and BSEP which were supposed to be important for NASH therapy. Moreover, compounds **13j** and **13z** were able to activate FXR in vivo and they all displayed strong induction to the target genes of FXR in C57BL/6J mice. More importantly, compounds **13j** and **13z** showed high liver/blood ratios in the pharmacokinetic experiment, which contribute to reducing the potential side effects which were caused by the prolonged systemic activation of FXR. Those characteristics indicated that our compounds **13j** and **13z** which provided a new framework for FXR agonists were worth further research in the therapy of NASH and other FXR correlative diseases.

4. Experimental section

4.1. Chemistry

All of the chemicals and solvents which were used directly without further purification were purchased from commercial suppliers. Compounds were dissolved in solvent (CDCl3, MeOH-d_4 or DMSO-d_6) with tetramethylsilane (TMS) which was used as an internal standard. ^{1}H NMR spectra were recorded on a Bruker Avance III 400 or a Bruker Avance III 500 NMR spectrometer. ^{13}C NMR spectra were recorded on a Bruker Avance III 126 NMR spectrometer. HR/MS (ESI) data were recorded on the Agilent G6520 Q-TOF system. Low-resolution Mass data were recorded using an Agilent liquid-chromatography mass spectrometer system that consisted of an Agilent 1260 infinity LC coupled to Agilent 6120 Quadrupole mass spectrometer (ESI). Column chromatography was performed with silica gel (200–400 mesh) or with prepacked silica cartridges (4–80 g) from Bonna-Agela Technologies Inc. (Tianjin, China) and was eluted with a CombiFlash@ Rf 200 from Teledyne Isco. Purity of final compounds were analyzed by HPLC and were greater than 95%.

4.1.1. (E)-2,6-dichlorobenzaldehyde oxime (2a)

A solution of sodium hydroxide (1.656 g, 41.41 mmol, 10 mL H_2O) was added to a solution of $NH_2OH{\cdot}HCl$ (3.940 g, 56.69 mmol, 20 mL H_2O) at 0 °C. Then, a solution of 2,6-dichlorobenzaldehyde (10.0 g, 57.14 mmol, 80 mL ethanol) was added to the mixture at 25 °C. After completion, the mixture was concentrated under vacuum to give crude product. The crude product was recrystallized in the solution (ethanol: H_2O = 1:1) to give 9.597 g of compound **2a** in 89% yield as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.69 (s, 1H), 7.60 (dt, J = 2.3, 1.1 Hz, 1H), 7.60–7.58 (m, 1H), 7.56–7.51 (m, 1H), 3.54–3.38 (m, 1H). MS(ESI) m/e $[M+H]^+$: 189.9.

4.1.2. (E)-2-(trifluoromethoxy)benzaldehyde oxime (2b)

Compound **2b** (8.3 g, a white solid) was synthesized from 2-(Trifluoromethoxy) benzaldehyde (10.0 g, 52.60 mmol) according to the procedure for **2a**, 77% yield. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.42 (s, 1H), 7.89 (dd, J = 7.8, 1.8 Hz, 1H), 7.43 (ddd, J = 8.2, 7.4, 1.8 Hz, 1H), 7.35–7.27 (m, 2H); MS(ESI)m/e$[M+H]^+$: 206.0.

4.1.3. (Z)-2,6-dichloro-N-hydroxybenzimidoyl chloride (3a)

NCS (2.124 g, 15.90 mmol) was added to the solution of compound **2a** (3.0 g, 15.87 mmol, 50 mL DMF) in three portions. The mixture was stirred for 5 h at 25 °C. Then the mixture was diluted with ethyl acetate (50 mL) and washed with brine (80 mL × 3). The combined organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum to give the crude product. The crude product was recrystallized in hexane to give 3.478 g of compound **3a** in 94% yield as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.64 (d, J = 1.7 Hz, 1H), 7.63 (d, J = 0.7 Hz, 1H), 7.58 (dd, J = 9.2, 6.8 Hz, 1H). MS(ESI)m/e$[M+H]^+$: 223.9.

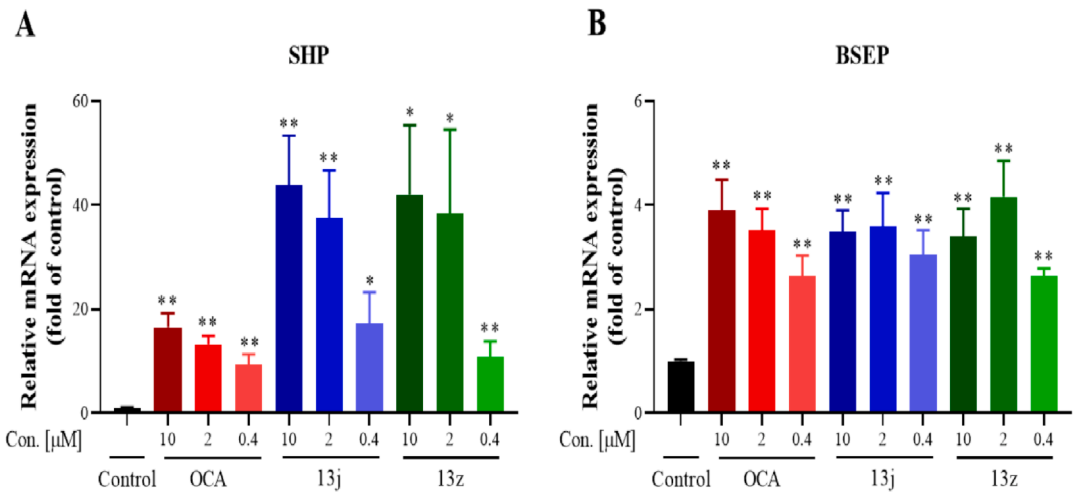


Fig. 3. Induction of FXR target gene SHP (A) and BSEP (B) in the primary C57 mouse hepatocytes by compounds **13j**, **13z** and OCA. Primary C57 mouse hepatocytes were treated for 24 h with vehicle only or the compounds at differing concentrations. Gene expression levels were determined by qRT-PCR. Values were shown as fold values of the vehicle control group (mean ± S.E.M, n = 3; *p < 0.05, **p < 0.01 vs control).

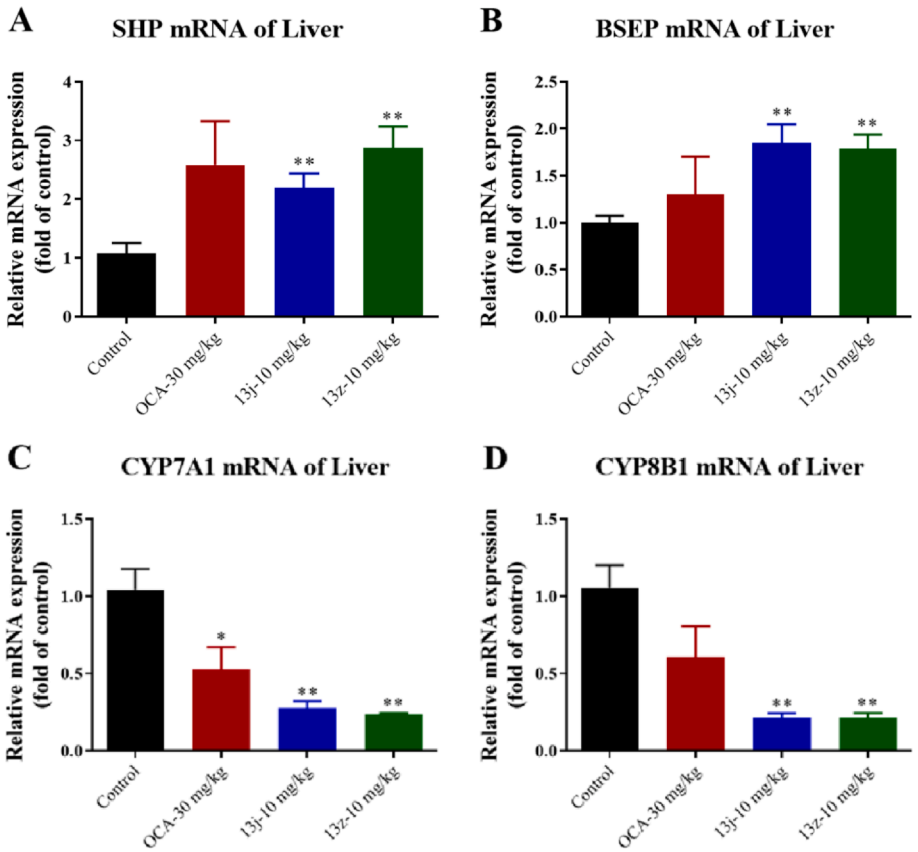


Fig. 4. Induction of FXR target genes SHP, BSEP, CYP7A1 and CYP8B1 in mouse livers by compounds **13j**, **13z** and OCA following a single oral dose. Gene expression levels were determined by qRT-PCR. Values were shown as a fold value in relation to the vehicle control group (mean ± S.E.M, n = 6; *p < 0.05, **p < 0.01 vs control).

*4.1.4. (Z)-N-hydroxy-2-(trifluoromethoxy)benzimidoyl chloride **(3b)***

Compound **3b** (7.0 g, a yellow oil) was synthesized from **2b** (7.0 g, 34.14 mmol) according to the procedure for **3a**. 86% yield. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.29 (s, 1H), 7.61 (dd, J = 7.7, 1.8 Hz, 1H), 7.50 (td, J = 7.9, 1.8 Hz, 1H), 7.41–7.32 (m, 2H); MS(ESI)m/e[M+H]$^+$: 240.0.

*4.1.5. Ethyl 5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazole-4-carboxylate **(4a)***

The TEA (1.137 g, 11.15 mmol) was added to the solution of ethyl 3-cyclopropyl-3-oxopropanoate (2.679 g, 17.16 mmol, 30 mL DMF). After stirred for five hours, the mixture was treated with the solution of compound **3a** (1.920 g, 8.61 mmol, 10 mL DMF). After completion, the mixture was diluted with ethyl acetate (40 mL) and washed with brine (80 mL × 3). The combined organic layer was dried over anhydrous

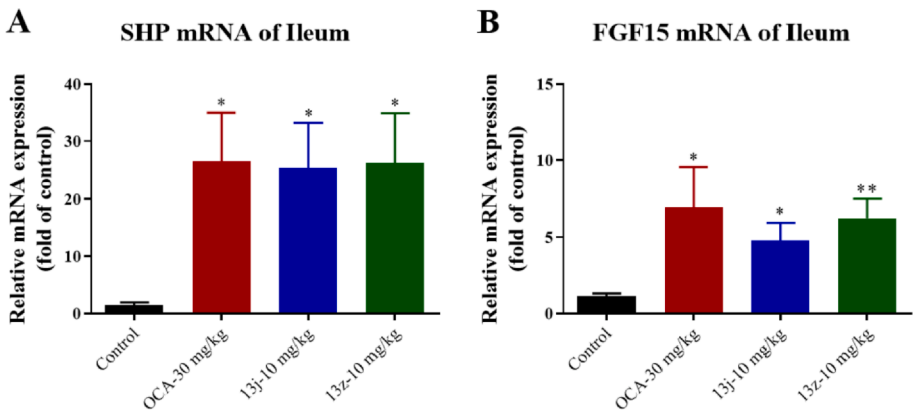


Fig. 5. Induction of FXR target genes SHP and FGF15 in the mouse ileum by compounds **13j**, **13z** and OCA following a single oral dose. Gene expression levels were determined by qRT-PCR. Values were shown as a fold value of the vehicle control group (mean ± SEM, n = 6; *p < 0.05, **p < 0.01 vs control).

Table 4
Liver/plasma concentration (L/P) ratios in ICR mice.

Compound	Time (h)	Liver conc (ng/g)	Plasma conc (ng/mL)	L/P ratio
13j	1.5	4534	124.0	37
	4.0	2610	45.4	57
	8.0	341	7.8	43
13z	1.5	4344	56.7	77
	4.0	3579	45.2	79
	8.0	941	8.4	111

PO, 15 mg/kg, each; male, n = 3 for each time point. l/P is short for liver/plasma.

sodium sulfate, concentrated under vacuum, and purified by flash chromatography with ethyl acetate and petroleum ether (PE:EA = 4:1) to give 1.651 g of compound **4a** in 59% yield as a white solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.41 (d, J = 1.9 Hz, 1H), 7.39 (d, J = 0.7 Hz, 1H), 7.33 (dd, J = 9.3, 6.6 Hz, 1H), 4.12 (q, J = 7.1 Hz, 2H), 2.93 (tt, J = 8.4, 5.1 Hz, 1H), 1.45–1.36 (m, 2H), 1.36–1.23 (m, 2H), 1.02 (t, J = 7.1 Hz, 3H); MS(ESI)m/e[M+H]$^+$: 326.0.

*4.1.6. Ethyl 5-cyclopropyl-3-(2-(trifluoromethoxy)phenyl)isoxazole-4-carboxylate **(4b)***

Compound **4b** (4.1 g, a yellow solid) was synthesized from 3b (5.0 g, 20.92 mmol) according to the procedure for **4a**. 57% yield. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.53–7.45 (m, 2H), 7.38–7.30 (m, 2H), 4.18–4.13 (m, 2H), 2.91–2.82 (m, 1H), 1.38–1.32 (m, 2H), 1.25–1.21 (m, 2H), 1.08 (t, J = 7.0 Hz, 3H); MS(ESI)m/e[M+H]$^+$: 342.0.

*4.1.7. (5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazol-4-yl) methanol **(5a)***

A solution of $LiAlH_4$ (128.2 mg, 3.38 mmol, 1.41 mL THF) was added to the solution of compound **4a** (1.0 g, 3.08 mmol, 20 mL THF) at 0 °C. The mixture was stirred at 25 °C for 2 h and then quenched with a saturated solution of NH_4Cl. The mixture was filtered through celite and extracted with ethyl acetate (30 mL × 3). The combined organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum to give the crude product. The crude product was recrystallized in hexane to obtain 657.0 mg of compound **5a** in 75% yield as a white solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.44 (d, J = 1.6 Hz, 1H), 7.42 (d, J = 0.7 Hz, 1H), 7.36 (dd, J = 9.2, 6.8 Hz, 1H), 4.41 (d, J = 5.8 Hz, 2H), 2.19 (tt, J = 8.4, 5.1 Hz, 1H), 1.28 (ddd, J = 6.7, 5.0, 4.1 Hz, 2H), 1.18–1.10 (m, 2H); MS(ESI)m/e[M+H]$^+$: 284.0.

*4.1.8. (5-cyclopropyl-3-(2-(trifluoromethoxy)phenyl)isoxazol-4-yl) methanol **(5b)***

Compound **5b** (2.5 g, a yellow oil) was synthesized from 4b (3.5 g, 10.26 mmol) according to the procedure for **5a**. 82% yield. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.56–7.54 (m, 2H), 7.40–7.39 (m, 2H), 4.51 (s, 2H), 2.22–2.20 (m, 1H), 1.71 (s, 1H) 1.11–1.28 (m, 4H); MS(ESI)m/e[M+H]$^+$: 300.0.

*4.1.9. 4-(bromomethyl)-5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazole **(6a)***

A solution of compound **5a** (400.0 mg, 1.41 mmol, 30 mL DCM) was added with PPh_3 (606.6 mg, 2.12 mmol) and then stirred for 10 min. After that, the mixture was added with CBr_4 (693.5 mg, 2.12 mmol) in three portions and stirred for another 2 h at 0 °C. After completion, the mixture was concentrated under vacuum and purified by flash chromatography with ethyl acetate and petroleum ether (PE:EA = 8:1) to give 420.0 mg of compound **6a** in 86% yield as a white solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.46 (d, J = 1.9 Hz, 1H), 7.44 (d, J = 0.7 Hz, 1H), 7.38 (dd, J = 9.3, 6.6 Hz, 1H), 4.23 (s, 2H), 2.13 (tt, J = 8.4, 5.1 Hz, 1H), 1.30 (ddd, J = 6.3, 5.0, 3.8 Hz, 2H), 1.23–1.16 (m, 2H); MS(ESI)m/e[M+H]$^+$: 345.9.

*4.1.10. 4-(bromomethyl)-5-cyclopropyl-3-(2-(trifluoromethoxy)phenyl) isoxazole**(6b)***

Compound **6b** (1.52 g, a yellow oil) was synthesized from **5b** (1.5 g, 5.02 mmol) according to the procedure for **6a**. 84% yield. 1H NMR (400 MHz, Chloroform-d) δ 7.59 (dd, J = 7.6, 1.8 Hz, 1H), 7.54 (dd, J = 8.0, 1.9 Hz, 1H), 7.46–7.39 (m, 2H), 4.34 (s, 2H), 2.12 (tt, J = 8.4, 5.1 Hz, 1H), 1.29–1.25 (m, 2H), 1.21–1.15 (m, 2H); MS(ESI)m/e[M+H]$^+$: 361.9.

*4.1.11. General procedure for the synthesis of intermediates **7a-7l**. 1-(4-((5-cyclopropyl-3-(2,6-dichlorophenyl)isoxazol-4-yl)methoxy)phenyl) ethan-1-one **(7a)***

A solution of compound **6a** (138.0 mg, 0.40 mmol, 10 mL DMF) was added with 4′-hydroxyacetophenone (54.4 mg, 0.40 mmol). Then the mixture was added with K_2CO_3 (110.4 mg, 0.80 mmol) and stirred at 25 °C. After completion, the mixture was diluted with ethyl acetate (10 mL) and washed with brine (20 mL × 3). The combined organic layer was dried over anhydrous sodium sulfate, concentrated under vacuum and purified by flash chromatography with ethyl acetate and petroleum ether (PE:EA = 3:1) to give 120.6 mg of compound **7a** in 75% yield as a white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.89–7.83 (m, 2H), 7.39 (dd, J = 8.1, 0.9 Hz, 2H), 7.31 (dd, J = 9.0, 7.2 Hz, 1H), 6.85–6.80 (m, 2H), 4.87 (s, 2H), 2.53 (s, 3H), 2.17 (tt, J = 8.4, 5.1 Hz, 1H), 1.30–1.26 (m, 2H), 1.18–1.12 (m, 2H); MS(ESI)m/e[M+H]$^+$: 402.0. Synthesis of intermediates **7b-7l**. Compound **6a** or **6b** was reacted with the derivates of 4′-hydroxyacetophenone using a procedure similar to the synthesis of **7a** to afford **7b-7l** (68–90% yield).

*4.1.12. General procedure for the synthesis of intermediates **9a-9g**. Methyl 4-(bromomethyl)benzoate **(9a)***

Compound **8a** (4.505 g, 30.00 mmol), NBS (5.339 g, 30.00 mmol) and AIBN (492.6 mg, 3.00 mmol) were dissolved in CCl_4 (60 mL). Then the mixture was stirred at 70 °C. After completion, the mixture was concentrated under vacuum and purified by flash chromatography with ethyl acetate and petroleum ether (PE:EA = 8:1) to give 4.582 g of compound **9a** in 67% yield as a white solid. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.02 (d, J = 1.7 Hz, 1H), 8.00 (d, J = 2.0 Hz, 1H), 7.46 (d, J = 1.8 Hz, 1H), 7.45 (d, J = 1.9 Hz, 1H), 4.50 (s, 2H), 3.92 (s, 3H). MS (ESI)m/e[M+H]$^+$: 228.9. Synthesis of intermediates **9b-9g**. The required compounds **8b-8g** were reacted using a procedure similar to the synthesis of **9a** to afford **9b-9g** (58–72% yield).

*4.1.13. General procedure for the synthesis of intermediates **10a-10g**. Methyl 4-(((1,3-dioxoisoindolin-2-yl)oxy)methyl)benzoate **(10a)***

A solution of compound **9a** (1.831 g, 8.00 mol, 60 mL DMF) was added with N-Hydroxyphthalimide (1.956 g, 12.00 mmol) and stirred for 10 min under an inert atmosphere of nitrogen. Then, the mixture was added with DIPEA (2.067 g, 16.00 mmol) and stirred at 70 °C. After completion, the mixture was added with water. The formed precipitate was filtered, washed with water, and then dried under vacuum at 50 °C to provide 1.518 g of compound **10a** in 61% yield as a white solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.09–8.03 (m, 2H), 7.84–7.79 (m, 2H), 7.75 (dd, J = 5.5, 3.1 Hz, 2H), 7.62 (d, J = 8.2 Hz, 2H), 5.27 (s, 2H), 3.92 (s, 3H); MS(ESI)m/e[M+H]$^+$: 312.0. Synthesis of intermediates **10b-10g**. The required compounds **9b-9g** were reacted using a procedure similar to the synthesis of **10a** to afford **10b-10g** (58–85% yield).

*4.1.14. General procedure for the synthesis of intermediates **11a-11g**. O-(4-(methoxycarbonyl)benzyl)hydroxylammonium chloride **(11a)***

Butan-1-amine (292.5 mg, 4.00 mmol) was added to a solution of compound **10a** (1.244 g, 4.00 mmol, 60 mL MeOH) under an inert atmosphere of nitrogen. The mixture was stirred at 25 °C. Upon completion, the mixture was added with ethanolic solution of HCl at 0 °C until the PH was adjusted to 3. Then, the mixture was concentrated under vacuum at 25 °C to obtain the crude product. The crude product was washed with MTBE to provide 703.0 mg of compound **11a** in 81% yield as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.00 (dd, J = 8.3, 4.0 Hz, 2H), 7.60–7.51 (m, 2H), 5.11 (d, J = 2.7 Hz, 2H), 3.87 (s, 3H). Synthesis of intermediates **11b-11g**. The required compounds **10b-10g** were reacted using a procedure similar to the synthesis of **11a** to afford **11b-11g** (78–85% yield).

*4.1.15. General procedure for the synthesis of intermediates **SC1-ME and 12a-12z**. methyl (E)-4-((((1-(4-((5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)benzoate **(12a)***

A solution of compound **7a** (60.1 mg, 0.15 mmol, 10 mL MeOH) was added with compound **11a** (39.0 mg, 0.18 mmol) and stirred at 25 °C. After completion, the mixture was concentrated under vacuum and purified by flash chromatography with ethyl acetate and petroleum ether (PE: EA = 4:1) to provide 63.4 mg of compound **12a** in 75% yield as a colorless oil. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.08–7.97 (m, 2H), 7.54–7.47 (m, 2H), 7.45 (d, J = 8.1 Hz, 2H), 7.42–7.35 (m, 2H), 7.30 (dd, J = 9.0, 7.0 Hz, 1H), 6.81–6.72 (m, 2H), 5.25 (s, 2H), 4.80 (s, 2H), 3.91 (s, 3H), 2.23 (s, 3H), 2.15 (tt, J = 8.4, 5.1 Hz, 1H), 1.28–1.24 (m, 2H), 1.17–1.08 (m, 2H); MS(ESI)m/e[M+H]$^+$: 565.1. Synthesis of intermediates **SC1-ME** and **12b-12z**. The required compounds **11a-11g** were reacted using a procedure similar to the synthesis of **12a** to afford compound **SC1-ME** and **12b-12z** (58–85% yield).

*4.1.16. General procedure for the synthesis of compound **SC1 and 13a-13z**. (E)-4-((((1-(4-((5-cyclopropyl-3-(2,6-dichlorophenyl)isoxazol-4-yl) methoxy)phenyl)ethylidene)amino)oxy)methyl)benzoic acid **(13a)***

A solution (7 mL, THF: MeOH: H_2O = 3:3:1) was added with Compound **12a** (56.4 mg, 0.10 mol) and LiOH·H_2O (12.5 mg, 0.30 mmol) and then stirred at 40 °C. Upon completion, the mixture was concentrated under vacuum and then added with water to dissolve. The pH was adjusted to 4 with 2 N HCl. The formed precipitate was filtered, washed with water, and dried under vacuum below 30 °C to give 45.6 mg of final compound **13a** in 83% yield as a white solid. The required intermediates **SC1-ME** and **12b-12z** were reacted using a procedure similar to the synthesis of **13a** to afford compound **SC1** and **13b-13z** (80–92%).

*4.1.17. (E)-3-((((1-(4-((5-cyclopropyl-3-(2,6-dichlorophenyl)isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)benzoic acid **(SC1)***

White solid, 83% yield; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.14 (s, 1H, H-35), 8.04 (d, J = 7.7 Hz, 1H, H-33), 7.65 (d, J = 7.7 Hz, 1H, H-31), 7.53–7.49 (m, 2H, H-21 and H-23), 7.47 (t, J = 7.7 Hz, 1H, H-32), 7.39 (d, J = 1.4 Hz, 1H, H-12 or H-14), 7.37 (d, J = 0.6 Hz, 1H,H-12 or H-14), 7.30 (dd, J = 9.0, 7.1 Hz, 1H, H-13), 6.80–6.74 (m, 2H, H-20 and H-24), 5.25 (s, 2H, H-28), 4.80 (s, 2H, H-1), 2.23 (s, 3H, H-29), 2.15 (tt, J = 8.5, 5.1 Hz, 1H, H-7), 1.28 (dt, J = 6.9, 4.7 Hz, 2H, H-8 or H-9), 1.16–1.09 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.45, 171.65, 159.35, 159.15, 154.92, 138.91, 135.75, 133.40, 131.26, 129.76, 129.57, 129.52, 129.47, 128.57, 128.09, 127.71, 127.40, 114.60, 110.44, 77.29, 77.04, 76.79, 75.30, 59.51, 12.85, 8.41, 7.78. HR/MS (ESI): m/z calcd $C_{29}H_{24}Cl_2N_2O_5$ (M+H$^+$) 551.1135, found 551.1141.

*4.1.18. (E)-4-((((1-(4-((5-cyclopropyl-3-(2,6-dichlorophenyl)isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)benzoic acid **(13a)***

White solid, 87% yield; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.12–8.05 (m, 2H, H-21 and H-23), 7.49 (t, J = 8.2 Hz, 4H, H-31, H-32, H-34, H-35), 7.39 (d, J = 1.4 Hz, 1H, H-12 or H-14), 7.37 (s, 1H, H-12 or H-14), 7.30 (dd, J = 9.1, 7.0 Hz, 1H, H-13), 6.81–6.72 (m, 2H, H-20 and H-24), 5.27 (s, 2H, H-18), 4.80 (s, 2H, H-1), 2.24 (s, 3H, H-29), 2.16 (tt, J = 8.4, 5.1 Hz, 1H, H-7), 1.31–1.26 (m, 2H, H-8 or H-9), 1.16–1.10 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.41, 171.30, 159.32, 159.16, 154.95, 144.61, 135.75, 131.22, 130.27, 129.46, 128.46, 128.06, 127.70, 127.60, 127.36, 114.57, 110.38, 77.25, 77.00, 76.75, 75.18, 59.49, 12.81, 8.38, 7.76. HR/MS (ESI): m/z calcd $C_{29}H_{24}Cl_2N_2O_5$ (M+H$^+$) 551.1135, found 551.1124.

*4.1.19. (E)-4-((((1-(2-chloro-4-((5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)benzoic acid **(13b)***

White solid, 82% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.14–8.07 (m, 2H, H-32 and H-34), 7.51–7.45 (m, 2H, H-31 and H-35), 7.40 (d, J = 1.1 Hz, 1H, H-12 or H-14), 7.38 (d, J = 0.5 Hz, 1H, H-12 or H-14), 7.31 (dd, J = 8.9, 7.2 Hz, 1H, H-13), 7.11 (d, J = 8.5 Hz, 1H, H-21), 6.81 (d, J = 2.5 Hz, 1H, H-24), 6.67 (dd, J = 8.6, 2.5 Hz, 1H, H-20), 5.28 (s, 2H, H-28), 4.78 (s, 2H, H-1), 2.24 (s, 3H, H-29), 2.13 (tt, J = 8.4, 5.1 Hz, 1H, H-7), 1.31–1.26 (m, 2H, H-8 or H-9), 1.18–1.13 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.57, 171.10, 159.26, 159.03, 156.75, 144.41, 135.71, 133.28, 131.32, 130.85, 130.29, 129.63, 128.42, 128.10, 127.56, 127.48, 116.10, 113.60, 110.00, 77.25, 77.00, 76.74, 75.14, 59.79, 29.68, 16.79, 8.42, 7.73. HR/MS (ESI): m/z calcd $C_{29}H_{23}Cl_3N_2O_5$ (M+H$^+$) 585.0745, found 585.0755.

*4.1.20. (E)-3-((((1-(2-chloro-4-((5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)benzoic acid **(13c)***

White solid, 86% yield; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.13 (s, 1H, H-35), 8.04 (d, J = 7.8 Hz, 1H, H-33), 7.64 (d, J = 7.6 Hz, 1H, H-31), 7.47 (t, J = 7.7 Hz, 1H, H-32), 7.40 (d, J = 1.5 Hz, 1H, H-12 or H-14), 7.38 (d, J = 0.6 Hz, 1H, H-12 or H-14), 7.31 (dd, J = 9.1, 6.9 Hz, 1H, H-13), 7.13 (d, J = 8.5 Hz, 1H, H-21), 6.81 (d, J = 2.5 Hz, 1H, H-24), 6.68 (dd, J = 8.5, 2.5 Hz, 1H, H-20), 5.26 (s, 2H, H-28), 4.78 (s, 2H, H-1), 2.23 (s, 3H, H-29), 2.17–2.09 (m, 1H, H-7), 1.31–1.27 (m, 2H, H-8 or H-9), 1.19–1.10 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.60,

171.41, 159.29, 159.03, 156.70, 138.72, 135.72, 133.31, 133.21, 131.36, 130.90, 129.73, 129.60, 129.53, 129.44, 128.59, 128.13, 127.56, 116.13, 113.61, 110.06, 77.28, 77.03, 76.78, 75.24, 59.80, 16.82, 8.45, 7.76. HR/MS (ESI): *m/z* calcd $C_{29}H_{23}Cl_3N_2O_5$ ($M+H^+$) 585.0745, found 585.0760.

*4.1.21. (E)-5-((((1-(2-chloro-4-((5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)furan-2-carboxylic acid (**13d**)*

White solid, 81% yield; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.62 (d, J = 1.5 Hz, 1H, H-12 or H-14), 7.60 (s, 1H, H-12 or H-14), 7.53 (dd, J = 9.2, 6.9 Hz, 1H, H-13), 7.22–7.13 (m, 2H, H-33 and H-21), 6.96 (d, J = 2.5 Hz, 1H, H-24), 6.78 (dd, J = 8.6, 2.6 Hz, 1H, H-20), 6.64 (d, J = 3.4 Hz, 1H, H-34), 5.11 (s, 2H, H-29), 4.93 (s, 2H, H-1), 2.48–2.41 (m, 1H, H-7), 2.09 (s, 3H, H-27), 1.18 (dt, J = 8.2, 2.8 Hz, 2H, H-8 or H-9), 1.14–1.08 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.62, 162.73, 159.29, 159.09, 157.25, 156.93, 143.73, 135.72, 133.27, 131.37, 130.89, 129.47, 128.13, 127.55, 120.57, 116.15, 113.62, 111.55, 110.04, 77.29, 77.03, 76.78, 67.80, 59.80, 16.75, 8.46, 7.76.HR/MS (ESI): *m/z* calcd $C_{27}H_{21}Cl_3N_2O_6$ ($M+H^+$) 575.0538, found 575.0546.

*4.1.22. (E)-5-((((1-(2-chloro-4-((5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)nicotinic acid (**13e**)*

White solid, 84% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.24 (d, J = 2.0 Hz, 1H, H-33), 8.86 (d, J = 2.2 Hz, 1H, H-31 or H-35), 8.39 (t, J = 2.1 Hz, 1H, H-31 or H-35), 7.40 (d, J = 1.2 Hz, 1H, H-12 or H-14), 7.38 (d, J = 0.6 Hz, 1H, H-12 or H-14), 7.31 (dd, J = 9.0, 7.2 Hz, 1H, H-13), 7.11 (d, J = 8.5 Hz, 1H, H-21), 6.81 (d, J = 2.5 Hz, 1H, H-24), 6.68 (dd, J = 8.6, 2.5 Hz, 1H, H-20), 5.29 (s, 2H, H-28), 4.78 (s, 2H, H-1), 2.23 (s, 3H, H-29), 2.16–2.10 (m, 1H, H-7), 1.30–1.26 (m, 2H, H-8 or H-9), 1.17–1.12 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.58, 159.25, 159.08, 157.40, 152.21, 149.76, 137.82, 135.69, 134.29, 133.26, 131.34, 130.79, 129.37, 128.10, 127.52, 116.14, 113.61, 109.99, 77.26, 77.00, 76.75, 72.64, 59.77, 29.68, 16.84, 8.43, 7.73. HR/MS (ESI): *m/z* calcd $C_{28}H_{22}Cl_3N_3O_5$ ($M+H^+$) 586.0698, found 586.0707.

*4.1.23. (E)-3-((((1-(5-((5-cyclopropyl-3-(2,6-dichlorophenyl)isoxazol-4-yl)methoxy)pyridin-2-yl)ethylidene)amino)oxy)methyl)benzoic acid (**13f**)*

White solid, 82% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.17 (dd, J = 2.9, 0.6 Hz, 1H, H-24), 8.14 (d, J = 1.8 Hz, 1H, H-35), 8.05 (dt, J = 7.8, 1.5 Hz, 1H, H-33), 7.75 (dd, J = 8.8, 0.7 Hz, 1H, H-21), 7.65 (dt, J = 7.8, 1.4 Hz, 1H, H-31), 7.47 (t, J = 7.7 Hz, 1H, H-32), 7.39 (d, J = 1.1 Hz, 1H, H-12 or H-14), 7.37 (d, J = 0.5 Hz, 1H, H-12 or H-14), 7.30 (dd, J = 8.9, 7.2 Hz, 1H, H-13), 7.06 (dd, J = 8.8, 2.9 Hz, 1H, H-20), 5.29 (s, 2H, H-28), 4.87 (s, 2H, H-1), 2.33 (s, 3H, H-29), 2.14 (tt, J = 8.4, 5.1 Hz, 1H, H-7), 1.31–1.26 (m, 2H, H-8 or H-9), 1.18–1.12 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.57, 171.05, 159.27, 155.98, 154.65, 147.33, 138.67, 136.45, 135.68, 133.25, 131.34, 129.69, 129.55, 129.51, 128.59, 128.11, 127.53, 122.14, 121.28, 109.95, 77.25, 77.00, 76.75, 75.58, 59.98, 11.47, 8.48, 7.74. HR/MS (ESI): *m/z* calcd $C_{28}H_{23}Cl_2N_3O_5$ ($M+H^+$) 552.1088, found 552.1090.

*4.1.24. (E)-5-((((1-(5-((5-cyclopropyl-3-(2,6-dichlorophenyl)isoxazol-4-yl)methoxy)pyridin-2-yl)ethylidene)amino)oxy)methyl)furan-2-carboxylic acid (**13g**)*

White solid, 85% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.18 (dd, J = 2.9, 0.7 Hz, 1H, H-24), 7.78 (dd, J = 8.8, 0.7 Hz, 1H, H-21), 7.43 (d, J = 1.2 Hz, 1H, H-12 or H-14), 7.41 (d, J = 0.6 Hz, 1H, H-12 or H-14), 7.34 (dd, J = 8.9, 7.1 Hz, 1H, H-13), 7.28 (s, 1H, H-32), 7.12–7.07 (m, 1H, H-20), 6.55 (d, J = 3.5 Hz, 1H, H-31), 5.24 (s, 2H, H-28), 4.91 (s, 2H, H-1), 2.32 (s, 3H, H-29), 2.17 (tt, J = 8.4, 5.1 Hz, 1H, H-7), 1.35–1.30 (m, 2H, H-8 or H-9), 1.23–1.15 (m, 2H, H-8 or H-9). ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.55, 161.92, 158.81, 155.82, 154.91, 153.35, 144.82, 143.65, 135.17, 133.66, 131.08, 127.73, 126.89, 124.98, 122.24, 119.86, 111.59, 109.01, 76.82, 76.61, 76.40, 67.89, 59.94, 11.30, 8.25, 7.30. HR/MS (ESI): *m/z* calcd $C_{26}H_{21}Cl_2N_3O_6$ ($M+H^+$) 542.0880, found 542.0873.

*4.1.25. (E)-3-((((1-(2-chloro-4-((5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)-5-methylbenzoic acid (**13h**)*

White solid, 89% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.94 (s, 1H, H-33 or H-35), 7.86 (s, 1H, H-33 or H-35), 7.45 (s, 1H, H-31), 7.40 (d, J = 1.1 Hz, 1H, H-12 or H-14), 7.38 (s, 1H, H-12 or H-14), 7.31 (dd, J = 8.9, 7.2 Hz, 1H, H-13), 7.14 (d, J = 8.5 Hz, 1H, H-21), 6.81 (d, J = 2.5 Hz, 1H, H-24), 6.68 (dd, J = 8.5, 2.5 Hz, 1H, H-20), 5.22 (s, 2H, H-28), 4.78 (s, 2H, H-1), 2.43 (s, 3H, H-40), 2.23 (s, 3H, H-29), 2.14 (tt, J = 8.4, 5.0 Hz, 1H, H-7), 1.31–1.26 (m, 2H, H-8 or H-9), 1.17–1.13 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.56, 159.27, 158.99, 156.55, 138.51, 138.45, 135.70, 134.03, 133.29, 131.32, 130.88, 130.10, 129.77, 128.10, 127.55, 126.88, 116.10, 113.58, 110.03, 77.25, 77.00, 76.74, 75.30, 59.78, 21.23, 16.81, 8.42, 8.19, 7.73. HR/MS (ESI): *m/z* calcd $C_{30}H_{25}Cl_3N_2O_5$ ($M+H^+$) 599.0902, found 599.0916.

*4.1.26. (E)-3-((((1-(3-chloro-4-((5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)benzoic acid (**13i**)*

White solid, 89% yield; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.13 (s, 1H, H-33), 8.04 (d, J = 7.9 Hz, 1H, H-31), 7.68–7.59 (m, 2H, H-21 and H-29), 7.47 (t, J = 7.7 Hz, 1H, H-30), 7.43–7.36 (m, 3H, H-12, H-14 and H-18), 7.32 (dd, J = 9.1, 6.9 Hz, 1H, H-13), 6.79 (d, J = 8.6 Hz, 1H, H-19), 5.26 (s, 2H, H-26), 4.91 (s, 2H, H-1), 2.26–2.20 (m, 1H, H-7), 2.21 (s, 3H, H-27), 1.27 (dt, J = 6.2, 4.4 Hz, 2H, H-8 or H-9), 1.17–1.08 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.81, 171.81, 159.02, 154.24, 153.78, 138.68, 135.78, 133.47, 131.32, 130.59, 129.79, 129.61, 129.35, 128.62, 128.19, 128.09, 127.44, 125.35, 123.65, 113.71, 110.00, 77.27, 77.02, 76.77, 75.45, 60.70, 12.67, 8.45, 7.82. HR/MS (ESI): *m/z* calcd $C_{29}H_{23}Cl_3N_2O_5$ ($M+H^+$) 585.0745, found 585.0750.

*4.1.27. (E)-3-((((1-(2-chloro-4-((5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)-4-cyanobenzoic acid (**13j**)*

White solid, 90% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.29–8.25 (m, 1H, H-33), 8.11 (dd, J = 7.9, 1.7 Hz, 1H, H-31), 7.79 (d, J = 8.0 Hz, 1H, H-30), 7.39 (d, J = 1.1 Hz, 1H, H-12 or H-14), 7.38 (d, J = 0.6 Hz, 1H, H-12 or H-14), 7.30 (dd, J = 8.9, 7.2 Hz, 1H, H-13), 7.13 (d, J = 8.5 Hz, 1H, H-19), 6.81 (d, J = 2.5 Hz, 1H, H-22), 6.68 (dd, J = 8.6, 2.5 Hz, 1H, H-18), 5.44 (s, 2H, H-26), 4.78 (s, 2H, H-1), 2.27 (s, 3H, H-27), 2.18–2.08 (m, 1H, H-7), 1.30–1.26 (m, 2H, H-8 or H-9), 1.17–1.12 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, DMSO) δ 172.80, 166.44, 159.53, 159.16, 156.96, 142.53, 135.20, 135.06, 134.09, 132.93, 132.49, 131.37, 130.31, 129.54, 129.10, 128.86, 127.35, 117.22, 116.14, 115.18, 114.50, 110.57, 73.06, 59.77, 17.00, 8.90, 7.70, 1.61. HR/MS (ESI): *m/z* calcd $C_{30}H_{22}Cl_3N_3O_5$ ($M+H^+$) 610.0698, found 610.0716.

*4.1.28. (E)-3-((((1-(2-bromo-4-((5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)benzoic acid (**13k**)*

White solid, 87% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.14 (d, J = 1.7 Hz, 1H, H-33), 8.05 (dt, J = 7.8, 1.5 Hz, 1H, H-31), 7.64 (dt, J = 7.6, 1.4 Hz, 1H, H-29), 7.48 (t, J = 7.7 Hz, 1H, H-30), 7.40 (d, J = 1.1 Hz, 1H, H-12 or H-14), 7.38 (s, 1H, H-12 or H-14), 7.31 (dd, J = 8.9, 7.2 Hz, 1H, H-13), 7.10 (d, J = 8.5 Hz, 1H, H-19), 7.00 (d, J = 2.5 Hz, 1H, H-22), 6.72 (dd, J = 8.5, 2.5 Hz, 1H, H-18), 5.26 (s, 2H, H-26), 4.78 (s, 2H, H-1), 2.23 (s, 3H, H-27), 2.17–2.11 (m, 1H, H-7), 1.30–1.27 (m, 2H, H-8 or H-9), 1.15 (dt, J = 8.5, 3.4 Hz, 2H, H-8 or H-9). ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.16, 158.85, 158.37, 157.10, 137.92, 135.22, 132.15, 131.28, 130.93, 130.45, 129.05, 129.02, 127.95, 127.67, 127.05, 121.69, 118.68, 113.67, 109.57, 76.81, 76.60, 76.38, 74.86, 59.32,

16.63, 8.09, 7.33. HR/MS (ESI): *m/z* calcd $C_{29}H_{23}BrCl_2N_2O_5$ ($M+H^+$) 629.0240, found 629.0225.

*4.1.29. (E)-3-((((1-(4-((5-cyclopropyl-3-(2,6-dichlorophenyl)isoxazol-4-yl)methoxy)-2-(trifluoromethyl)phenyl)ethylidene)amino)oxy)methyl) benzoic acid **(13l)***

White solid, 92% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.11 (d, *J* = 1.7 Hz, 1H, H-33), 8.04 (dt, *J* = 7.8, 1.5 Hz, 1H, H-31), 7.62 (dt, *J* = 7.6, 1.4 Hz, 1H, H-29), 7.47 (t, *J* = 7.7 Hz, 1H, H-30), 7.39 (d, *J* = 1.1 Hz, 1H, H-12 or H-14), 7.38 (s, 1H, H-12 or H-14), 7.31 (dd, *J* = 8.9, 7.2 Hz, 1H, H-13), 7.17 (d, *J* = 8.5 Hz, 1H, H-19), 7.05 (d, *J* = 2.7 Hz, 1H, H-22), 6.92 (dd, *J* = 8.5, 2.6 Hz, 1H, H-18), 5.24 (s, 2H, H-26), 4.84 (s, 2H, H-1), 2.19 (s, 3H, H-27), 2.17–2.10 (m, 1H, H-7), 1.31–1.26 (m, 2H, H-8 or H-9), 1.18–1.13 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 172.56, 171.59, 159.26, 158.15, 156.14, 138.69, 135.68, 133.16, 131.40 (d, *J* = 13.8 Hz), 129.61, 129.50, 129.36, 129.29, 128.54, 128.10, 127.54, 124.55, 122.37, 117.83, 113.23–113.00 (m), 109.93, 75.21, 59.89, 17.19, 8.44, 7.72. HR/MS (ESI): *m/z* calcd $C_{30}H_{23}Cl_2F_3N_2O_5$ ($M+H^+$) 619.1009, found 619.1016.

*4.1.30. (E)-3-((((1-(4-((5-cyclopropyl-3-(2,6-dichlorophenyl)isoxazol-4-yl)methoxy)-2-fluorophenyl)ethylidene)amino)oxy)methyl)benzoic acid **(13m)***

White solid, 88% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.13 (s, 1H, H-33), 8.05 (d, *J* = 7.9 Hz, 1H, H-31), 7.64 (d, *J* = 7.6 Hz, 1H, H-29), 7.47 (t, *J* = 7.7 Hz, 1H, H-30), 7.40 (d, *J* = 1.1 Hz, 1H, H-12 or H-14), 7.38 (s, 1H, H-12 or H-14), 7.31 (td, *J* = 8.8, 8.1, 1.7 Hz, 2H, H-22 and H-13), 6.56 (dd, *J* = 8.7, 2.5 Hz, 1H, H-18 or H-19), 6.50 (dd, *J* = 12.5, 2.4 Hz, 1H, H-18 or H-19), 5.25 (s, 2H, H-26), 4.78 (s, 2H, H-1), 2.24 (d, *J* = 2.6 Hz, 3H, H-27), 2.14 (tt, *J* = 8.3, 5.1 Hz, 1H, H-7), 1.28 (dt, *J* = 6.8, 4.6 Hz, 2H, H-8 or H-9), 1.18–1.10 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 172.53, 171.29, 162.14, 160.12, 160.03, 159.27, 153.79, 138.68, 135.71, 133.34, 131.31, 130.09 (d, *J* = 5.4 Hz), 129.71, 129.55, 129.36, 128.58, 128.09, 127.57, 118.09 (d, *J* = 12.4 Hz), 110.72, 110.01, 102.95, 102.75, 75.32, 59.78, 15.64 (d, *J* = 5.3 Hz), 8.42, 7.74. HR/MS (ESI): *m/z* calcd $C_{29}H_{23}Cl_2FN_2O_5$ ($M+H^+$) 569.1041, found 569.1054.

*4.1.31. (E)-2-((((1-(2-chloro-4-((5-cyclopropyl-3-(2,6-dichlorophenyl) isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)benzoic acid **(13n)***

White solid, 88% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.93 (d, *J* = 7.8 Hz, 1H, H-32), 7.54 (d, *J* = 5.8 Hz, 2H, H-29, H-30), 7.37 (d, *J* = 8.0 Hz, 3H, H-12, H-14, H-31), 7.30 (dd, *J* = 9.0, 7.2 Hz, 1H, H-13), 7.07 (d, *J* = 8.5 Hz, 1H, H-19), 6.78 (d, *J* = 2.5 Hz, 1H, H-22), 6.63 (dd, *J* = 8.6, 2.5 Hz, 1H, H-18), 5.51 (s, 2H, H-26), 4.75 (s, 2H, H-1), 2.25 (s, 3H, H-27), 2.12 (tt, *J* = 8.4, 5.1 Hz, 1H, H-7), 1.31–1.26 (m, 2H, H-8 or H-9), 1.14 (dt, *J* = 8.4, 3.4 Hz, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.59, 159.26, 159.14, 157.03, 139.16, 135.68, 133.21, 132.36, 131.35, 131.00, 130.83, 129.12, 128.78, 128.11, 127.62, 127.51, 116.17, 113.53, 109.97, 77.25, 77.00, 76.74, 73.72, 59.75, 29.68, 16.92, 8.43, 7.72. HR/MS (ESI): *m/z* calcd $C_{29}H_{23}Cl_3N_2O_5$ ($M+H^+$) 585.0745, found 585.0748.

*4.1.32. (E)-5-((((1-(2-chloro-4-((5-cyclopropyl-3-(2-(trifluoromethoxy) phenyl)isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl) nicotinic acid **(13o)***

White solid, 88% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.30 (s, 1H, H-32), 8.91 (s, 1H, H-30 or H-34), 8.46 (s, 1H, H-30 or H-34), 7.54 (ddd, *J* = 15.8, 7.8, 1.8 Hz, 2H, two of H-12, H-13, H-14, H-15), 7.40 (tt, *J* = 7.7, 1.7 Hz, 2H, two of H-12, H-13, H-14, H-15), 7.17 (d, *J* = 8.5 Hz, 1H, H-20), 6.84 (d, *J* = 2.4 Hz, 1H, H-23), 6.71 (dd, *J* = 8.5, 2.5 Hz, 1H, H-19), 5.33 (s, 2H, H-27), 4.88 (s, 2H, H-1), 2.27 (s, 3H, H-28), 2.21–2.09 (m, 1H, H-7), 1.27 (dt, *J* = 7.0, 4.5 Hz, 2H, H-8 or H-9), 1.21–1.11 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.95, 168.40, 159.33, 159.03, 157.40, 152.40, 149.88, 146.79, 137.72, 134.21, 133.31, 131.78, 131.34, 130.82, 129.37, 127.12, 122.52, 120.92, 116.17, 113.55, 109.74, 77.25, 77.00, 76.74, 72.66, 59.86, 16.82, 8.22, 7.60. HR/MS (ESI): *m/z* calcd $C_{29}H_{23}ClF_3N_3O_6$ ($M+H^+$) 602.1300, found 602.1309.

*4.1.33. (E)-3-((((1-(2-chloro-4-((5-cyclopropyl-3-(2-(trifluoromethoxy) phenyl)isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)-5-methylbenzoic acid **(13p)***

White solid, 80% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.94 (s, 1H, H-32 or H-34), 7.87 (s, 1H, H-32 or H-34), 7.60–7.47 (m, 2H, two of H-12, H-13, H-14, H-15), 7.44 (s, 1H, H-30), 7.39 (td, *J* = 7.7, 1.1 Hz, 2H, two of H-12, H-13, H-14, H-15), 7.17 (d, *J* = 8.5 Hz, 1H, H-20), 6.83 (d, *J* = 2.5 Hz, 1H, H-23), 6.69 (dd, *J* = 8.5, 2.5 Hz, 1H, H-19), 5.22 (s, 2H, H-27), 4.87 (s, 2H, H-1), 2.41 (s, 3H, H-43), 2.24 (s, 3H, H-28), 2.20–2.07 (m, 1H, H-7), 1.29–1.24 (m, 2H, H-8 or H-9), 1.19–1.11 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.91, 159.34, 158.91, 156.46, 146.79, 138.29, 133.66, 133.32, 131.78, 131.31, 130.92, 130.09, 129.77, 127.12, 126.82, 122.56, 120.92, 116.11, 113.51, 109.76, 77.25, 76.99, 76.74, 75.38, 59.85, 21.19, 16.78, 8.20, 7.60. HR/MS (ESI): *m/z* calcd $C_{31}H_{26}ClF_3N_2O_6$ ($M+H^+$) 615.1504, found 615.1516.

*4.1.34. (E)-3-((((1-(2-chloro-4-((5-cyclopropyl-3-(2-(trifluoromethoxy) phenyl)isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl) benzoic acid **(13q)***

White solid, 83% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.15 (s, 1H, H-34), 8.07 (d, *J* = 7.7 Hz, 1H, H-32), 7.65 (d, *J* = 7.6 Hz, 1H, H-30), 7.54 (ddd, *J* = 16.3, 8.0, 1.8 Hz, 2H, two of H-12, H-13, H-14, H-15), 7.50–7.45 (m, 1H, H-31), 7.40 (td, *J* = 7.7, 1.1 Hz, 2H, two of H-12, H-13, H-14, H-15), 7.17 (d, *J* = 8.5 Hz, 1H, H-20), 6.83 (d, *J* = 2.6 Hz, 1H, H-23), 6.70 (dd, *J* = 8.5, 2.6 Hz, 1H, H-19), 5.27 (s, 2H, H-27), 4.87 (s, 2H, H-1), 2.26 (s, 3H, H-28), 2.14 (tt, *J* = 8.4, 5.1 Hz, 1H, H-7), 1.28–1.24 (m, 2H, H-8 or H-9), 1.18–1.12 (m, 2H, H-8 or H-9). ^{13}C NMR (151 MHz, $CDCl_3$) δ 172.58, 171.11, 158.91, 158.51, 156.39, 146.33, 138.23, 132.92, 132.86, 131.34, 130.96, 130.47, 129.17, 129.14, 128.82, 128.18, 126.73, 122.01, 120.52, 115.66, 113.07, 109.35, 76.79, 76.58, 76.37, 74.76, 59.38, 16.43, 7.87, 7.20. HR/MS (ESI): *m/z* calcd $C_{30}H_{24}ClF_3N_2O_6$ ($M+H^+$) 601.1348, found 601.1355.

*4.1.35. (E)-5-((((1-(4-((5-cyclopropyl-3-(2-(trifluoromethoxy)phenyl) isoxazol-4-yl)methoxy)-3-fluorophenyl)ethylidene)amino)oxy)methyl) nicotinic acid **(13r)***

White solid, 91% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.30 (d, *J* = 2.1 Hz, 1H, H-32), 8.90 (d, *J* = 2.2 Hz, 1H, H-30 or H-34), 8.47 (t, *J* = 2.1 Hz, 1H, H-30 or H-34), 7.55 (dd, *J* = 7.8, 1.8 Hz, 1H, H-12, H-13, H-14 or H-15), 7.49 (td, *J* = 7.9, 1.8 Hz, 1H, H-12, H-13, H-14 or H-15), 7.40–7.32 (m, 3H, two of H-12, H-13, H-14, H-15; H-19, H-20 or H-22), 7.25–7.20 (m, 1H, H-19, H-20 or H-22), 6.79 (t, *J* = 8.4 Hz, 1H, H-19, H-20 or H-22), 5.31 (s, 2H, H-27), 4.95 (s, 2H, H-1), 2.21 (s, 3H, H-28), 2.15 (tt, *J* = 8.4, 5.1 Hz, 1H, H-7), 1.21 (dt, *J* = 6.6, 3.5 Hz, 2H, H-8 or H-9), 1.10 (dt, *J* = 8.5, 3.5 Hz, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.13, 168.36, 159.33, 154.51, 152.50, 149.90, 146.77, 137.94, 134.19, 131.90, 131.28, 130.37, 127.07, 126.31, 122.47, 122.06, 120.79, 115.78, 114.13, 113.97, 109.84, 77.25, 76.99, 76.74, 72.85, 61.25, 12.61, 8.24, 7.61. HR/MS (ESI): *m/z* calcd $C_{29}H_{23}F_4N_3O_6$ ($M+H^+$) 586.1596, found 586.1594.

*4.1.36. (E)-3-((((1-(4-((5-cyclopropyl-3-(2-(trifluoromethoxy)phenyl) isoxazol-4-yl)methoxy)-3-fluorophenyl)ethylidene)amino)oxy)methyl)-5-methylbenzoic acid **(13s)***

White solid, 81% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.86 (s, 1H, H-32 or H-34), 7.79 (s, 1H, H-32 or H-34), 7.53 (dd, *J* = 7.9, 1.7 Hz, 1H, H-12, H-13, H-14 or H-15), 7.47 (td, *J* = 7.7, 7.2, 1.7 Hz, 1H, H-12, H-13, H-14 or H-15), 7.33 (ddd, *J* = 10.4, 6.6, 2.6 Hz, 4H, two of H-12, H-13, H-14, H-15; H-30; H-19, H-20 or H-22), 7.20 (d, *J* = 8.5 Hz, 1H, H-19, H-20 or H-22), 6.76 (t, *J* = 8.5 Hz, 1H, H-19, H-20 or H-22), 5.13 (s,

2H, H-27), 4.92 (s, 2H, H-1), 2.31 (s, 3H, H-43), 2.24–1.94 (m, 4H, H-7 and H-28), 1.21 (dt, $J = 6.6$, 4.4 Hz, 2H, H-8 or H-9), 1.14–1.04 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 173.11, 159.34, 153.78, 153.44, 151.82, 146.76, 146.66 (d, $J = 11.1$ Hz), 138.06 (d, $J = 16.2$ Hz), 133.36, 131.89, 131.24, 130.81 (d, $J = 6.5$ Hz), 127.06, 122.49, 121.94, 120.78, 115.78, 113.95 (d, $J = 20.0$ Hz), 109.86, 75.71, 61.24, 21.13, 12.49, 8.23, 7.59. HR/MS (ESI): *m/z* calcd $C_{31}H_{26}F_4N_2O_6$ ($M+H^+$) 599.1800, found 599.1800.

4.1.37. (E)-3-((((1-(4-((5-cyclopropyl-3-(2-(trifluoromethoxy)phenyl) isoxazol-4-yl)methoxy)-3-methoxyphenyl)ethylidene)amino)oxy)methyl)-5-methylbenzoic acid (13t)

White solid, 83% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.98 (d, $J = 1.7$ Hz, 1H, H-32 or H-34), 7.87 (t, $J = 1.3$ Hz, 1H, H-32 or H-34), 7.56 (dd, $J = 7.6$, 1.8 Hz, 1H, H-12, H-13, H-14 or H-15), 7.47 (tt, $J = 3.1$, 1.5 Hz, 2H, H-12, H-13, H-14 or H-15; H-30), 7.35 (ddd, $J = 8.5$, 3.0, 1.3 Hz, 2H, two of H-12, H-13, H-14, H-15), 7.22 (d, $J = 2.0$ Hz, 1H, H-22), 7.03 (dd, $J = 8.3$, 2.1 Hz, 1H, H-20), 6.72 (d, $J = 8.4$ Hz, 1H, H-19), 5.23 (s, 2H, H-27), 4.93 (s, 2H, H-1), 3.77 (s, 3H, H-43), 2.51–2.41 (m, 3H, H-44), 2.23 (s, 3H, H-28), 2.22–2.15 (m, 1H, H-7), 1.24–1.20 (m, 2H, H-8 or H-9), 1.10–1.05 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 172.86, 171.60, 159.38, 154.77, 150.00, 148.46, 146.83, 138.55 (d, $J = 17.9$ Hz), 134.39, 132.03, 131.05, 130.66, 130.15, 129.26, 127.27, 126.92, 122.69, 120.68, 118.99, 114.74, 110.34, 109.37, 75.46, 60.98, 55.73, 21.24, 12.80, 8.21, 7.66. HR/MS (ESI): *m/z* calcd $C_{32}H_{29}F_3N_2O_7$ ($M+H^+$) 611.2000, found 611.2006.

4.1.38. (E)-3-((((1-(4-((5-cyclopropyl-3-(2-(trifluoromethoxy)phenyl) isoxazol-4-yl)methoxy)-3-methylphenyl)ethylidene)amino)oxy)methyl)-5-methylbenzoic acid (13u)

White solid, 85% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.95 (d, $J = 1.7$ Hz, 1H, H-32 or H-34), 7.86 (d, $J = 2.0$ Hz, 1H, H-32 or H-34), 7.55 (dd, $J = 7.7$, 1.8 Hz, 1H, H-12, H-13, H-14 or H-15), 7.52–7.45 (m, 2H, H-12, H-13, H-14 or H-15; H-30), 7.41 (dd, $J = 2.3$, 0.9 Hz, 1H, H-22), 7.35 (tdd, $J = 7.9$, 4.0, 1.3 Hz, 3H, two of H-12, H-13, H-14, H-15; H-20), 6.73 (d, $J = 8.5$ Hz, 1H, H-19), 5.22 (s, 2H, H-27), 4.90 (s, 2H, H-1), 2.43 (s, 3H, H-43), 2.23 (s, 3H, H-28), 2.14 (tt, $J = 8.4$, 5.1 Hz, 1H, H-7), 1.96 (s, 3H, H-42), 1.26–1.23 (m, 2H, H-8 or H-9), 1.15–1.07 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 172.39, 171.59, 159.39, 157.20, 155.08, 146.87, 138.57 (d, $J = 35.8$ Hz), 134.21, 131.75, 131.17, 130.07, 129.26, 129.09, 128.49, 127.03 (d, $J = 3.4$ Hz), 124.78, 122.88, 120.78, 110.63, 110.46, 75.34, 59.65, 21.24, 15.90, 12.91, 8.08, 7.63. HR/MS (ESI): *m/z* calcd $C_{32}H_{29}F_3N_2O_6$ ($M+H^+$) 595.2050, found 595.2051.

4.1.39. (E)-5-((((1-(4-((5-cyclopropyl-3-(2-(trifluoromethoxy)phenyl) isoxazol-4-yl)methoxy)-3-methylphenyl)ethylidene)amino)oxy)methyl) nicotinic acid (13v)

White solid, 89% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 9.27 (s, 1H, H-32), 8.92–8.82 (m, 1H, H-30 or H-34), 8.45 (s, 1H, H-30 or H-34), 7.54 (dd, $J = 7.6$, 1.8 Hz, 1H, H-12, H-13, H-14 or H-15), 7.48 (td, $J = 7.9$, 1.8 Hz, 1H, H-12, H-13, H-14 or H-15), 7.35 (ddd, $J = 18.9$, 9.2, 2.1 Hz, 4H, two of H-12, H-13, H-14, H-15; H-20 and H-22), 6.72 (d, $J = 8.6$ Hz, 1H, H-19), 5.29 (s, 2H, H-27), 4.90 (s, 2H, H-1), 2.22 (s, 3H, H-28), 2.14 (tt, $J = 8.4$, 5.1 Hz, 1H, H-7), 1.94 (s, 3H, H-42), 1.23 (td, $J = 4.7$, 1.7 Hz, 2H, H-8 or H-9), 1.11 (dt, $J = 8.4$, 3.4 Hz, 2H, H-8 or H-9). ^{13}C NMR (151 MHz, Chloroform-*d*) δ 171.99, 168.68, 162.59, 158.94, 156.89, 155.41, 149.84, 146.39, 138.17, 131.27, 130.78, 128.12, 128.02, 126.61, 124.41, 122.38, 120.70, 120.35, 118.98, 110.02 (d, $J = 22.2$ Hz), 75.13, 59.17, 15.43, 12.49, 7.71, 7.19. HR/MS (ESI): *m/z* calcd $C_{30}H_{26}F_3N_3O_6$ ($M+H^+$) 582.1846, found 582.1851.

4.1.40. (E)-4-cyano-3-((((1-(4-((5-cyclopropyl-3-(2-(trifluoromethoxy) phenyl)isoxazol-4-yl)methoxy)-3-fluorophenyl)ethylidene)amino)oxy) methyl)benzoic acid (13w)

White solid, 91% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.27 (s, 1H, H-33), 8.11 (dd, $J = 8.0$, 1.7 Hz, 1H, H-31), 7.80 (d, $J = 8.1$ Hz, 1H, H-30), 7.54 (dd, $J = 7.9$, 1.8 Hz, 1H, H-12, H-13, H-14 or H-15), 7.52–7.46 (m, 1H, H-12, H-13, H-14 or H-15), 7.39–7.33 (m, 3H, two of H-12, H-13, H-14, H-15; H-18, H-19 or H-21), 7.24 (s, 1H, H-18, H-19 or H-21), 6.79 (t, $J = 8.5$ Hz, 1H, H-18, H-19 or H-21), 5.42 (s, 2H, H-26), 4.95 (s, 2H, H-1), 2.24 (s, 3H, H-27), 2.15 (tt, $J = 8.4$, 5.0 Hz, 1H, H-7), 1.24–1.19 (m, 2H, H-8 or H-9), 1.14–1.07 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 173.16, 169.51, 159.33, 154.82, 153.79, 151.83, 146.92 (d, $J = 11.2$ Hz), 146.77, 142.44, 133.17, 133.02, 131.90, 131.29, 130.78, 130.27 (d, $J = 6.5$ Hz), 129.64, 127.08, 122.45, 122.13 (d, $J = 3.2$ Hz), 120.80, 116.71, 116.47, 115.80, 114.11 (d, $J = 20.3$ Hz), 109.85, 73.12, 61.24, 12.55, 8.25, 7.61. HR/MS (ESI): *m/z* calcd $C_{31}H_{23}F_4N_3O_6$ ($M+H^+$) 610.1596, found 610.1593.

4.1.41. (E)-4-cyano-3-((((1-(4-((5-cyclopropyl-3-(2-(trifluoromethoxy) phenyl)isoxazol-4-yl)methoxy)-3-methoxyphenyl)ethylidene)amino)oxy) methyl)benzoic acid (13x)

White solid, 91% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.31 (d, $J = 1.6$ Hz, 1H, H-33), 8.11 (dd, $J = 8.1$, 1.7 Hz, 1H, H-31), 7.80 (d, $J = 8.0$ Hz, 1H, H-30), 7.55 (dd, $J = 7.6$, 1.8 Hz, 1H, H-12, H-13, H-14 or H-15), 7.51–7.45 (m, 1H, H-12, H-13, H-14 or H-15), 7.37–7.30 (m, 2H, two of H-12, H-13, H-14, H-15), 7.24 (d, $J = 2.1$ Hz, 1H, H-21), 7.01 (dd, $J = 8.3$, 2.1 Hz, 1H, H-19), 6.71 (d, $J = 8.4$ Hz, 1H, H-18), 5.43 (s, 2H, H-26), 4.93 (s, 2H, H-1), 3.76 (s, 3H, H-41), 2.25 (s, 3H, H-27), 2.19 (tt, $J = 8.4$, 5.1 Hz, 1H, H-7), 1.24–1.18 (m, 2H, H-8 or H-9), 1.10–1.04 (m, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 172.91, 169.56, 159.36, 155.77, 150.02, 148.64, 146.81, 142.56, 133.08, 133.01, 132.01, 131.07 (d, $J = 5.0$ Hz), 130.09, 129.61, 126.93, 122.62, 120.68, 119.08, 116.91, 116.58, 114.63, 110.31, 109.31, 72.95, 60.94, 55.79, 12.64, 8.22, 7.66. HR/MS (ESI): *m/z* calcd $C_{32}H_{26}F_3N_3O_7$ ($M+H^+$) 622.1796, found 622.1793.

4.1.42. (E)-4-cyano-3-((((1-(4-((5-cyclopropyl-3-(2-(trifluoromethoxy) phenyl)isoxazol-4-yl)methoxy)-3-methylphenyl)ethylidene)amino)oxy) methyl)benzoic acid (13y)

White solid, 88% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.32 (d, $J = 1.7$ Hz, 1H, H-33), 8.13 (dd, $J = 8.0$, 1.7 Hz, 1H, H-31), 7.82 (d, $J = 8.0$ Hz, 1H, H-30), 7.57 (dd, $J = 7.6$, 1.8 Hz, 1H, H-12, H-13, H-14 or H-15), 7.52 (td, $J = 8.0$, 1.8 Hz, 1H, H-12, H-13, H-14 or H-15), 7.43 (d, $J = 2.3$ Hz, 1H, H-21), 7.38 (tt, $J = 8.0$, 3.7 Hz, 3H, two of H-12, H-13, H-14, H-15; H-19), 6.75 (d, $J = 8.5$ Hz, 1H, H-18), 5.46 (s, 2H, H-26), 4.93 (s, 2H, H-1), 2.29 (s, 3H, H-27), 2.17 (tt, $J = 8.4$, 5.1 Hz, 1H, H-7), 1.98 (s, 3H, H-40), 1.29–1.26 (m, 2H, H-8 or H-9), 1.14 (dt, $J = 8.4$, 3.5 Hz, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, Chloroform-*d*) δ 172.43, 169.58, 159.37, 157.36, 156.14, 146.86, 142.78, 133.09, 132.97, 131.73, 131.19, 130.69, 129.48, 128.56 (d, $J = 4.5$ Hz), 127.04 (d, $J = 4.8$ Hz), 124.87, 122.83, 120.78, 116.53 (d, $J = 9.0$ Hz), 110.63, 110.42, 72.89, 59.64, 15.88, 12.82, 8.09, 7.62. HR/MS (ESI): *m/z* calcd $C_{32}H_{26}F_3N_3O_6$ ($M+H^+$)606.1846, found 606.1856.

4.1.43. (E)-3-((((1-(2-chloro-4-((5-cyclopropyl-3-(2-(trifluoromethoxy) phenyl)isoxazol-4-yl)methoxy)phenyl)ethylidene)amino)oxy)methyl)-4-cyanobenzoic acid (13z)

White solid, 84% yield; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.31 (dd, $J = 1.7$, 0.7 Hz, 1H, H-34), 8.14 (dd, $J = 8.0$, 1.7 Hz, 1H, H-32), 7.82 (d, $J = 8.0$ Hz, 1H, H-31), 7.57–7.51 (m, 2H, two of H-12, H-13, H-14, H-15), 7.40 (tt, $J = 7.7$, 1.4 Hz, 2H, two of H-12, H-13, H-14, H-15), 7.18 (d, $J = 8.5$ Hz, 1H, H-20), 6.83 (d, $J = 2.5$ Hz, 1H, H-23), 6.71 (dd, $J = 8.6$, 2.5 Hz, 1H, H-19), 5.47 (s, 2H, H-27), 4.87 (s, 2H, H-1), 2.32 (s, 3H, H-28), 2.17–2.10 (m, 1H, H-7), 1.26 (dt, $J = 5.1$, 3.1 Hz, 2H, H-8 or H-9), 1.16 (dt, $J = 8.4$, 3.5 Hz, 2H, H-8 or H-9). ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.22, 166.44, 159.40, 159.24, 156.98, 146.38, 142.54, 135.21, 134.08, 132.53, 132.42, 132.10, 131.42, 130.30, 129.54, 129.13, 128.28, 122.59, 121.87, 117.22, 116.28, 115.17, 114.53, 110.22, 73.07, 59.83, 16.99, 8.69, 7.57. HR/MS (ESI): *m/z* calcd $C_{31}H_{23}ClF_3N_3O_6$ ($M+H^+$) 626.1300, found 626.1294.

4.2. Biological studies

4.2.1. FXR activation assay

The Huh7 cells were kindly provided by stem cell bank, Chinese Academy of Sciences. Cells were grown in DMEM supplemented with 10% FBS, 1% glutamax, 1% non-essential amino acids at 37 °C in a humidified atmosphere of 5% CO_2. The cells were seeded in 100-mm dish one day before the transfection. The expression plasmid encoding human FXR (Genecopoeia, Guangdong, China) and FXR response element (FXRE)-Luc reporter plasmid (Genomeditech, Shanghai, China) were transiently co-transfected according to the manufacturer's instruction of FuGENE 6. Six hours after transfection, medium was replaced with Phenol-Red-free DMEM supplemented with 10% charcoal stripped FBS (Biological Industries, Kibbutz Beit-Haemek, Israel), 1% glutamax, 1% non-essential amino acids and incubated overnight. Cells were then planted into 96-well plates with a density of 2×10^4 cells per well. Six hours after plating, cells were treated with vehicle (0.5% DMSO) or different concentrations of compounds for another 16 h. Subsequently, luciferase activity was determined by using Steady-Glo luciferase assay system (Promega, WI, USA). The efficacy of OCA (10 μM) is set as 100%. Experiments were performed in triplicate. EC_{50} of final compounds were determined by nonlinear regression analysis (Graphpad Prism, CA, USA).

4.2.2. hTGR5 agonist assay

Agonistic effect of compounds on hTGR5 were evaluated by TGR5-CRE-driven luciferase assay. hTGR5/CRE/HEK293 stable cell line was obtained by transfection of HEK293 cells with hTGR5-pcDNA3.1 and CRE-driven luciferase reporter plasmid (pGL4.29, Promega, Madison, WI, USA). Cells were plated into 96-well plates and incubated at 37 °C, 5% CO_2 overnight. Then, cells were treated with fresh medium which contained different concentrations of compounds, positive control (20 μM INT-777) or vehicle control (0.5% DMSO) for 5.5 h. Luciferase activity of each well was then determined by Steady-Glo luciferase assay system. Value of the vehicle control was set as 0%, and value of positive control was set as 100%. EC_{50} was determined by nonlinear regression analysis (Graphpad Prism).

4.2.3. Animals

The animals were housed under a 12-h light and 12-h dark cycle and were allowed free access to regular chow and water. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC), Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

4.2.4. FXR target gene expression in primary mouse hepatocytes

Primary mouse hepatocytes were isolated from overnight-fasted male C57BL/6J mice (8–12 weeks old) using a previously described protocol.[35] Isolated hepatocytes were then plated in 24 well plates and incubated 4 h to allow their adhesion. Cells were then treated with vehicle (0.5% DMSO) or different doses of compounds for 24 h.

4.2.5. FXR target gene expression in mouse liver and intestine

Male C57BL/6J mice (7–8 weeks) mice were fasted for 3 h before oral dosing with compound **13j** (10 mg/kg), **13z** (10 mg/kg), OCA (30 mg/kg) or vehicle (0.25% CMC-Na, wt/vol). Animals were sacrificed six hours after dosing, and the samples of liver and ileum were collected, snap-frozen and stored at −80 °C for gene expression analysis.

4.2.6. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

RNA was extracted from the primary hepatocytes or tissues using TRIzol reagent (Life Technologies, CA, USA). The cDNA was generated by a Primer Script RT reagent kit with gDNA eraser (TaKaRa Biotechnology, Dalian, China) and analyzed via quantitative real-time PCR using SYBR Premix Ex Taq Kit. The relative amount of individual mRNA was normalized to the expression of GAPDH mRNA using the ΔΔCt method. The primer sequences used in this study were listed in Table 5.

Table 5
Primers used for qRT-PCR analysis.

Gene	Forward (5′–3′)	Reverse (5′–3′)
SHP	TGAGCTGGGTCCCAAGGA	CCTGGCACATCTGGGTTGA
BSEP	TCTGACTCAGTGATTCTTCGCA	CCCATAAACATCAGCCAGTTGT
CYP7A1	CAAGAACCTGTACATGAGGGAC	CACTTCTTCAGAGGCTGCTTTC
CYP8B1	CCTCTGGACAAGGGTTTTGTG	GCACCGTGAAGACATCCCC
FGF15	GAGGACCAAAACGAACGAAATT	ACGTCCTTGATGGCAATCG
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

4.2.7. Pharmacokinetics

Compound **13j** and **13z** (15 mg/kg) were orally administrated to overnight-fasted ICR mice (male, n = 3 per time point) separately. 2 h after the administration, the mice were refed. Blood samples were collected before oral administration and 1.5 h, 4 h and 8 h after oral administration, and then centrifuged at 4500 rpm for 10 min at 4 °C to obtain serums. Livers were collected before oral administration and 1.5 h, 4 h and 8 h after oral administration. Liver samples were then snap-frozen and stored at −80 °C. 100 μL of methanol/acetonitrile (1:1, v/v) was added to 10 μL of serum. Then the mixture was precipitated, vortex for 1 min, and centrifuged (11,000 rpm) for 5 min to obtain the supernatant. After that, 20 μL of supernatant was redissolved in 20 μL of ACN/H_2O (1:1, v/v) and the mixture was analyzed in LC-MS/MS. Liver sample was added with ten times the weight of methanol/acetonitrile (1:1, v/v) and the mixture was homogenized at 50 Hz for 120 s in homogenizer to obtain the homogenate. The homogenate was centrifugated (11,000 rpm) for 5 min and the supernatant was collected. Then 20 μL of supernatant was redissolved in 20 μL of ACN/H_2O (1:1, v/v) and the mixture was analyzed in LC-MS/MS.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

All authors participated in this study and they approved to the final version. There is no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2021.116280.

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