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Discovery and structural development of small molecules that enhance transport activity of bile salt export pump mutant associated with progressive familial intrahepatic cholestasis type 2

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ABSTRACT

Progressive familial intrahepatic cholestasis type 2 (PFIC2) is caused by hereditary mutations of bile salt export pump (BSEP), such as E297G BSEP, which is a folding-defective mutant that is unable to traffic beyond the endoplasmic reticulum (ER). 4-Phenylbutyric acid (4-PBA) enhances the cell surface expression and transport capacity of E297G BSEP, but has a relatively high dose (1 mM or more) is required to show the effect. Here, we show that bile acids possibly act as pharmacological chaperones, promoting the proper folding and trafficking of E297G BSEP. We also describe the discovery and structural development of non-steroidal compounds with potent pharmacological chaperone activity for E297G BSEP.

1. Introduction

Progressive familial intrahepatic cholestasis type 2 (PFIC2) is a recessive hereditary liver disease, characterized by cholestasis and jaundice in the first year of life and leading to cirrhosis and death before adulthood, unless liver transplantation is carried out.[1] Although ursodeoxycholic acid has been widely used in treatment of the disease, it is considered to be a symptomatic treatment which is effective only in a small proportion of patients (approx 10%).[2] Therefore, alternative agents for the treatment of PFIC2 are urgently needed. Many studies have been performed in PFIC2 patients, and the cause of the disease was shown to be a hereditary defect in the expression of bile salt export pump (BSEP/ABCB11). BSEP is a member of the ATP-binding cassette transmembrane transporter family and mediates the excretion of monovalent bile acids (such as taurocholate) from liver cells. Genomic analysis of PFIC2 patients has revealed many kinds of missense, premature termination, frame-shift and splicing-junction mutations in the BSEP gene.[3] Among them, two missense mutations, E297G (Glu297Gly) and D482G (Asp482Gly), are frequently observed in PFIC2 patients.[4,5] Generally, wild-type (WT) BSEP is synthesized and folded at the endoplasmic reticulum (ER). Then, correctly folded BSEP acquires Golgi-related glycosylation and is trafficked to the plasma membrane. But, the E297G mutant is considered to be folding-defective (misfolded). As a result, it is retained within the cell on endoplasmic reticulum (ER), and does not acquire the Golgi-related glycosylation. Hayashi et al. reported that the E297G and D482G mutations cause a reduction of BSEP on plasma membrane, but importantly, they found that the transport function of both mutated BSEPs is almost normal if they localized correctly.[5]

Thus, the folding-defective nature, linked to abnormal trafficking (retention at ER), of E297G mutant lies at the core of the etiology of PFIC2, at least in part. If E297G BSEP could be induced to fold properly, the mutant protein could be transported to the correct localization, where it should be functional, providing a promising strategy for treatment of E297G BSEP-related PFIC2. Hayashi et al. reported that sodium 4-phenylbutylic acid (4-PBA) enhanced the cell-surface expression and transport capacity of E297G BSEP by prolonging the half-life of cell surface-resident BSEP, through modulating its ubiquitination status and AP2-mediated internalization.[5–7] Although 4-PBA can be beneficial for the treatment for PFIC2, a relatively high dose (1 mM or more) is required to rescue the function of the mutant BSEP in vivo. Therefore, the development of more potent compounds is a pivotal work to improve clinical application of the medical therapy for PFIC2. Based on this background, we searched for novel compounds able to enhance the transport capacity of E297G BSEP at lower doses.

Previously, we and others have investigated the usefulness of pharmacological chaperones for treatment of diseases caused by folding-defective membrane proteins.[8–12] A pharmacological chaperone is defined as a small molecule that specifically binds to its

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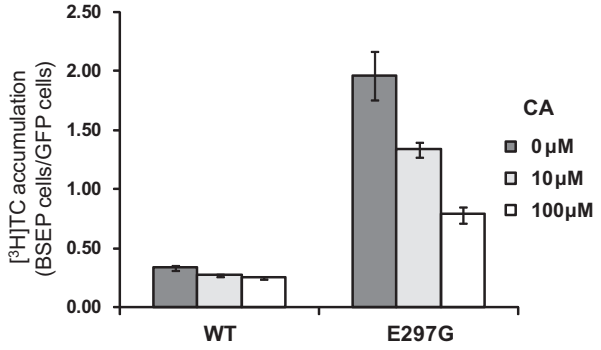


Figure 1. Concentration-dependence of the effect of cholic acid (CA) on [^{3}H]TC accumulation in MDCK II cells expressing wild type (WT) BSEP or E297G BSEP. Vertical scale: The amounts of [^{3}H]TC accumulated in the MDCK II cells transfected with WT BSEP, E297G BSEP or GFP were measured by means of radioactivity (dpm). The ratio between the [^{3}H]TC accumulated in the BSEP-transfected MDCK II cells and that in the GFP-transfected cells are presented.

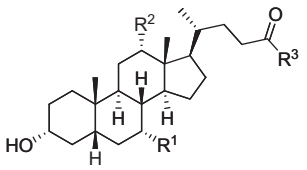
target protein and induces or promotes proper folding and trafficking of the protein.[13] The advantages of pharmacological chaperones are considered to include high specificity and efficacy at much lower dose levels than 4-PBA.[13] In the cases of some folding-defective mutant proteins, including mutated rhodopsin, it has been shown that their ligands (substrates, inhibitors, modulators, etc.) act as pharmacological chaperones.[8] Therefore, we hoped that bile acids, which are substrates of BSEP, might act as pharmacological chaperones of E297G BSEP, consequently enhancing the cell-surface expression and transport capacity of E297G BSEP. Here, we show that bile acids do act as pharmacological chaperones of E297G BSEP. We also describe the discovery and structural development of non-steroidal compounds with potent pharmacological chaperone activity for E297G BSEP.

2. Results and discussion

2.1. Effects of bile acids on [^{3}H]TC accumulation

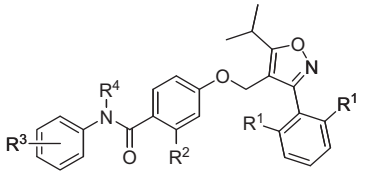
To screen compounds that enhance the transport capacity of E297G BSEP, we established a functional assay system, using Madin-Darby canine kidney II (MDCKII) cells expressing Na^+-taurocholate cotransporting polypeptide (NTCP) and WT or E297G BSEP. The amount of [^{3}H]taurocholate ([^{3}H]TC) accumulation in cells expressing E297G BSEP was higher than that in cells

Table 1
Decreasing effect of bile acids on [^{3}H]TC accumulation



Compd	R^1	R^2	R^3	Accumulation of [^{3}H] TC (%)	
				100 μM	10 μM
Cholic acid (CA)	OH	OH	OH	40	73
Glycocholic acid (GC)	OH	OH	Glycine	108	
Taurocholic acid (TC)	OH	OH	Taurine	105	
Deoxycholic acid (DCA)	H	OH	OH	65	
Chenodeoxycholic acid (CDCA)	β-OH	H	OH	46	50
Ursodeoxycholic acid (UDCA)	α-OH	H	OH	43	76

Table 2
Decreasing effect of GW4064 and derivatives on [^{3}H]TC accumulation



Compd	R^1	R^2	R^3	R^4	Accumulation of [^{3}H]TC (%) at 10 μM
CDCA					50
GW4064					33
6g	Cl	Cl	3-COOH	H	111
6a	Cl	Cl	3-COOH	Me	66
6b	Cl	Cl	3-COOH	n-Bu	49
6c	Cl	Cl	3-COOH	Bn	31
6d	Cl	Cl	3-COOH	Phenethyl	53
6e	Cl	Cl	3-COOH	1-Naphthyl	59
6f	Cl	Cl	3-COOH	2-Naphthyl	70
15a	H	Cl	3-COOH	Bn	64
15b	Cl	H	3-COOH	Bn	85
15c	Me	Cl	3-COOH	Bn	93
15d	Cl	Cl	2-COOH	Bn	80
15e	Cl	Cl	4-COOH	Bn	35
5c	Cl	Cl	3-COOMe	Bn	93

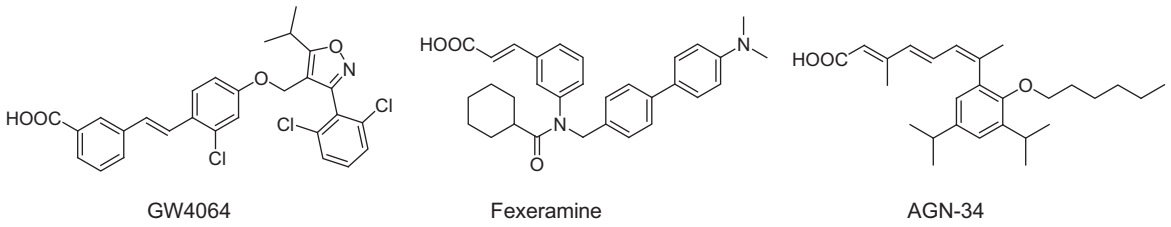


Figure 2. Structures of representative FXR ligands.

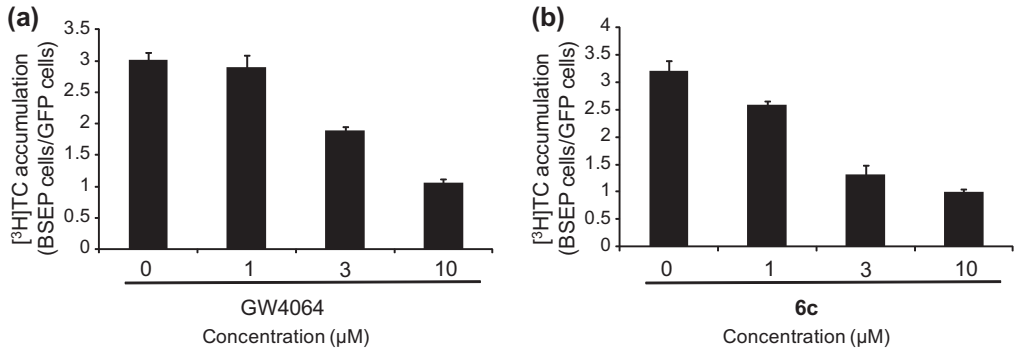
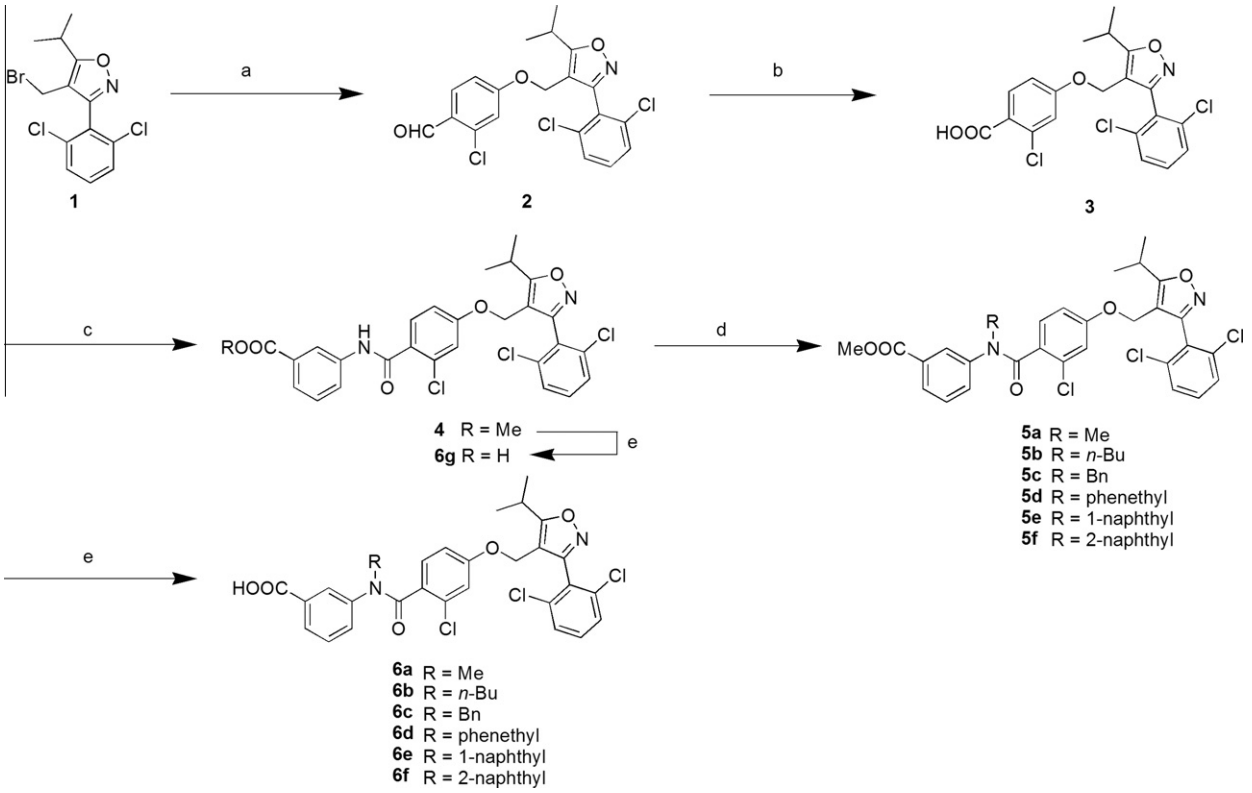
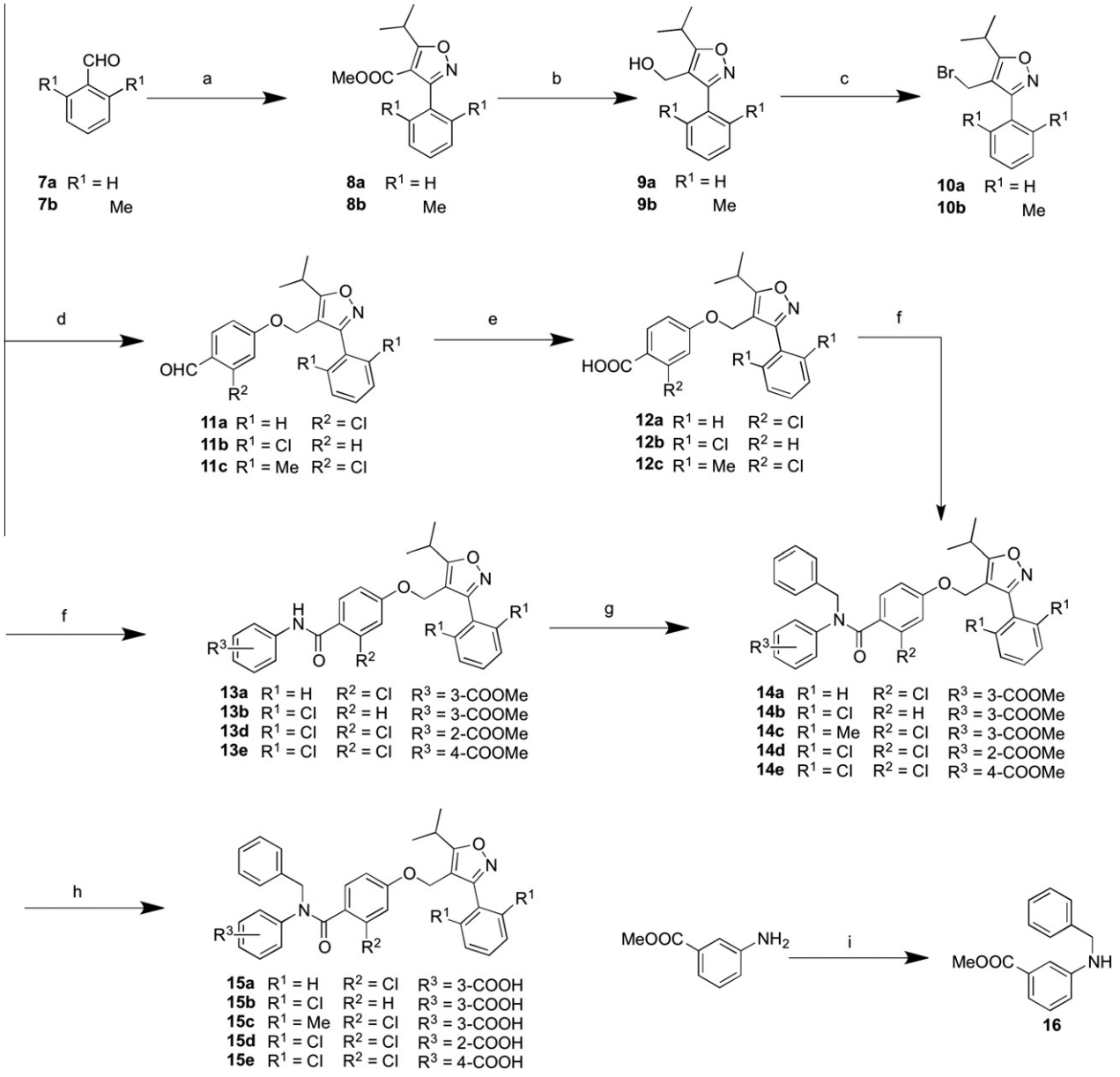


Figure 3. Concentration-dependence of the effects of GW4064 (a), and **6c** (b) on [^{3}H]TC accumulation in MDCK II cells expressing E297G BSEP. Vertical scale: same as the legend of Figure 1.



Scheme 1. Synthetic routes to the present series of amide compounds. Reagents and conditions: (a) 2-chloro-4-hydroxybenzaldehyde, K_2CO_3, DMF, rt; (b) 2-methyl-2-butene, $NaClO_2$, NaH_2PO_4, *t*-BuOH, H_2O, rt; (c) (i) methyl 3-aminobenzoate, MsCl, Et_3N, THF, rt; (d) NaH, RBr, DMF, rt; (e) LiOH, H_2O, THF, rt.

expressing WT BSEP because of the cellular efflux function of [^{3}H]TC by BSEP and the lower cell-surface expression of E297G BSEP compared with WT BSEP (Fig. 1).[14] We first examined the effect of several BSEP substrates and other related bile acids, that is, cholic acid (CA), glycocholic acid (GC), taurocholic acid (TC), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA), on the function of E297G BSEP. As shown in Table 1, the bile acids examined, with the exceptions of GC and



Scheme 2. Synthetic routes to the present series of compounds. Reagents and conditions: (a) (i) NH_2OH–HCl, NaOH, EtOH, 90 °C; (ii) NCS, DMF, rt; (iii) 4-methyl-3-oxopentanoic acid methyl ester, Et_3N, NaOMe, MeOH, rt; (b) DIBAL, THF, 0 °C; (c) CBr_4, PPh_3, DCM, rt; (d) 2-chloro-4-hydroxybenzaldehyde or 4-hydroxybenzaldehyde, K_2CO_3, DMF, rt; (e) 2-methyl-2-butene, $NaClO_2$, NaH_2PO_4, *t*-BuOH, H_2O, rt; (f) (i) methyl aminobenzoates (for **13a**, **b**, **d**, **e**) or **16** (for **14c**), MsCl, Et_3N, THF, rt; (g) NaH, BnBr, DMF, rt; (h) LiOH, H_2O, THF, rt; (i) benzaldehyde, AcOH, $NaBH(OAc)_3$, DCM, rt.

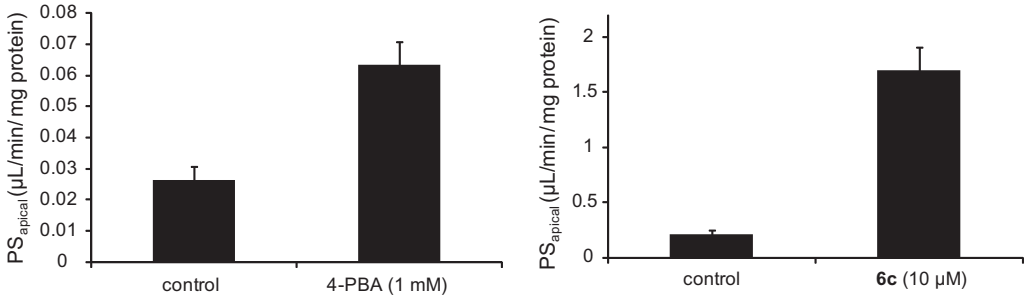


Figure 4. Effect of 4-PBA and **6c** on [^{3}H]TC transport across the apical membrane in monolayers of MDCK II cells expressing E297G BSEP. The clearance (PS_{apical}) was determined as described in the Experimental section. Each bar represents the mean ± SE of triplicate determinations.

TC, possessed moderate to potent BSEP-function promoting activity. These compounds dose-dependently decreased the amount of [^{3}H]TC accumulation in the concentration range of 1–100 μM (Fig. 1). CDCA showed the most potent activity among the tested

bile acids, decreasing[^{3}H]TC accumulation by 50% at 10 μM (Table 2). We speculated that bile acids increased the cell-surface expression of E297G BSEP by acting as pharmacological chaperones, thereby decreasing [^{3}H]TC accumulation in cells expressing E297G BSEP.

2.2. Molecular design for novel non-steroidal compounds

Though CDCA showed potent activity, it has steroid skeleton, and so might influence other proteins, such as nuclear receptors. Therefore, we searched for active compounds with a non-steroidal skeleton. For this purpose, we focused on farnesoid X receptor (FXR) ligands. FXR is a well-characterized member of the so-called metabolic subfamily of nuclear receptors, and is a transcriptional sensor for bile acids. CDCA is an endogenous FXR ligand,[15,16] and acts as a signaling molecule that governs lipid, steroid, and cholesterol homeostasis.[17] FXR is involved in possesses such as glucose utilization, inflammation, and carcinogenesis.[18,19] After deorphanization of FXR in 1999, extensive studies were directed to the creation of FXR ligands that could modulate FXR-mediated specific gene expression, and several steroidal or non-steroidal FXR ligands have been reported (Fig. 2).[20–22] We hypothesized that non-steroidal FXR ligands might act as a structural equivalent of CDCA and enhance the transport capacity of E297G BSEP. First, we investigated whether FXR ligands enhance the transport capacity of E297G BSEP. We found that GW4064, which is a potent non-steroidal agonist for FXR, dose-dependently decreased [^{3}H]TC accumulation (Fig. 3**a**). GW4064 has the advantage that its basic architecture is distinct from that of CDCA, and it shows no activity towards other nuclear receptors, including the retinoic acid receptor.[20] GW4064 seemed to be superior to CDCA in its efficacy to decrease [^{3}H]TC accumulation in cells expressing E297G BSEP (Table 2), and was selected as a lead compound for further structural development.

2.3. Structure–activity relationship of GW4064 derivatives

Kainuma and co-workers reported that the double bond of GW4064 can be replaced with a carbamoyl moiety without loss of FXR activity.[23] Similarly, we designed and prepared secondary amide **6g**, *N*-methyl amide **6a**, *N*-butyl amide **6b**, *N*-benzyl amide **6c**, *N*-phenethyl amide **6d**, and *N*-naphthyl amide **6e** and **6f** derivatives, and examined whether these derivatives decrease [^{3}H]TC accumulation in cells expressing E297G BSEP. Synthetic routes to the present series of compounds are outlined in Scheme 1. Substituted isoxazoles (**1**), which were prepared as described in the literature,[20] were reacted with 2-chloro-4-hydroxybenzaldehyde (**2**). The resulting aldehydes were oxidized to the carboxylic acids **3**, which were condensed with ethyl aminobenzoate to afford the amide linker derivatives **4**. *N*-Alkylation (**5a**–**f**) and saponification of the ester yielded carboxylic acid derivatives **6a**–**g**.

We evaluated the effect of the prepared compounds on [^{3}H]TC accumulation. As shown in Table 2, secondary amide derivative **6g** was ineffective at 10 μM, but introduction of an alkyl chain or a benzyl/phenethyl/naphthyl group at the amide linkage resulted in BSEP-function-promoting activity. *N*-Benzyl derivative **6c** showed the most potent activity among the amide derivatives, and dose-dependently decreased [^{3}H]TC accumulation (Fig. 3**b**). The pharmacological chaperone-like efficiency of the *N*-alkyl/*N*-benzyl amide derivatives **6a, b, c, g** decreased in the order of **6c** > **6b** > **6a** >> **6g**, and that of *N*-benzyl/phenethyl/naphthylamide derivatives **6c–f** decreased in the order of **6c** > **6d** > **6e** > **6f**. These results suggest that the size of the binding pocket hosting the central region of GW4064 derivatives might be limited to accommodate a benzyl or smaller group.

Next, we turned our attention to SAR of the carboxyl group and substituents such as Cl. Synthetic routes to the present series of the compounds are outlined in Scheme 2. Substituted isoxazoles **10a** and **10b**, which were prepared as described in the literature,[20] were reacted with 4-hydroxybenzaldehyde (**11a**) or 2-chloro-4-hydroxybenzaldehydes (**11b**–**c**). The resulting aldehydes were oxidized to the carboxylic acids **12**, which were condensed with methyl aminobenzoate to afford the amide linker derivatives **13a–b**, **13d–e**, and **14c**. *N*-Alkylation (**14a–b** and **14d–e**) and saponification of the ester yielded carboxylic acid derivatives **15a–e**.

In the case of GW4064, a brief SAR report from GSK researchers disclosed that a carboxyl group is essential and preferably located at the 3-position (*meta*-position) for FXR-agonistic activity.[20] We evaluated the BSEP-function-promoting activity of regioisomers, that is, the carboxyl group was shifted to the *ortho*-(**15d**) or *para*-position (**15e**) from the *meta*-position, as well as the effect of esterification of the *para*-carboxyl group (**5c**). As shown in Table 2, ester derivative **5c** had no effect on [^{3}H]TC accumulation in cells expressing E297G BSEP at 10 μM. On the other hand, *ortho*-substitution (**15d**) decreased the BSEP function-promoting activity, while *para*-substitution (**15e**) or *meta*-substitution (**6c**) did not affect the BSEP function-promoting activity. These results indicate that a carboxyl substituent at the *meta*- or *para*- position is important for interaction with E297G BSEP.

Next, we turned our attention to SAR of the Cl substituent. We prepared derivatives in which one of the Cl groups was substituted with a hydrogen (**15a** and **15b**) or a methyl group (**15c**), but these derivatives showed only weak BSEP function-promoting activity.

2.4. Transcellular transport assay

Finally, we evaluated the effectiveness of **6c** in a transcellular transport assay with MDCK II cells expressing E297G BSEP. [^{3}H]TC accumulation is determined by several parameters, including cellular efflux of [^{3}H]TC mediated by BSEP, but in the transcellular transport assay with MDCK II monolayers, the function of BSEP can be directly evaluated by measuring the efflux of [^{3}H]TC across the apical membrane after addition of [^{3}H]TC on the basal side, because BSEP is localized on apical membrane of MDCK II monolayers.[5] To directly confirm the effect of compounds on the function of BSEP, PS_{apical}, a kinetic parameter representing [^{3}H]TC transport across the apical membrane, was calculated. Treatment with 1 mM 4-PBA and 10 μM **6c** increased PS_{apical} by 3- and 7-fold, respectively (Fig. 4). Thus, **6c** enhanced the transport capacity at much lower concentration than 4-PBA.

3. Conclusion

We focused on the idea that the transport capacity of E297G BSEP might be increased by pharmacological chaperones that promote proper trafficking of the mutant BSEP. First, we established that bile acids decrease [^{3}H]TC accumulation in cells expressing E297G BSEP. Moreover, we discovered that non-steroidal GW4064 and its analogs also show this activity. Compound **6c** increased PS_{apical} by 7-fold at 10 μM. Further studies to examine in detail the molecular mechanism(s) of the enhancement of E297G BSEP trafficking and the effects of these compounds on other BSEP mutants are in progress.

4. Experimental

4.1. General

Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded on a JEOL JNMGX500 (500 MHz) spectrometer in the indicated solvent. Chemical shifts (δ) are reported in parts per

million relative to the internal standard tetramethylsilane. High-resolution mass spectra (HRMS) and fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMA-HX110 mass spectrometer. Bile acids were purchased from Aldrich, Tokyo Kasei Kogyo, Wako Pure Chemical Industry, and Kanto Kagaku, and used without purification. Flash column chromatography was performed using silica gel 60 (particle size 0.060–0.210 mm) supplied by Kanto Kagaku.

4.2. Biology

4.2.1. Materials and cell culture

[^{3}H]Taurocholate (TC) (2 Ci/mmol) was purchased from NEN Life Sciences (Boston MA). The BD Adeno-X-Adenoviral Expression System (BD Biosciences, Palo Alto, CA) was used to establish human WT and E297G BSEP recombinant adenoviruses as previously described.[14] As a control, recombinant adenovirus containing green fluorescence protein (GFP) was used. Madin-Darby canine kidney II (MDCK II) cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) containing 10% FBS, penicillin and streptomycin mixture at 37 °C in a humidified atmosphere of 5% CO_2 in air.

4.2.2. Generation of recombinant adenovirus

The recombinant adenoviruses containing wild-type BSEP, E297G BSEP, or GFP were constructed as described previously.[14] The BSEP cDNA was amplified via polymerase chain reaction(PCR) with Ex-Taq (Takara Bio, Shiga, Japan) from the commercially available cDNA library (Clontech, PaloAlto, CA). Cryptic bacterial promoter was inactivated as previously described[24] before being cloned into the adenovirus shuttle vector pShuttle (Clontech). Construction of the PFIC2 mutants was performed using a QuikChangeXL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The BSEP cDNA was sequenced in an ABI 377DNA Sequencer (Applied Biosystems, Foster City, CA). The sequence of the wild-type BSEP cDNA was identical to that published (accession number, AF091582).

The cloned wild-type and two mutated cDNAs were introduced into the adenovirus vector (Clontech). There combinant adenoviruses were produced using the adenovirus expression system, and the titer was checked with an Adeno-X Rapid Titer Kit (Clontech). As a control, recombinant adenoviruses containing green fluorescence protein (GFP) were used.

4.2.3. Transcellular transport assay

MDCK II cells were seeded on Transwell membrane inserts (pore size 3 μM; Falcon, Bedford, MA) in 24-well plates at a density of 1.5×10^5 cells per insert. After 2 days' culture, confluent cells were infected with recombinant adenovirus containing cDNA for WT, E297G, BSEP or GFP at 50 MOI. Cells were cultured for 24 hours after infection and subsequently treated with compounds. Then, 24 hours after treatment, transcellular transport assays were performed as described.[5] The apparent efflux clearance across the apical membrane (PS_{apical}) was calculated by dividing the steady-state rate for the transcellular transport of [^{3}H]TC determined over 2 h by the cellular concentration of [^{3}H]TC determined at the end of the experiment (2 h).

4.2.4. [^{3}H]TC accumulation assay

MDCK II cells were seeded on 24-well plates at a density of 1.5×10^5 cells. After 1 day of culture, confluent cells were infected with recombinant adenovirus containing cDNA for WT, E297G BSEP or GFP at 50 MOI. Cells were cultured for 24 h after infection and subsequently treated with compounds. Then, 24 h after treatment, the medium was removed and cells were washed with Krebs–Henseleit buffer (118 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.53 mM $CaCl_2$, adjust to pH 7.4). After incubation with [^{3}H]TC solution for 2 h, cells were washed three times with ice-cold Krebs–Henseleit buffer. Cells were dissolved in 2 N HCl and neutralized with 4 N NaOH, and [^{3}H]TC was measured by liquid scintillation counting.

4.3. Chemistry

4.3.1. 2-Chloro-4-{[5-isopropyl-3-(2,6-dichlorophenyl)isoxazol-4-yl]methoxy}benzaldehyde (2)

To a solution of **1** (261 mg, 0.748 mmol) in DMF (5.0 mL) were added 2-chloro-4-hydroxybenzaldehyde (120 mg, 0.766 mmol) and potassium carbonate (210 mg, 1.53 mmol), and the mixture was stirred for 3.0 h at room temperature. Then H_2O and AcOEt were added to it. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by silica gel chromatography (*n*-hexane/AcOEt = 4/1 v/v) to afford **2** (245 mg, 77%) as a colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.30 (s, 1H), 7.82 (d, J = 8.6 Hz, 1H), 7.41 (d, J = 7.6 Hz, 2H), 7.33 (t, J = 7.6 Hz, 1H), 6.79 (s, 1H), 6.75 (d, J = 8.6 Hz), 4.80 (s, 2H), 3.32 (septet, J = 7.3 Hz, 1H), 1.44 (d, J = 7.3 Hz, 6H); MS (FAB, $[M+H]^+$) m/z 424.

4.3.2. 2-Chloro-4-{[5-isopropyl-3-(2,6-dichlorophenyl)isoxazol-4-yl]methoxy}benzoic acid (3)

To a solution of **2** (795 mg, 1.82 mmol) in *t*-BuOH (15. 0 mL) were added NaH_2PO_4 (267 mg, 2.25 mmol), 2-methyl-2-butene (830 μL, 8.0 mmol), $NaClO_2$ (674 mg, 7.45 mmol) and H_2O (5.0 mL), and the mixture was stirred overnight at room temperature. Then H_2O and AcOEt were added to it. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by silica gel chromatography (*n*-hexane/AcOEt = 2/1 v/v) to afford **3** (797 mg, 97%) as a colorless solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.96 (d, J = 8.6 Hz, 1H), 7.41 (d, J = 7.6 Hz, 2H), 7.33 (t, J = 7.6 Hz, 1H), 6.85 (s, 1H), 6.71 (d, J = 8.6 Hz), 4.78 (s, 2H), 3.32 (septet, J = 7.3 Hz, 1H), 1.44 (d, J = 7.3 Hz, 6H); MS (FAB, $[M+H]^+$) m/z 440.

4.3.3. Methyl3-{2-chloro-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}-benzoylamino}benzoate (4)

To a solution of **3** (230 mg, 0.523 mg) in dry THF (20 mL) were added Et_3N (150 μL, 0.725 mmol), MsCl (50.0 μL, 0.628 mmol) and methyl 3-aminobenzoate (100 mg, 0.654 mmol) at 0 °C, and the mixture was stirred for 2.5 h at room temperature. Then H_2O and AcOEt were added to it. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by silica gel chromatography (*n*-hexane/AcOEt = 2/1 v/v) to afford **4** (255 mg, 85%) as a colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.12 (d, J = 15.2 Hz, 2H), 8.02 (d, J = 8.0 Hz, 1H), 7.83 (d, J = 8.0 Hz, 1H), 7.77 (d, J = 8.6 Hz, 2H), 7.47–7.40 (m, 2H), 7.34 (t, J = 8.0 Hz, 1H), 6.82 (s, 1H), 6.77 (d, J = 8.6 Hz, 1H), 4.78 (s, 2H), 3.92 (s, 3H), 3.33 (septet, J = 7.3 Hz, 1H), 1.44 (d, J = 7.3 Hz, 6H); MS (FAB, $[M+H]^+$) m/z 573.

4.3.4. Methyl3-{*N*-methyl-2-chloro-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}-benzamido}benzoate (5a)

Sixty percent Sodium hydride (2.2 mg, 0.055 mmol) was rinsed twice with *n*-hexane under an argon atmosphere to remove mineral oil, and DMF (0.50 mL) was added to it. To the suspension was added **4** (29 mg, 0.050 mmol) in DMF (0.50 mL), and the whole was stirred for 30 min at 0 °C. Methyl iodide (6.3 μL, 0.10 mmol) was added to the mixture and stirring was continued for 12 h at ambient temperature. Then 10 mL of water was added and the whole was extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (*n*-hexane/AcOEt = 3:1 to 2:1

v/v) to afford **5a** (29 mg, 98%) as a colorless oil; ^{1}H NMR (500 MHz, DMSO-d_6, 100 °C) δ 7.77–7.41 (m, 5H), 7.39 (t, J = 7.4 Hz, 1H), 7.18 (d, J = 8.5 Hz, 1H), 6.72 (s, 1H), 6.62 (d, J = 6.7 Hz, 1H), 4.81 (s, 2H), 3.84 (s, 3H), 3.42 (m, 1H), 3.31 (s, 3H), 1.31 (d, J = 6.7 Hz, 6H); MS (FAB, $[M+H]^+$) m/z 587.

4.3.5. Methyl 3-{*N*-butyl-2-chloro-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}-benzamido}benzoate (5b)

This compound was prepared from **4** (50 mg, 87.1 μmol) by means of a procedure similar to that used for **5a**. Compound **5b** (164 mg, 78%) as a pale yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78 (s, 2H), 7.37 (d, J = 7.9 Hz, 2H), 7.31 (t, J = 7.9 Hz, 1H), 7.22 (s, 2H), 6.94 (d, J = 8.6 Hz, 2H), 6.55 (s, 1H), 6.42 (d, J = 8.6 Hz, 1H), 4.61 (s, 2H), 3.90 (s, 2H), 4.78 (s, 2H), 3.24 (septet, J = 7.3 Hz, 1H), 1.36 (d, J = 7.3 Hz, 9H), 0.92 (t, J = 7.3 Hz, 3H); MS (FAB, $M+H^+$) m/z 629.

4.3.6. Methyl 3-{*N*-phenylmethyl-2-chloro-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]-methoxy}-benzamido}benzoate (5c)

This compound was prepared from **4** (255 mg, 0.444 mmol) by means of a procedure similar to that used for **5a**. Compound **5c** (217 mg, 74%) as a colorless solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74–6.97 (m, 13H), 6.56 (s, 1H), 6.42 (d, J = 8.5 Hz, 1H), 5.12 (s, 2H), 4.61 (s, 2H), 3.87 (s, 3H), 3.27–3.21 (m, 1H), 1.36 (d, J = 6.7 Hz, 6H); FAB:MS m/z 663 $(M+H)^{+\cdot}$

4.3.7. Methyl 3-{*N*-(2-phenylethyl)-2-chloro-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]-methoxy}benzamido}benzoate (5d)

This compound was prepared from 4 (78.0 mg, 0.136 mmol) by means of a procedure similar to that used for **5a**. Compound **5d** (67.0 mg, 73%) as a pale yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76–6.40 (m, 15H), 4.61 (s, 2H), 4.16 (s, 2H), 3.94 (s, 3H), 3.24 (br, 1H), 3.00 (s, 2H), 1.36 (d, J = 6.7 Hz, 6H); MS (FAB, [M+H]+) m/z 677.

4.3.8. Methyl 3-{*N*-(1-naphtylmethyl)-2-chloro-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]-methoxy}benzamido}benzoate (5e)

This compound was prepared from **4** (52.0 mg, 90.6 μmol) by means of a procedure similar to that used for **5a**. **5e** (27.0 mg, 37%) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.28 (d, J = 8.0 Hz, 1H), 7.84 (d, J = 8.0 Hz, 1H), 7.73 (d, J = 8.0 Hz, 1H), 7.64–7.50 (m, 4H), 7.35 (d, J = 7.3 Hz, 2H), 7.30 (d, J = 7.3 Hz, 1H), 7.23 (t, J = 7.3 Hz, 1H), 7.14 (s, 1H), 6.93 (d, J = 8.6 Hz, 2H), 6.72 (d, J = 7.3 Hz, 1H), 6.54 (s, 1H), 6.39 (d, J = 8.6 Hz, 1H), 5.61 (s, 2H), 3.83 (s, 3H), 3.22 (septet, J = 7.0 Hz, 1H), 1.34 (d, J = 7.0 Hz, 6H); MS (FAB, $[M+H]^+$) m/z 713.

4.3.9. Methyl 3-{*N*-(2-naphtylmethyl)-2-chloro-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]-methoxy}benzamido}benzoate (5f)

This compound was prepared from **4** (50.4 mg, 87.1 μmol) by means of a procedure similar to that used for **5a**. Compound **5f** (36.1 mg, 58%) as a pale yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.84–7.67 (m, 4H), 7.52–7.50 (m, 2H), 7.47–7.43 (m, 2H), 7.36 (d, J = 8.0 Hz, 2H), 7.33–7.28 (m, 2H), 7.05 (t, J = 8.0 Hz, 1H), 7.00 (s, 2H), 6.56 (s, 1H), 6.44 (d, J = 8.0 Hz, 1H), 5.28 (s, 2H), 4.62 (s, 2H), 3.84 (s, 3H), 3.24 (septet, J = 7.0 Hz, 1H), 1.36 (d, J = 7.0 Hz, 6H); MS (FAB, $[M+H]^+$) m/z 713.

4.3.10. 3-{2-Chloro-{4-[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}-*N*-methylbenzamido}benzoic acid (6a)

To a solution of **5a** (124 mg, 0.216 mmol) in THF (2.20 mL) was added 1 M LiOH (1.30 mL). The reaction mixture was stirred overnight at room temperature, then acidified with 2 M HCl and the whole was extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by silica gel chromatography (n-hexane/AcOEt = 1/1 v/v) to afford **6a** (104 mg, 86%) as pale yellow solid; mp 89–92 °C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.17 (d, J = 14.6 Hz, 2H), 8.09 (d, J = 8.0 Hz, 1H), 7.89 (d, J = 8.0 Hz, 1H), 7.78 (d, J = 8.5 Hz, 1H), 7.50 (t, J = 8.0 Hz, 1H), 7.42 (d, J = 7.9 Hz, 2H), 7.35 (t, J = 7.9 Hz, 1H), 6.83 (s, 1H), 6.78 (d, J = 8.5 Hz, 1H), 4.79 (s, 2H), 3.35–3.32 (m, 1H), 1.44 (d, J = 6.7 Hz, 6H); HRMS (FAB, $[M+H]^+$) calcd for $C_{27}H_{22}Cl_3N_2O_5$ 559.0594, found 559.0579.

4.3.11. 3-{2-Chloro-{4-[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}-*N*-butylbenzamido}benzoic acid (6b)

This compound was prepared from **5b** (6.30 mg, 10.0 μmol) by means of a procedure similar to that used for **6a**. Compound **6b** (4.50 mg, 73%) as pale yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 (s, 2H), 7.36 (d, J = 7.9 Hz, 2H), 7.30 (t, J = 7.9 Hz, 3H), 6.96 (d, J = 8.6 Hz, 1H), 6.55 (s, 1H), 6.44 (d, J = 8.6 Hz, 1H), 4.62 (s, 2H), 3.94 (s, 2H), 3.24 (septet, J = 7.3 Hz, 1H), 1.36 (d, J = 7.3 Hz, 9H), 0.92 (t, J = 7.3 Hz, 3H); MS (FAB, $[M+H]^+$) m/z 615. HRMS (FAB, $[M+H]^+$) calcd $C_{31}H_{30}Cl_3N_2O_5$ 615.1220, found 615.1171.

4.3.12. 3-{2-Chloro-{4-[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}-*N*-benzylbenzamido}benzoic acid (6c)

This compound was prepared from **5c** (6.3 mg, 10.0 μmol) by means of a procedure similar to that used for **6a**. Compound **6c** (4.5 mg, 73%) as pale yellow solid; mp 73–76 °C, ^{1}H NMR (500 MHz, DMSO-d_6, 100 °C) δ 7.65–7.22 (m, 12H), 7.16 (d, J = 8.5 Hz, 1H), 6.69 (s, 1H), 6.56 (d, J = 8.5 Hz, 1H), 5.03 (s, 2H), 4.79 (s, 2H), 3.40–3.35 (m, 1H), 1.31 (d, J = 6.5 Hz, 6H); HRMS (FAB, $[M+H]^+$) calcd for $C_{34}H_{28}Cl_3N_2O_5$ 649.1064, found 649.1072.

4.3.13. 3-{2-Chloro-{4-[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}-*N*-[2-phenylethyl]benzamido}benzoic acid (6d)

This compound was prepared from **5d** (30.5 mg, 44.2 μmol) by means of a procedure similar to that used for **6a**. Compound **6d** (20.2 mg, 68%) as a pale yellow solid; mp 52–55 °C, ^{1}H NMR (500 MHz, DMSO-d_6, 100 °C) δ 7.84–6.42 (m, 15H), 4.62 (s, 2H), 4.18 (s, 2H), 3.23 (br, 1H), 3.02 (s, 2H), 1.36 (d, J = 6.7 Hz, 6H); HRMS (FAB, $[M+H]^+$) calcd for $C_{35}H_{23}Cl_3N_2O_5$ 663.1220, found 663.1225.

4.3.14. 3-{2-Chloro-{4-[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}-*N*-[1-naphtylmethyl]benzamido}benzoic acid (6e)

This compound was prepared from **5e** (27.2 mg, 37.8 μmol) by means of a procedure similar to that used for **6a**. Compound **6e** (14.7 mg, 53%) as a pale yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.29 (d, J = 8.0 Hz, 1H), 7.84 (d, J = 8.0 Hz, 1H), 7.73 (d, J = 8.0 Hz, 1H), 7.70–7.67 (m, 3H), 7.57 (t, J = 7.3 Hz, 1H), 7.52 (t, J = 7.3 Hz, 1H), 7.34 (d, J = 7.3 Hz, 2H), 7.28 (d, J = 7.3 Hz, 1H), 7.24 (t, J = 7.3 Hz, 1H), 7.15 (s, 1H), 6.96 (d, J = 7.3 Hz, 2H), 6.78 (d, J = 7.3 Hz, 1H), 6.54 (s, 1H), 6.40 (d, J = 8.0 Hz, 1H), 5.63 (s, 2H), 4.60 (s, 2H), 3.22 (septet, J = 7.0 Hz, 1H), 1.34 (d, J = 7.0 Hz, 6H); FAB:MS m/z 698 $(M)^+$. HRMS (FAB, $[M+H]^+$) calcd for $C_{38}HC_{30}ClN_2O_5$ 699.1220, found 699.1213.

4.3.15. 3-{2-Chloro-{4-[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}-*N*-[2-naphtylmethyl]benzamido}benzoic acid (6f)

This compound was prepared from **8f** (36.0 mg, 50.4 μmol) by means of a procedure similar to that used for **6a**. Compound **6f** (20.8 mg, 57%) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.81–7.73 (m, 4H), 7.67 (s, 1H), 7.51 (d, J = 8.0 Hz, 2H), 7.47–7.43

(m, 2H), 7.35 (d, *J* = 8.0 Hz, 2H), 7.29 (d, *J* = 8.0 Hz, 2H), 7.12–7.01 (m, 3H), 6.56 (s, 2H), 6.44 (d, *J* = 8.0 Hz, 1H), 5.30 (s, 2H), 4.62 (s, 2H), 3.23 (septet, *J* = 7.0 Hz, 1H), 1.35 (d, *J* = 7.0 Hz, 6H); HRMS (FAB, $[M+H]^+$) calcd for $C_{38}HC_{30}ClN_2O_5$ 699.1220, found 699.1196.

4.3.16. 3-{2-chloro-4-[(3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl)methoxy]benzamido}benzoic acid (6g)

This compound was prepared from **4** (60.7 mg, 0.105 mmol) by means of a procedure similar to that used for **6a**. Compound **6g** (36.1 mg, 61%) as a pale yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.26 (s, 1H), 8.15–8.08 (m, 2H), 7.88 (d, *J* = 8.0 Hz, 1H), 7.74 (d, *J* = 8.6 Hz, 2H), 7.48 (t, *J* = 8.0 Hz, 1H), 7.41–7.31 (m, 3H),, 6.81 (s, 1H), 6.75 (d, *J* = 8.6 Hz, 1H), 4.77 (s, 2H), 3.33 (septet, *J* = 7.3 Hz, 1H), 1.44 (d, *J* = 7.3 Hz, 6H); HRMS (FAB, $[M+H]^+$) calcd for $C_{28}H_{24}Cl_3N_2O_5$ 573.0751, found 573.0720.

4.3.17. 2-Chloro-4-[(5-isopropyl-3-phenyl-isoxazol-4-yl)methoxy]benzaldehyde (11a)

This compound was prepared from **10a** (92.7 mg, 0.650 mmol) by means of a procedure similar to that used for **2**. Compound **11a** (218 mg, 94%) as a pale yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.35 (s, 1H), 7.91 (d, *J* = 8.5 Hz, 1H), 7.61 (dd, *J* = 7.9, 2.4 Hz, 2H), 7.47–7.42 (m, 3H), 6.97 (d, *J* = 2.4 Hz, 1H), 6.93 (dd, *J* = 8.5, 2.4 Hz, 1H), 4.92 (s, 2H), 3.27 (septet, *J* = 7.4 Hz, 1H), 1.41 (d, *J* = 7.4 Hz, 6H); MS (FAB, $[M+H]^+$) *m/z* 356.

4.3.18. 4-{[3-(2,6-Dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}benzaldehyde (11b)

This compound was prepared from **10b** (349 mg, 1.00 mmol) by means of a procedure similar to that used for **2**. Compound **11b** (406 mg, quant.) as a pale yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.85 (s, 1H), 7.77 (d, *J* = 8.5 Hz, 2H), 7.39 (d, *J* = 8.5 Hz, 2H), 7.32 (t, *J* = 8.5 Hz, 1H), 6.87 (d, *J* = 8.5 Hz, 1H), 4.81 (s, 2H), 3.33 (septet, *J* = 7.4 Hz, 1H), 1.43 (d, *J* = 7.4 Hz, 6H); MS (FAB, $[M+H]^+$) *m/z* 390.

4.3.19. 2-Chloro-4-{[3-(2,6-dimethylphenyl)-5-isopropylisoxazol-4-yl]methoxy}benzaldehyde (11c)

This compound was prepared from **10c** (100 mg, 0.324 mmol) by means of a procedure similar to that used for **2**. Compound **11c** (96.9 mg, 77%) as a pale yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.30 (s, 1H), 7.82 (d, *J* = 9.1 Hz, 1H), 7.23 (t, *J* = 7.9 Hz, 1H), 7.09 (d, *J* = 7.9 Hz, 1H), 6.78 (d, *J* = 2.4 Hz, 1H), 6.74 (dd, *J* = 9.1, 2.4 Hz, 1H), 4.64 (s, 2H), 3.31 (septet, *J* = 7.4 Hz, 1H), 2.11 (s, 6H), 1.41 (d, *J* = 7.4 Hz, 6H); MS (FAB, $[M+H]^+$) *m/z* 384.

4.3.20. 2-Chloro-4-[(5-isopropyl-3-phenylisoxazol-4-yl)methoxy]benzoic acid (12a)

This compound was prepared from **11a** (218 mg, 0.613 mmol) by means of a procedure similar to that used for **3**. Compound **12a** (187 mg, 82%) as a pale yellow powder; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (d, *J* = 8.6 Hz, 1H), 7.64–7.62 (m, 2H), 7.47–7.43 (m, 3H), 7.04 (d, *J* = 2.5 Hz, 1H), 6.89 (dd, *J* = 8.6, 2.5 Hz, 1H), 4.90 (s, 1H), 3.28 (septet, *J* = 6.7 Hz, 1H), 1.41 (d, *J* = 7.4 Hz, 6H).

4.3.21. 4-{[3-(2,6-Dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}benzoic acid (12b)

This compound was prepared from **11b** (390 mg, 1.00 mmol) by means of a procedure similar to that used for **3**. Compound **12b** (330 mg, 81%) as a colorless powder; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.97 (d, *J* = 8.5 Hz, 2H), 7.40 (d, *J* = 8.5 Hz, 2H), 7.32 (t, *J* = 8.5 Hz, 1H), 6.81 (d, *J* = 8.5 Hz, 2H), 4.80 (s, 2H), 3.33 (septet, *J* = 7.4 Hz, 1H), 1.43 (d, *J* = 7.4 Hz, 6H).

4.3.22. 2-Chloro-4-{[3-(2,6-dimethylphenyl)-5-isopropylisoxazol-4-yl]methoxy}benzoic acid (12c)

This compound was prepared from **11c** (95.9 mg, 0.247 mmol) by means of a procedure similar to that used for **3**. Compound **12c** (90.3 mg, 91%) as a colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.95 (d, *J* = 8.6 Hz, 1H), 7.22 (t, *J* = 7.9 Hz, 1H), 7.09 (d, *J* = 7.9 Hz, 1H), 6.85 (d, *J* = 2.4 Hz, 1H), 6.70 (dd, *J* = 8.6, 2.4 Hz, 1H), 4.62 (s, 2H), 3.30 (septet, *J* = 7.4 Hz, 1H), 2.11 (s, 6H), 1.43 (d, *J* = 7.4 Hz, 6H).

4.3.23. Methyl 3-{2-chloro-4-[(5-isopropyl-3-phenylisoxazol-4-yl)methoxy]benzamido}benzoate (13a)

This compound was prepared from **12a** (187 mg, 0.503 mmol) by means of a procedure similar to that used for **4**. Compound **13a** (186 mg, 94%) as a colorless powder; ^{1}H NMR (500 MHz, $CDCl_3$) 8.17 (s, 1H), 8.13 (s, 1H), 8.04 (d, *J* = 9.1 Hz, 1H), 7.88–7.83 (m, 2H), 7.65–7.63 (m, 2H), 7.47–7.45 (m, 3H, 7.01 (d, *J* = 2.4 Hz, 1H), 6.96 (dd, *J* = 9.1, 2.4 Hz, 1H), 4.90 (s, 2H), 3.93 (s, 3H), 3.29 (septet, *J* = 6.9 Hz, 1H), 1.41 (d, *J* = 6.9 Hz, 6H).

4.3.24. Methyl 3-{4-[(5-isopropyl-3-phenylisoxazol-4-yl)methoxy]benzamido}benzoate (13b)

This compound was prepared from **12b** (300 mg, 0.738 mmol) by means of a procedure similar to that used for **4**. Compound **13b** (230 mg, 68%) as a colorless powder; ^{1}H NMR (500 MHz, $CDCl_3$) 8.10 (s, 1H), 8.02 (d, *J* = 8.0 Hz, 1H), 7.77 (d, *J* = 8.6 Hz, 3H), 7.45 (t, *J* = 8.0 Hz, 1H), 7.41 (d, *J* = 8.0 Hz, 1H), 7.33 (t, *J* = 8.0 Hz, 1H), 6.85 (d, *J* = 8.6 Hz, 2H), 4.80 (s, 2H), 3.93 (s, 3H), 3.34 (septet, *J* = 6.8 Hz, 1H), 1.43 (d, *J* = 6.8 Hz, 6H).

4.3.25. Methyl 2-{2-chloro-4-[(3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl)methoxy]- benzamido}benzoate (13d)

This compound was prepared from **3** (147 mg, 0.256 mmol) by means of a procedure similar to that used for **4**. Compound **13d** (108 mg, 56%) as a pale yellow solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.85 (d, *J* = 8.6 Hz, 1H), 8.05 (d, *J* = 8.0 Hz, 1H), 7.61–7.55 (m, 2H), 7.41 (d, *J* = 8.0 Hz, 2H), 7.33 (t, *J* = 8.6 Hz, 1H), 7.12 (t, *J* = 8.0 Hz, 1H), 6.84 (d, *J* = 8.0 Hz, 1H), 6.75 (d, *J* = 8.6 Hz, 1H), 4.76 (s, 2H), 3.89 (s, 3H), 3.33 (septet, *J* = 7.9 Hz, 1H), 1.44 (d, *J* = 7.9 Hz, 6H); MS (FAB, $[M+H]^+$) *m/z* 574.

4.3.26. Methyl 4-{2-chloro-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}- benzamido}benzoate (13e)

This compound was prepared from **6** (147 mg, 0.256 mmol) by means of a procedure similar to that used for **4**. Compound **13e** (107 mg, 56%) as a pale yellow solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.21 (s, 1H), 8.04 (d, *J* = 8.6 Hz, 1H), 7.76 (d, *J* = 8.6 Hz, 1H), 7.70 (d, *J* = 8.0 Hz, 2H), 7.42 (d, *J* = 8.0 Hz, 2H), 7.35 (t, *J* = 8.6 Hz, 1H), 6.82 (d, *J* = 8.0 Hz, 1H), 6.77 (d, *J* = 8.0 Hz, 1H), 4.78 (s, 2H), 3.91 (s, 3H), 3.33 (septet, *J* = 7.9 Hz, 1H), 1.44 (d, *J* = 7.9 Hz, 6H); MS (FAB, $[M+H]^+$) *m/z* 574.

4.3.27. Methyl 3-{*N*-benzyl-2-chloro-4-[(5-isopropyl-3-phenylisoxazol-4-yl)methoxy]benzamido}- benzoate (14a)

This compound was prepared from **13a** (53.7 mg, 0.105 mmol) by means of a procedure similar to that used for **5a**. Compound **14a** (12.0 mg, 20%) as a colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78 (d, *J* = 8.6 Hz, 1H), 7.74 (s, 1H), 7.56 (d, *J* = 7.3 Hz, 2H), 7.44 (t, *J* = 7.3 Hz, 1H), 7.38 (t, *J* = 8.6 Hz, 1H), 7.31–7.27 (m, 5H), 7.16 (t, *J* = 8.6 Hz, 1H), 7.07 (t, *J* = 8.6 Hz, 2H), 6.75 (s, 2H), 6.61 (d, *J* = 8.6 Hz, 1H), 5.15 (s, 2H), 4.72 (s, 2H), 3.87 (s, 3H), 3.20 (septet, *J* = 6.9 Hz, 1H), 1.34 (t, *J* = 6.9 Hz, 1H).

4.3.28. Methyl 3-{*N*-benzyl-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}- benzamido}benzoate (14b)

This compound was prepared from **13b** (53.0 mg, 98.3 μmol) by means of a procedure similar to that used for **5a**. Compound **14b** (35.9 mg, 57%) as a colorless oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.9 Hz, 1H), 7.71 (s, 1H), 7.36 (d, *J* = 7.9 Hz, 2H), 7.30–7.27 (m, 8H), 7.22 (d, *J* = 8.6 Hz, 3H), 7.16 (t, *J* = 7.9 Hz, 1H), 6.94

(d, J = 7.3 Hz, 1H), 6.51 (d, J = 8.6 Hz, 2H), 5.12 (s, 2H), 4.66 (s, 2H), 3.87 (s, 3H), 3.26 (septet, J = 7.3 Hz, 1H), 1.36 (d, J = 7.3 Hz, 6H); MS (FAB, $[M+H]^+$) m/z.629.

4.3.29. Methyl 3-(benzylamino)benzoate (16)

To a solution of methyl 3-aminobenzoate (150 mg, 1.00 mmol) in dry DCM (5.0 mL) was added benzaldehyde (200 mg), acetic acid (200 μL) and $NaBH(OAC)_3$ (600 mg, 2.83 mmol) at 0 °C. The reaction mixture was stirred at rt overnight, and partitioned between AcOEt and water. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was purified by silica gel chromatography (AcOEt/hexane = 1/10) to afford **16** (138 mg, 57%) as a colorless powder; ^{1}H NMR (500 MHz, $CDCl_3$) 7.39–7.32 (m, 6H), 7.29 (t, J = 8.0 Hz, 1H), 7.22 (t, J = 8.0 Hz, 1H), 6.79 (d, J = 8.0 Hz, 1H), 4.37 (s, 2H), 4.16 (s, 1H), 3.88 (s, 3H); MS (FAB, $[M]^+$) m/z 241.

4.3.30. Methyl 3-{*N*-benzyl-2-chloro-4-{[3-(2,6-dimethylphenyl)-5-isopropylisoxazol-4-yl]methoxy}-benzamido}benzoate (14c)

This compound was prepared from **12c** (25.3 mg, 0.104 mmol) by means of a procedure similar to that used for **4**. Compound **14c** (45.5 mg, 78%) as a pale yellow foam; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74–7.71 (m, 2H), 7.31–7.27 (m, 6H), 7.20 (t, J = 7.9 Hz, 2H), 7.11 (t, J = 7.9 Hz, 2H), 7.05 (d, J = 7.3 Hz, 2H), 6.99 (t, J = 8.6 Hz, 2H), 6.54 (s, 1H), 6.42 (d, J = 7.9 Hz, 1H), 5.12 (s, 2H), 4.45 (s, 2H), 3.86 (s, 3H), 3.23 (septet, J = 6.9 Hz, 1H), 2.04 (s, 6H), 1.36 (d, J = 6.9 Hz, 6H).

4.3.31. Methyl 2-{N-benzyl-2-chloro-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}-benzamido}benzoate (14d)

This compound was prepared from **12c** (50 mg, 87.1 μmol) by means of a procedure similar to that used for **4**. Compound **14d** (35 mg, 61%) as pale yellow foam;^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79 (d, J = 8.0 Hz, 1H), 7.38–7.34 (m, 2H), 7.30 (t, J = 8.6 Hz, 2H), 7.29 (d, J = 8.0 Hz, 1H), 7.20–7.13 (m, 4H), 7.00 (d, J = 8.0 Hz, 1H), 6.84 (d, J = 8.6 Hz, 1H), 6.55 (d, J = 2.5 Hz, 1H), 6.36 (dd, J = 8.0, 2.5 Hz, 1H), 5.89 (d, J = 14.6 Hz, 1H), 4.59 (s, 2H), 4.13 (d, J = 14.6 Hz, 1H), 3.95 (s, 3H), 3.22 (septet, J = 7.9 Hz, 1H), 1.34 (dd, J = 16.4, 7.9 Hz, 6H); MS (FAB, $[M+H]^+$) m/z 663.

4.3.32. Methyl 4-{N-benzyl-2-chloro-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}-benzamido}benzoate (14e)

This compound was prepared from **13e** (43.1 mg, 74.9 μmol) according to **4**. Compound **14e** (41.8 mg, 82%) as a pale yellow solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, J = 8.0 Hz, 2H), 7.37–7.27 (m, 8H), 6.99 (d, J = 7.3 Hz, 3H), 6.59 (s, 1H), 6.45 (d, J = 7.3 Hz, 1H), 5.10 (s, 2H), 4.62 (s, 2H), 3.84 (s, 3H), 3.25 (septet, J = 7.9 Hz, 1H), 1.37 (d, J = 7.9 Hz, 6H); MS (FAB, $[M+H]^+$) m/z 663.

4.3.33. 3-{*N*-Benzyl-2-chloro-4-{[5-isopropyl-3-phenylisoxazol-4-yl]methoxy}benzamido}benzoic acid (15a)

This compound was prepared from **14a** (12.8 mg, 20.5 μmol) by means of a procedure similar to that used for **6a**. Compound **15a** (3.90 mg, 33%) as a pale yellow foam; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 (d, J = 7.3 Hz, 1H), 7.77 (s, 1H) 7.55 (d, J = 6.7 Hz, 2H), 7.42 (d, J = 7.3 Hz, 1H), 7.38 (d, J = 7.3 Hz, 2H), 7.31–7.27 (m, 5H), 7.20 (t, J = 8.6 Hz, 1H), 7.13–7.08 (m, 2H), 6.74 (s, 1H), 6.62 (d, J = 7.9 Hz, 1H), 5.16 (s, 2H), 4.72 (s, 2H), 3.20 (septet, J = 6.9 Hz, 1H), 1.33 (d, J = 6.9 Hz, 6H); HRMS (FAB, $[M+H]^+$) calcd $C_{34}H_{30}Cl_2N_2O_5$ 581.1843, found 581.1888.

4.3.34. 3-{*N*-Benzyl-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}benzamido}- benzoic acid (15b)

This compound was prepared from **14b** (35 mg, 55.6 μmol) by means of a procedure similar to that used for **6a**. Compound **15b** (26 mg, 74%) as pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 (d, J = 7.9 Hz, 1H), 7.75 (s, 1H), 7.35 (d, J = 7.9 Hz, 2H), 7.28–7.27 (m, 5H), 7.24–7.19 (m, 4H), 7.02 (d, J = 7.9 Hz, 1H), 6.53 (d, J = 7.9 Hz, 2H), 5.14 (s, 2H), 4.67 (s, 2H), 3.26 (septet, J = 7.3 Hz, 1H), 1.35 (d, J = 7.3 Hz, 6H); HRMS (FAB, $[M+H]^+$) calcd $C_{34}H_{29}Cl_2N_2O_5$ 615.1454, found 615.1434.

4.3.35. 3-(*N*-Benzyl-2-chloro-4-((3-(2,6-dimethylphenyl)-5-isopropylisoxazol-4-yl)methoxy)- benzamido)benzoic acid (15c)

This compound was prepared from **14c** (45.6 mg, 72.3 μmol) by means of a procedure similar to that used for **6a**. Compound **15c** (33.1 mg, 75%) as a pale yellow foam; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.81 (d, J = 6.1 Hz, 1H), 7.76 (s, 1H), 7.31–7.28 (m, 5H), 7.21–7.15 (m, 2H), 7.08–6.99 (m, 4H), 6.54 (s, 1H), 6.44 (d, J = 7.3 Hz, 1H), 5.14 (s, 2H), 4.46 (s, 2H), 3.23 (septet, J = 6.9 Hz, 1H), 2.04 (s, 6H), 1.36 (d, J = 6.9 Hz, 6H); HRMS (FAB, $[M]^+$) calcd $C_{36}H_{33}ClN_2O_5$ 608.2078, found 608.2086.

4.3.36. 2-{*N*-Benzyl-2-chloro-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}- benzamido}benzoic acid (15d)

This compound was prepared from **14d** (35.9 mg, 52.7 μmol) by means of a procedure similar to that used for **6a.** Compound **15d** (19.1 mg, 55%) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.93 (d, J = 8.0 Hz, 1H), 7.37–7.31 (m, 4H), 7.30–7.27 (m, 3H), 7.19 (t, J = 8.0 Hz, 2H), 7.10 (d, J = 8.6 Hz, 1H), 6.85 (d, J = 8.6 Hz, 1H), 6.56 (d, J = 2.5 Hz, 1H), 6.38 (dd, J = 8.0, 2.5 Hz, 1H), 5.99 (d, J = 14.6 Hz, 1H), 4.59 (s, 2H), 4.15 (d, J = 14.6 Hz, 1H), 3.21 (septet, J = 7.9 Hz, 1H), 1.34 (dd, J = 16.4, 7.9 Hz, 6H);HRMS (FAB, $[M+H]^+$) calcd for $C_{34}H_{27}Cl_3N_2O_5$ 649.1064, found 649.1074.

4.3.37. 4-{*N*-Benzyl-2-chloro-4-{[3-(2,6-dichlorophenyl)-5-isopropylisoxazol-4-yl]methoxy}- benzamido}benzoic acid (15e)

This compound was prepared from **6e** (40.1 mg, 60.2 μmol) by means of a procedure similar to that used for **6a.** Compound **15e** (25.4 mg, 55%) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.81 (d, J = 7.3 Hz, 2H), 7.34 (d, J = 8.0 Hz, 2H), 7.29–7.21 (m, 6H), 7.00 (d, J = 7.3 Hz, 3H), 6.59 (s, 1H), 6.45 (d, J = 7.3 Hz, 1H), 5.10 (s, 2H), 4.62 (s, 2H), 3.24 (septet, J = 7.9 Hz, 1H), 1.35 (d, J = 7.9 Hz, 6H);HRMS (FAB, $[M+H]^+$) calcd $C_{34}H_{27}Cl_3N_2O_5$ 649.1064, found 649.1020.

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