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Identification of *trans*-4-[1-[[7-fluoro-2-(1-methyl-3-indolyl)-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid as a potent, orally active VLA-4 antagonist

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ABSTRACT

For the purpose of obtaining orally potent VLA-4 inhibitors, we have carried out structural modification of the (*N*′-phenylureido)phenyl group in compound **1**, where the group was found to be attributed to poor pharmacokinetic profile in our previous research. Through modification, we have identified several compounds with both potent in vitro activity and improved oral exposure. In particular, compound **7e** with 7-fluoro-2-(1-methyl-1*H*-indol-3-yl)-1,3-benzoxazolyl group as a novel replacement of the (*N*′-phenylureido)phenyl group significantly inhibited eosinophil infiltration into bronchoalveolar lavage fluid at 15 mg/kg in an *Ascaris*-antigen-induced murine bronchial inflammatory model, and its efficacy was comparable to that of the *anti*-mouse α_4 antibody (R1–2).

1. Introduction

The very late antigen-4 (VLA-4, integrin $\alpha_4\beta_1$, CD49d/CD29) expressed on the surface of most leukocytes is a heterodimeric glycoprotein receptor consisting of α_4 and β_1 chains.[1] It has been well-established that VLA-4 plays a pivotal role in the process of adhesion, migration, invasion, and proliferation of inflammatory cells at the site of inflammation via interactions with its natural ligands, the vascular cell adhesion molecule-1 (VCAM-1, CD106) expressed on cytokine-stimulated endothelial cells and the alternatively spliced portion of the type III connecting segment of fibronectin (FN).[2,3] The inappropriate continuation of the cellular process causes tissue damage and dysfunction, resulting in the pathogenesis of inflammatory and autoimmune diseases such as asthma,[4] rheumatoid arthritis,[5] multiple sclerosis (MS)[6] and Crohn's disease (CD).[7] So far, anti-α_4 antibodies and small-molecule VLA-4 antagonists have demonstrated efficacy in a wide variety of inflammatory animal models, validating the blockade of the VLA-4/VCAM or FN interaction as a therapeutic target.[8] Furthermore, the humanized monoclonal anti-α_4 antibody natalizumab was approved for the treatment of relapsing forms of MS in the US and relapsing and remitting MS in the EU. On top of that, natalizumab has recently been a therapeutic option approved for adult patients with moderately to severely active CD in the US.[9] Several small-molecule VLA-4 antagonists have been advanced into clinical trials for the treatment of MS and CD, however, none of these candidates has yet reached the marketplace. Thus, from a safety and cost point of view, the next hurdle to overcome in this field should be to develop an oral small-molecule VLA-4 antagonist with similar efficacy to natalizumab and an appropriate half-life.

We have previously reported that we identified *trans*-4-[(4*S*)-substituted-(2*S*)-pyrrolidinylmethyloxy]cyclohexanecarboxylic acid derivative **1** as a potent VLA-4 antagonist[10] and worked on structural modification of the lipophilic moiety for the purpose of improvement of poor pharmacokinetic (PK) profile. As a result, we made clear that modifications to reduce the polar surface area (PSA) value and the number of hydrogen bond donors (HBD) in the lipophilic moiety lead to improvement of PK profile, resulting in the discovery of 1-methyl-3-indolyl based compound **2** as a clinical candidate which exhibits potent oral efficacy in animal models of asthma with good oral bioavailability.[11]

From these results, we decided to conduct further structural modifications of the lipophilic moiety in compound **1**. In the aforementioned modifications, we incorporated the urea in (*N*′-phenylureido)phenyl group into the cyclic structures leading to 2-(arylamino)benzoxazolyl (I), 4-(2-benzoxazolylamino)phenyl (II), and 4-(heteroarylcarboxamide)phenyl (III) groups as depicted

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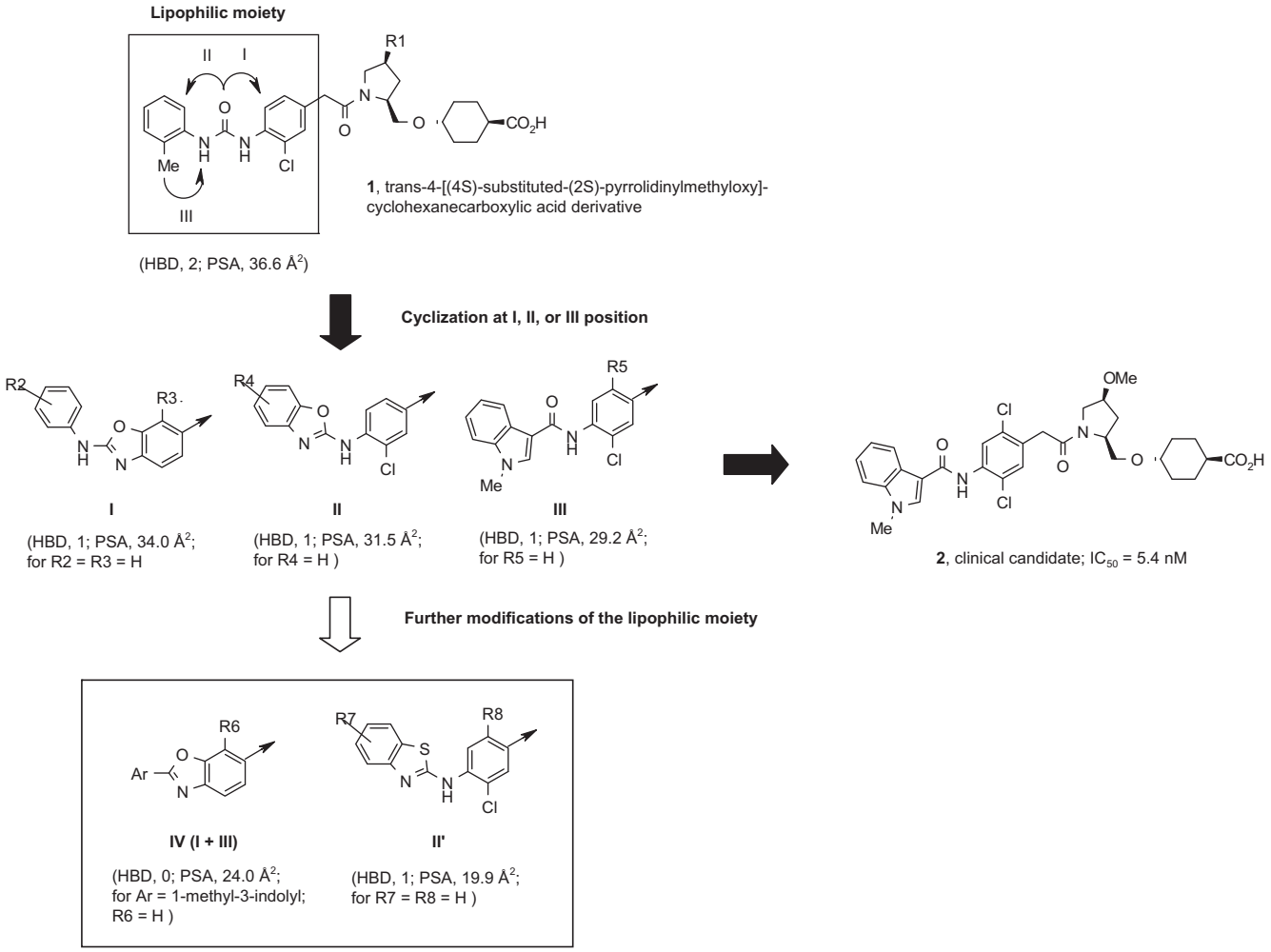


Figure 1. Drug designs of lipophilic moiety.

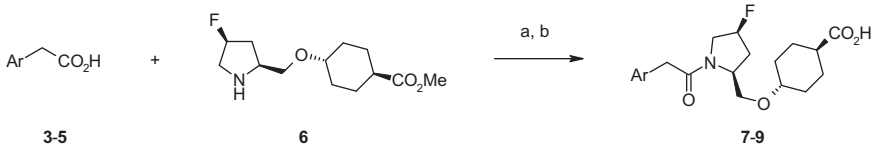
in Figure 1.[11] In this study, we attempted the following two modifications as shown in Figure 1, that one modification leads to 2-aryl-1,3-benzoxazolyl (IV) groups double cyclized at I and III positions, and the other leads to 4-(2-benzothiazolylamino)phenyl (II′) groups cyclized at II position and replaced the oxygen atom with a sulfur atom, which PSA values (24.0 Å^2 for IV, Ar = 1-methyl-3-indolyl, R6 = H; 19.9 Å^2 for II′, R7 = R8 = H) are less than that of the lipophilic moiety III used for compound **2** (29.2 Å^2). In addition, we fixed a fluorine atom as R1 of **1** in the modification based on the structure–activity relationships (SAR) that we previously reported.[10]

Herein, we report the synthesis and SAR of a series of compounds that we modified the lipophilic moiety as well as in vivo evaluation results of some representative compounds.

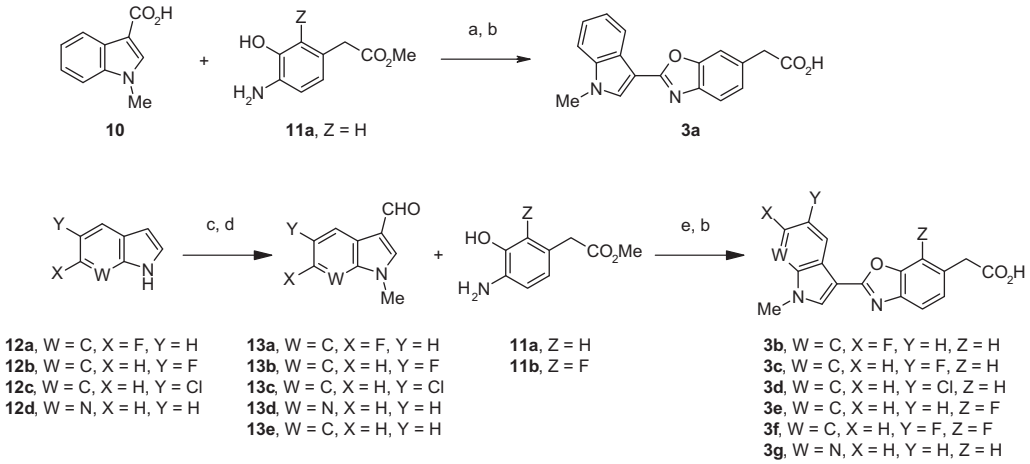
2. Chemistry

The target compounds were synthesized according to the general procedure outlined in Scheme 1. Thus, arylacetic acids **3**–**5** were condensed with pyrrolidine **6**[10,11] using EDC·HCl and HOBt. The resulting amides were subjected to basic hydrolysis to afford *trans*-4-substituted cyclohexanecarboxylic acids **7**–**9**. The syntheses of the intermediates **3**–**5** in Scheme 1 are fully presented in Schemes 2–4.

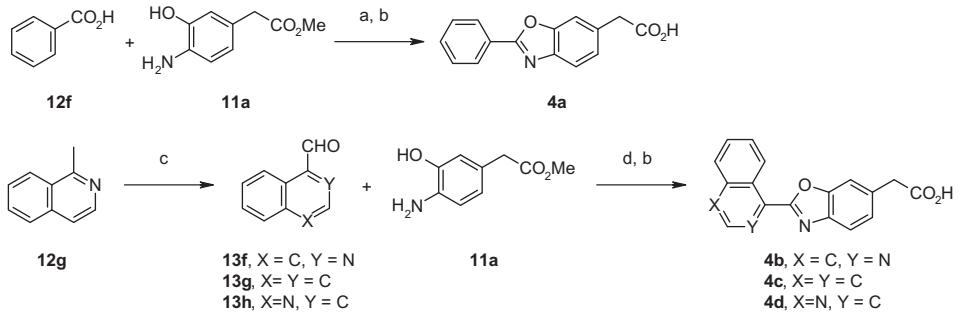
The preparation of (6-benzoxazolyl)acetic acids **3a**–**g** is depicted in Scheme 2. 1-Methylindole-3-carboxylic acid (**10**) was refluxed in xylene with methyl 4-amino-3-hydroxyphenylacetate (**11a**)[12] in the presence of boric acid to give the corresponding benzoxazole, which was hydrolyzed under a basic condition to provide [2-(1-methyl-1*H*-indol-3-yl)-1,3-benzoxazol-6-yl]acetic acid (**3a**). Indoles **12a**–**c** or azaindole **12d** were subjected to *N*-methylation using NaH and MeI, followed by Vilsmeier–Haack reaction to afford the aldehydes **13a**–**d**.[13,14] Treatment of the aldehydes **13a**–**e** with methyl 4-amino-3-hydroxyphenylacetate (**11a**) or methyl 4-amino-2-fluoro-3-hydroxyphenylacetate (**11b**)[11] in the presence of iodobenzene diacetate furnished the corresponding benzoxazoles, which were hydrolyzed under a basic condition to provide the (2-aryl-6-benzoxazolyl)acetic acids **3b**–**g**.



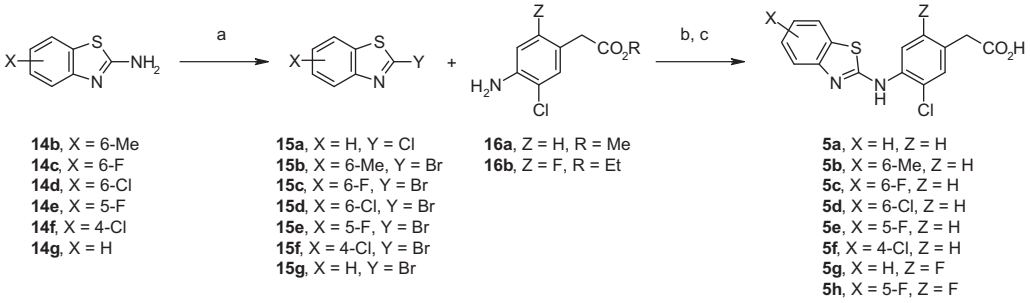
Scheme 1. Reagents and conditions: (a) EDC·HCl, HOBt, DMAP or Et_3N, DMF; (b) aq NaOH, THF.



Scheme 2. Reagents and conditions: (a) $B(OH)_3$, xylene, reflux; (b) 0.25 N NaOH, THF; (c) NaH, MeI, DMF; (d) $POCl_3$, DMF; (e) methyl 4-amino-3-hydroxyphenylacetate or methyl 4-amino-2-fluoro-3-hydroxyphenylacetate, $PhI(OAc)_2$, EtOH.



Scheme 3. Reagents and conditions: (a) $B(OH)_3$, xylene, reflux; (b) 0.25 N NaOH, THF; (c) SeO_2, dioxane, reflux; (d) $PhI(OAc)_2$, EtOH.



Scheme 4. Reagents and conditions: (a) CuBr, ^{t}BuONO, MeCN, 60 °C; (b) PPTS, xylene, reflux; (c) 0.5 N or 1 N NaOH, THF.

(6-Benzoxazolyl)acetic acids **4a–d** were synthesized as shown in Scheme 3. Intermediate **4a**[15] was prepared from **11a** and benzoic acid (**12f**) in two steps as described for the preparation of **3a** in Scheme 2. On the other hand, aldehydes **13f–h** in which aldehyde **13f**[16] was prepared by oxidation of **12g** using SeO_2, were converted to **4b–d** by the same procedure as that used for the preparation of **3b–g** in Scheme 2.

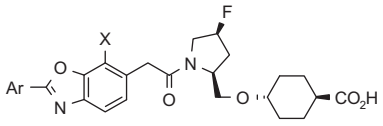
The preparation of [4-(2-benzothiazolyl)aminophenyl]acetic acids **5a–h** was shown in Scheme 4. When 2-Aminobenzothiazoles **14b–g** were treated with *tert*-butyl nitrite, followed by CuBr, the 2-bromobenzothiazoles **15b–g** were successfully produced.[17] Nucleophilic displacement of the resulting **15b–g** or commercially available 2-chlorobenzothiazole **15a** with 4-aminophenylacetates **16a**[18] or **16b**[19] in the presence of PPTS and subsequent basic hydrolysis afforded [4-(2-benzothiazolyl)aminophenyl]acetic acids **5a–h**.

3. Results and discussion

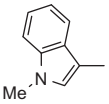
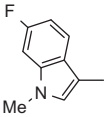
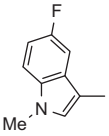
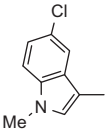
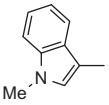
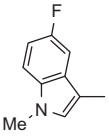
3.1. In vitro activity

The synthesized compounds (**7–9**) were evaluated for their VLA-4 inhibitory activities in an established receptor binding assay (VLA-4 overexpressed Chinese hamster ovary cells/Human VCAM-1 labeled with Europium) with or without the addition of 3% human serum albumin (HSA).

Table 1
Inhibitory activity of 2-(1-methyl-1*H*-indol-3-yl)-1,3-benzoxazole derivatives **7a–f**

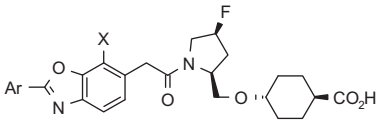


7a-f

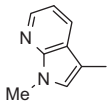
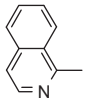
Compound	Ar	X	IC_{50} (nM) (±3% HSA)	Ratio (±)
7a		H	2.3/121	53
7b		H	22/977	44
7c		H	7.3/350	48
7d		H	30/n.d.	n.d.
7e		F	4.7/156	33
7f		F	10/432	43

n.d., not determined.

Table 2
Inhibitory activity of 2-aryl-1,3-benzoxazole derivatives **8a–e**

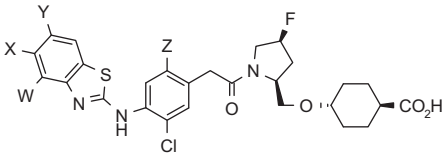


8a-e

Compound	Ar	X	IC_{50} (nM) (±3% HSA)	Ratio (±)
8a		H	200/n.d.	n.d.
8b		H	487/n.d.	n.d.
8c		H	43/n.d.	n.d.
8d		H	900/n.d.	n.d.
8e		H	59/1468	25

n.d., not determined.

Table 3
Inhibitory activity of 4-(2-benzothiazolyl)aminophenyl derivatives **9a–h**



9a-h

Compound	W	X	Y	Z	IC_{50} (nM) (±3% HSA)	Ratio (±)
9a	H	H	H	H	2.5/137	55
9b	H	H	Me	H	65/36% inhibition at 1 μg/mL	n.d.
9c	H	H	F	H	9.2/619	67
9d	H	H	Cl	H	9.0/514	57
9e	H	F	H	H	4.0/190	48
9f	Cl	H	H	H	7.0/293	42
9g	H	H	H	F	2.6/62	24
9h	H	F	H	F	3.6/127	35

n.d., not determined.

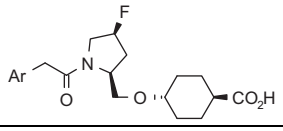
The evaluation results of 2-(1-methyl-1*H*-indol-3-yl)-1,3-benzoxazole derivative **7a–f** are shown in Table 1. At first, compound **7a** with 2-(1-methyl-1*H*-indol-3-yl)-1,3-benzoxazole as a lipophilic moiety showed potent activity with IC_{50} values of 2.3 and 121 nM (+3% HSA). We next investigated the effect of substituents (F or Cl) on the benzene rings of **7a** on the inhibitory activity. The introduction of a fluorine atom at the C-6 position in the indole ring (**7b**) decreased the activity by 1/10 as compared with **7a**. The introduction of a fluorine atom at the C-5 position in the indole ring (**7c**) relatively retained the potency, however, a chlorine atom at the same position (**7d**) caused a significant loss of the potency. Concerning the introduction of a fluorine atom at the C-7 position in the benzoxazole ring, **7e** and **7f** relatively retained the activity as compared with the corresponding compounds **7a** and **7c**. In addition, of all the 2-(1-methyl-1*H*-indol-3-yl)-1,3-benzoxazole derivatives, compound **7a** and **7e** were found to show acceptable activities with IC_{50}s of less than 200 nM even in the presence of 3% HSA.

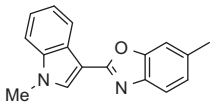
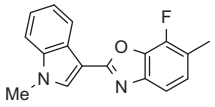
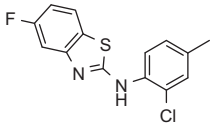
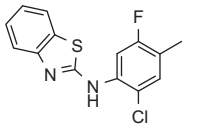
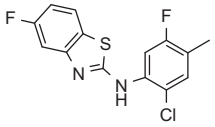
Next, we examined replacement of the 1-methyl-1*H*-indole ring in **7a** with 1-methyl-1*H*-pyrrolo[2,3-*b*]pyridine, benzene, naphthalene, quinoline and isoquinoline (Table 2). However, all of the modifications were detrimental to the potency.

The evaluation results of 4-(2-benzothiazolyl)aminophenyl derivatives **9a–h** are summarised in Table 3. In this modification, we fixed a chlorine atom at the 3-position on the central benzene ring based on a positive effect on pharmacokinetic properties in rodents and dogs that we previously reported.[20]

Compound **9a** showed preferable activity with IC_{50} values of 2.5 and 137 nM (+3% HSA). The introduction of a methyl group at the C-6 position in the benzothiazole ring (**9b**) caused a significant loss of the potency as compared with **9a**. In the case of a fluorine (**9c**) or chlorine atom (**9d**) at the same position, those compounds were slightly less potent than **9a**. The introduction of a fluorine atom at the C-5 position (**9e**) and a chlorine atom at the C-4 position (**9f**) in the benzothiazole ring relatively retained the potency. On the other hand, the introduction of a fluorine atom at the C-2 position in the central benzene ring (**9g** and **9h**) retained comparable activity to the corresponding compounds **9a** and **9e**, which seem to be in line with the SAR we mentioned in Table 1. Additionally, it was found that compound **9a**, **9e**, **9g**, and **9h** in the 4-(2-benzothiazolyl)aminophenyl derivatives exhibited promising

Table 4
Estimated serum concentration of compounds **7a**, **7e**, **9e**, **9g**, and **9h**



Compound	Ar	Estimated serum concentration (ng/mL) 15 min/60 min
7a		461/Not tested
7e		2211/406
9e		3196/284
9g		3316/691
9h		8551/619

activities with IC_{50}s of less than 200 nM even in the presence of 3% HSA.

We selected compounds **7a**, **7e**, **9e**, **9g**, and **9h** based on the potency in the presence of 3% HSA and structural character, and measured the activities in the serum at 15 and 60 min after oral dosing of those compounds at 10 mg/kg in mice, calculating the estimated serum concentrations to predict the oral absorption. These results are summarized in Table 4.

Almost all compounds except for **7a** exhibited relatively high estimated serum concentrations. Among them, compound **9h** with two fluorine atoms in the lipophilic moiety showed the highest concentration values of 8551 ng/ml (15 min) and 619 ng/ml (60 min). From the results previously reported,[21] we consider that the introduction of a fluorine atom into the lipophilic moiety would make the compound easier to use certain serum protein as carrier, resulting in the high serum concentration.

3.2. In vivo efficacy

On the basis of the values of the estimated serum concentration, we selected compounds **7e**, **9g**, and **9h** for further biological evaluation. These compounds were evaluated for their anti-inflammatory effect in an *Ascaris*-antigen-induced murine bronchial inflammatory model by measuring the level of eosinophils in bronchoalveolar lavage fluid (BALF) at 48 h after the antigen challenge. The compounds were administered orally to the mice at 5 mg/kg, bid and 15 mg/kg, bid. As shown in Figure 2, compound **7e** significantly reduced eosinophil infiltration into BALF by 46% at 15 mg/kg, and its efficacy was comparable to that of compound **2**[11] and the *anti*-mouse α_4 antibody[22] (R1–2, 5 mg/kg once a day) used as the positive control in this experiment. However, compounds **9g** and **9h** were not effective in this inflammatory model (data not shown). The basis of the discrepancy remains currently unresolved, but might be attributed to retention of the serum concentration after oral dosing.

3.3. Pharmacokinetic properties

Concerning compound **7e** exhibiting excellent efficacy in a bronchial inflammatory model, we determined the pharmacokinetic profile in rats in order to make sure how this modification of the lipophilic moiety in **7e** affects pharmacokinetic properties. As shown in Table 5, compound **7e** showed good oral exposure (AUC = 1000 ng h/ml) and iv plasma clearance (CL = 3.6 ml/min/kg). In addition, the profiles seemed to be more preferable as compared with those of compound **2** (AUC = 975 ng h/ml, CL = 19.3 ml/min/kg).[11]

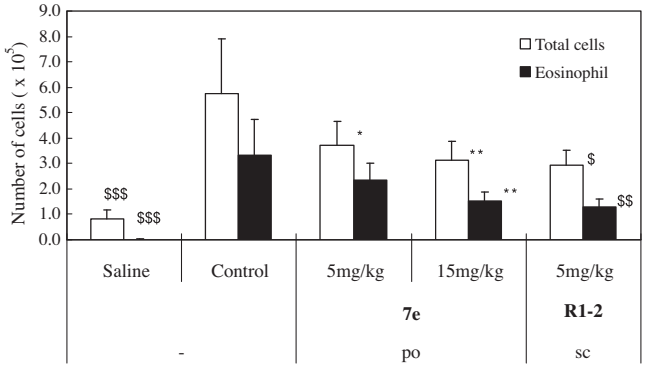


Figure 2. Effect of compound **7e** in an *Ascaris*-antigen-induced Murine Bronchial Inflammatory Model. Compound **7e** was given to the mice twice a day for 2 days. The *anti*-mouse α4 antibody (R1–2) was given to the mice once a day for 2 days. Each column represents mean ± S.D. of seven animals. $^{\$}p$ <0.05, $^{\$\$}p$ <0.01, $^{\$\$\$}p$ <0.001; significant difference from the control (Aspin-Welch's *t*-test), $^{*}p$ <0.05, $^{**}p$ <0.01; significant difference from the control (Dunnett's multiple comparison test).

Table 5
Pharmacokinetic properties of **7e** in rats ($n = 3$)

F[a] (%)	po (1 mg/kg)			iv (1 mg/kg)			
	AUC[b] (ng h/ml)	C_{max}[c] (ng/ml)	$T_{1/2}$[d] (h)	AUC (ng h/ml)	CL[e] (ml/min/kg)	V_{dss}[f] (l/kg)	$T_{1/2}$ (h)
21	1000	643	2.2	4668	3.6	0.173	0.7

[a] Oral bioavailability.
[b] Pharmacokinetic area under curve.
[c] Pharmacokinetic maximum concentration.
[d] Plasma half-life.
[e] Pharmacokinetic clearance.
[f] Volume of distribution.

4. Conclusion

For the purpose of obtaining orally potent VLA-4 inhibitors, we have worked on structural exploration of the lipophilic moiety in compound **1** based on our previous result that reduction of PSA value and HBD in the moiety improved PK profile. As a result, we found out several novel lipophilic moieties maintaining potent VLA-4 inhibitory activity. Moreover, this study has led to the identification of compound **7e** with 7-fluoro-2-(1-methyl-1*H*-indol-3-yl)-1,3-benzoxazolyl group as a lipophilic moiety, which demonstrated excellent efficacy at oral dosing in a bronchial inflammatory model as well as favorable PK profile.

5. Experimental

5.1. General

All starting materials and synthesis reagents were obtained commercially. Column chromatography was performed with a Merck Silica Gel 60 (particle size 0.060–0.200 or 0.040–0.063). Flash column chromatography was performed with Biotage FLASH Si or YAMAZEN Hi-Flash packed columns. Thin-layer chromatography (TLC) was performed on Merck precoated TLC glass sheets with Silica Gel 60 F254. Yields were of purified products and were not optimized. The ^{1}H NMR spectra were recorded on a JEOL JNM-EX-400 spectrometer, and chemical shifts are given in ppm (δ) from tetramethylsilane as an internal standard. The special splitting patterns are designated as follows: s, singlet; d, doublet; dd, double of doublet; t, triplet; td, triple of doublet; q, quartet; m, multiple. The IR spectra were recorded on a HORIBA FT-720 spectrometer. The mass spectra were recorded on a SCIEX API-150EX spectrometer (ESI). The high-resolution mass (HRMS) spectra were recorded on a JEOL JMS-100LP spectrometer. Elemental analysis was performed using a PerkinElmer CHNS/O 2400II, a Leco CHNS-932 and a YOKOKAWA analysis IC7000RS.

5.2. General procedure A: preparation of *trans*-4-[1-[[2-(1-methyl-3-indolyl)-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid (7a)

To a solution of [2-(1-methyl-3-indolyl)-6-benzoxazolyl]acetic acid (**3a**) (251 mg, 0.82 mmol), methyl *trans*-4-[(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylate (**6**)[10,11] (212 mg, 0.82 mmol), HOBt (22.0 mg, 0.16 mmol), and DMAP (20.0 mg, 0.16 mmol) in DMF (5.0 mL) was added EDC·HCl (204 mg, 1.07 mmol). After being stirred for 4 h, the reaction mixture was diluted with water and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4, and concentrated to dryness. The residue was purified by column chromatography with EtOAc as eluent to give methyl *trans*-4[-1-[[2-(1-methyl-3-indolyl)-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylate (431 mg, 96%) as a yellow amorphous solid. ^{1}H NMR ($CDCl_3$) δ 1.21–1.34 (m, 2H), 1.40–1.53 (m, 2H), 1.96–2.15 (m, 4H), 2.19–2.50 (m, 3H), 3.26 (m, 1H), 3.35 and 3.50 (each m, total 1H), 3.64 and 3.66 (each s, total 3H), 3.69–3.87 (m, 3H), 3.90 (s, 3H), 3.94–4.06 (m, 2H), 4.25 and 4.39 (each m, total 1H), 5.23 (m, 1H), 7.19 (m, 1H), 7.34–7.41 (m, 3H), 7.49 (m, 1H), 7.64 (m, 1H), 7.93 (s, 1H), 8.44 (m, 1H); MS (ESI) m/z 548 $[M+H]^+$.

To a solution of methyl *trans*-4-[1-[[2-(1-methyl-3-indolyl)-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylate (431 mg, 0.79 mmol) in THF (8.0 ml) was added 0.25 N NaOH (5.00 ml, 1.25 mmol) at room temperature. After being stirred for 5 h, the reaction mixture was poured into 1 N HCl (10 ml), and the precipitate was collected, washed with water, and dried to give the title compound (396 mg, 94%) as a colorless solid. IR (ATR) 2938, 2861, 1718, 1644, 1627, 1575, 1523, 1423 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.15–1.27 (m, 2H), 1.32–1.45 (m, 2H), 1.89–2.11 (m, 4H), 2.14–2.29 (m, 3H), 3.23 (m, 1H), 3.55 (m, 1H), 3.75–3.95 (m, 6H), 3.97 (s, 3H), 4.11 and 4.38 (each m, total 1H), 5.35 and 5.40 (each m, total 1H), 7.22 (m, 1H), 7.31–7.39 (m, 2H), 7.55–7.67 (m, 3H), 8.35 (s, 1H), 12.03 (br s, 1H); MS (ESI) m/z 534 $[M+H]^+$; Anal. Calcd for $C_{30}H_{32}FN_3O_5$: C, 67.53; H, 6.04; N, 7.87. Found: C, 67.33; H, 6.06; N, 7.70.

Compound **7b–d** and **8a** were prepared according to general procedure A.

5.3. *trans*-4-[1-[[2-(6-Fluoro-1-methyl-3-indolyl)-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexane carboxylic acid (7b)

Yield 84% (two steps). A yellow solid. IR (ATR) 2938, 1712, 1616, 1577, 1434 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.09–1.39 (m, 4H), 1.84–1.93 (m, 4H), 2.06–2.44 (m, 3H), 3.16–3.99 (m, 10H, including singlet, 3H, at δ 3.89), 4.13 and 4.33 (each m, total 1H), 5.21–5.46 (m, 1H), 7.12–7.21 (m, 2H), 7.49 (dd, J = 9.8, 2.2 Hz, 1H), 7.51 and 7.53 (each s, total 1H), 7.60 and 7.61 (each d, J = 8.1 Hz, total 1H), 8.28 (dd, J = 8.1, 5.6 Hz, 1H), 8.30 and 8.31 (each s, total 1H), 12.03 (br s, 1H); HRMS (ESI) Calcd for $C_{30}H_{31}F_2N_3O_5$+H: 552.23100. Found: 552.23598.

5.4. *trans*-4-[1-[[2-(5-Fluoro-1-methyl-3-indolyl)-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexane carboxylic acid (7c)

Yield 74% (two steps). A yellow solid. IR (ATR) 2938, 1716, 1616, 1631, 1577, 1484, 1434 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.09–1.39 (m, 4H), 1.84–1.97 (m, 4H), 2.03–2.28 (m, 3H), 3.15–4.00 (m, 7H), 3.93 (s, 3H), 4.15 and 4.34 (each m, total 1H), 5.30 and 5.36 (each m, total 1H), 7.16–7.21 (m, 2H), 7.52 and 7.54 (each s, total 1H), 7.61–7.64 (m, 2H), 7.97 (dd, J = 9.8, 2.9 Hz, 1H), 8.36 and 8.37 (each s, total 1H), 12.03 (br s, 1H); HRMS (ESI) Calcd for $C_{30}H_{31}F_2N_3O_5$+H: 552.23100. Found: 552.23543.

5.5. *trans*-4-[1-[[2-(5-Chloro-1-methyl-3-indolyl)-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid (7d)

Yield 65% (two steps). A yellow solid. IR (ATR) 2938, 2863, 1720, 1629, 1581, 1434 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.13–1.40 (m, 4H), 1.85–2.07 (m, 4H), 2.11–2.44 (m, 3H), 3.19–3.52 (m, 3H), 3.72–3.90 (m, 4H), 3.94 (s, 3H), 4.07 and 4.35 (each m, total 1H), 5.31 and 5.37 (each m, total 1H), 7.20 (m, 1H), 7.35 (dd, J = 8.8, 2.0 Hz, 1H), 7.53–7.57 (m, 1H), 7.64–7.66 (m, 2H), 8.29–8.31 (m, 1H), 8.38–8.41 (m, 1H), 12.02 (br s, 1H); MS (ESI) m/z 568 $[M+H]^+$; HRMS (ESI) Calcd for $C_{30}H_{31}ClFN_3O_5$+H: 568.20145. Found: 568.19918.

5.6. *trans*-4-[1-[[2-[1-Methyl-3-(1*H*-pyrrolo[2,3-*b*]pyridinyl)]-6-benzoxazolyl]acethyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid (8a)

Yield 66% (two steps). A yellow solid. IR (ATR) 2940, 2861, 1716, 1631, 1569, 1525, 1486, 1442 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.13–1.37 (m, 4H), 1.85–2.09 (m, 4H), 2.11–2.21 (m, 3H), 3.17–3.31 (m, 2H), 3.45 and 3.58 (each m, total 1H), 3.72–3.92 (m, 4H), 3.96 (s, 3H), 4.15 and 4.35 (each m, total 1H), 5.32 and 5.37 (each m, total 1H), 7.21 (m, 1H), 7.35 and 7.36 (each d, J = 8.1 Hz, total 1H), 7.55 and 7.57 (each s, total 1H), 7.61 and 7.62 (each d, J = 8.1 Hz, total 1H), 8.44 (dd, J = 4.7, 1.5 Hz, 1H), 8.53 (s, 1H), 8.61 (dd, J = 7.8, 1.5 Hz, 1H), 11.99 (br s, 1H); MS (ESI) m/z 535 $[M+H]^+$; Anal. Calcd for $C_{29}H_{31}FN_4O_5{\cdot}0.75H_2O$: C, 63.55; H, 5.98; N, 10.22. Found: C, 63.67; H, 5.91; N, 10.22.

5.7. General procedure B: preparation of *trans*-4-[1-[(2-phenyl-6-benzoxazolyl)acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid (8b)

To a solution of 2-phenyl-6-benzoxazolylacetic acid (**4a**) (57 mg, 0.23 mmol) and methyl *trans*-4-[(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylate (**6**) (58 mg, 0.23 mmol) in DMF (5 ml) were added HOBt (58 mg, 0.43 mmol), DMAP (catalytic amount) and EDC·HCl (65 mg, 0.34 mmol). The reaction mixture was stirred at room temperature for 18 h. The reaction mixture was poured in 1 N HCl and extracted with EtOAc three times. The combined extracts were washed with brine and dried over anhydrous $MgSO_4$. The solvent was evaporated under reduced pressure. The residue was dissolved in THF–MeOH (4 ml, 1:1, v/v) then 1 N NaOH (1 ml) was added. The reaction mixture was stirred for 18 h at room temperature and poured in 1 N HCl. The mixture was extracted with $CHCl_3$–MeOH and the combined extracts were dried over anhydrous $MgSO_4$. The mixture was evaporated and the residue was purified with TLC ($CHCl_3$–MeOH, 20:1, v/v) to give the title compound (42 mg, 39%) as a colorless solid. ^1H NMR (DMSO-d_6) δ 1.15–1.40 (m, 4H), 1.90 (m, 3H), 2.20 (m, 2H), 3.20–3.60 (m, 6H), 3.70–4.00 (m, 3H), 4.20 and 4.40 (m, 1H), 5.32 and 5.48 (each d, J = 7.0 Hz, total 1H), 7.30 (m, 1H), 7.70 (s, 4H), 7.74 (d, J = 2.0 Hz, 1H), 8.20 (m, 2H). MS (ESI) m/z 481 $[M+H]^+$; Anal. Calcd for $C_{27}H_{29}FN_2O_5{\cdot}1.0H_2O$: C, 65.05; H, 6.27; N, 5.62. Found: C, 64.86; H, 5.89; N, 5.37.

Compound **8d** was prepared according to general procedure B.

5.8. *trans*-4-[1-[[2-(4-Quinolinyl)-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid (8d)

Yield 56%. A colorless solid. ^1H NMR (DMSO-d_6) δ 1.20 (m, 2H), 1.32 (m, 2H), 1.90 (m, 4H), 2.18 (m, 4H), 3.20 (m, 1H), 3.45–3.60 (m, 1H), 3.72–4.12 (m, 4H), 4.15 and 4.40 (each m, total 1H), 5.35 (m, 1H), 7.36 (m, 1H), 7.75 (d, J = 9.3 Hz, 1H), 7.85 (m, 1H), 7.90 (m, 2H), 8.19 (d, J = 7.3 Hz, 1H), 8.30 (dd, J = 1.5, 4.5 Hz, 1H), 9.16 (d, J = 4.5 Hz, 1H), 9.42 (d, J = 8.6 Hz, 1H); MS (ESI) m/z 532 $[M+H]^+$; Anal. Calcd for $C_{30}H_{30}FN_3O_5{\cdot}0.5H_2O$: C, 66.65; H, 5.78; N, 7.77. Found: C, 66.53; H, 5.61; N, 7.63.

5.9. General procedure C: preparation of *trans*-4-[1-[[7-fluoro-2-(1-methyl-3-indolyl)-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid (7e)

A mixture of [7-fluoro-2-(1-methyl-3-indolyl)-6-benzoxazolyl]acetic acid (**3e**) (350 mg, 1.08 mmol), methyl *trans*-4-[(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylate (**6**) (280 mg, 1.08 mmol), EDC·HCl (311 mg, 1.62 mmol), HOBt (219 mg, 1.62 mmol) and Et_3N (0.75 ml, 5.40 mmol) in DMF (10 ml) was stirred at room temperature for 17 h. The mixture was poured into ice water and extracted with EtOAc. The combined extracts were washed with ice water and brine. After dried over Na_2SO_4, the extracts were concentrated in vacuo. The residue was purified by column chromatography on silica gel with *n*-hexane–EtOAc (3:1, v/v) as eluent to give methyl *trans*-4-[1-[[7-fluoro-2-(1-methyl-3-indolyl)-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylate (360 mg, 59%) as a brown oil. ^1H NMR ($CDCl_3$) δ 1.21–1.65 (m, 4H), 1.95–2.56 (m, 7H), 3.21–3.39 (m, 2H), 3.56 (dd, J = 8.8, 6.8 Hz, 1H), 3.64 and 3.67 (each s, total 3H, amide isomers), 3.68–3.90 (m, 3H), 3.92 (s, 3H), 4.00 and 4.03 (each s, total 1H), 4.31–4.42 (m, 1H), 5.19–5.39 (m, 1H), 7.15–7.23 (m, 1H), 7.36–7.48 (m, 4H), 7.98 (s, 1H), 8.45 (m, 1H); MS (ESI) m/z 566 $[M+H]^+$.

To a solution of methyl *trans*-4-[1-[[7-fluoro-2-(1-methyl-3-indolyl)-6- benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylate (130 mg, 0.23 mmol) in THF–MeOH (15 ml, 2:1, v/v) was added 1 N NaOH (4 ml). After stirring at room temperature for 13 h, the mixture was concentrated under reduced pressure and acidified with 1 N HCl. The precipitate was collected, washed with water and dried under reduced pressure to give the title compound (93 mg, 74%) as a brown solid. IR (ATR) 2941, 2864, 1716, 1628, 1583, 1504, 1442, 1369 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.13–1.43 (m, 5H), 1.82–2.36 (m, 7H), 3.24 (m, 1H), 3.46–3.93 (m, 4H), 3.95 (s, 3H), 4.11 and 4.15 (each s, total 1H, amide isomers), 4.42 (m, 1H), 5.25–5.50 (m, 1H), 7.22 (m, 1H), 7.35 (m, 2H), 7.48 and 7.50 (each t, J = 4.4 and 3.6 Hz respectively, total 1H, amide isomers), 7.63 (d, J = 6.6 Hz, 1H), 8.29 (d, J = 7.3 Hz, 1H), 8.44 and 8.45 (each s, total 1H, amide isomers); MS (ESI) m/z 552 $[M+H]^+$; Anal. Calcd for $C_{30}H_{31}F_2N_3O_5{\cdot}1H_2O$: C, 63.26; H, 5.84; N, 7.38. Found: C, 63.52; H, 5.77; N, 7.25.

Compound **8c, 8e** and **9a**–**h** were prepared according to general procedure C.

5.10. *trans*-4-[1-[[2-(1-Naphthyl)-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid (8c)

Yield 29% (two steps). A colorless solid. ^1H NMR (DMSO-d_6) δ 1.11–1.36 (m, 4H), 1.83–2.20 (m, 7H), 3.19–4.01 (m, 7H), 4.09–4.19 and 4.32–4.42 (each m, total 1H), 5.23–5.47 (m, 1H), 7.28–7.32 (m, 1H), 7.64–7.85 (m, 5H), 8.09 (d, J = 8.0 Hz, 1H), 8.21 (d, J = 8.0 Hz, 1H), 8.42–8.44 (m, 1H), 9.40–9.42 (m, 1H); MS (ESI), m/z 531 $[M+H]^+$; Anal. Calcd for $C_{31}H_{31}FN_2O_5{\cdot}0.5H_2O$: C, 69.00; H, 5.98; N, 5.19. Found: C, 69.11; H, 5.76; N, 5.17.

5.11. *trans*-4-[1-[[2-(1-Isoquinolinyl)-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylic acid (8e)

Yield 72% (two steps). A pale yellow solid. ^1H NMR (DMSO-d_6) δ 1.12–1.37 (m, 4H), 1.84–2.11 (m, 4H), 2.14–2.21 (m, 3H), 3.17–4.04

(m, 7H), 4.10–4.20 and 4.33–4.43 (each m, total 1H), 5.23–5.47 (m, 1H), 7.33–7.37 (m, 1H), 7.72–7.74 (m, 1H), 7.86–7.93 (m, 3H), 8.11–8.15 (m, 2H), 8.74–8.76 (m, 1H), 9.51–9.53 (m, 1H), 12.02 (br s, 1H); MS (ESI) *m/z* 532 $[M+H]^+$; Anal. Calcd for $C_{30}H_{30}FN_3O_5{\cdot}H_2O$: C, 65.56; H, 5.87; N, 7.65. Found: C, 65.39; H, 5.94; N, 7.51.

5.12. *trans*-4-[1-[[4-(2-Benzothiazolyl)amino-3-chlorophenyl] acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecar boxylic acid (9a)

Yield 70% (two steps). A pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.10–1.39 (m, 4H), 1.84–1.94 (m, 4H), 2.06–2.20 (m, 3H), 3.15–3.87 (m, 7H), 4.07–4.17 and 4.28–4.38 (each m, total 1H), 5.22–5.47 (m, 1H), 7.11–7.15 (m, 1H), 7.19–7.23 (m, 1H), 7.27–7.31 (m, 1H), 7.36–7.38 (m, 1H), 7.50–7.52 (m, 1H), 7.77–7.79 (m, 1H), 8.06–8.10 (m, 1H); MS (ESI) *m/z* 546 $[M+H]^+$; HRMS (ESI) Calcd for $C_{27}H_{29}ClFN_3O_4S$+H: 546.16296. Found: 546.15858.

5.13. *trans*-4-[1-[[3-Chloro-4-(6-methyl-2-benzothiazolyl) aminophenyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy] cyclohexanecarboxylic acid (9b)

Yield 59% (two steps). A brown solid. ^{1}H NMR (DMSO-d_6) δ 1.16–1.36 (m, 4H), 1.76–1.94 (m, 4H), 2.14–2.40 (m, total 6H, including s, 3H, at δ 2.34), 3.15–3.86 (m, 7H), 4.07–4.17 and 4.27–4.37 (each m, total 1H), 5.22–5.47 (m, 1H), 7.09–7.11 (m, 1H), 7.19–7.22 (m, 1H), 7.35–7.49 (m, 3H), 7.57 (s, 1H), 8.10–8.12 (m, 1H); MS (ESI) *m/z* 560 $[M+H]^+$; Anal. Calcd for $C_{28}H_{31}ClFN_3O_4S{\cdot}1.5H_2O$: C, 57.28; H, 5.84; N, 7.16; S, 5.46. Found: C, 57.20; H, 5.66; N, 6.91; S, 5.39.

5.14. *trans*-4-[1-[[3-Chloro-4-(6-fluoro-2-benzothiazolyl) aminophenyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy] cyclohexanecarboxylic acid (9c)

Yield 73% (two steps). A pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.17–1.38 (m, 4H), 1.74–2.21 (m, 7H), 3.18–3.87 (m, 7H), 4.09–4.19 and 4.29–4.39 (each m, total 1H), 5.23–5.47 (m, 1H), 7.12–7.23 (m, 2H), 7.37–7.38 (m, 1H), 7.51–7.54 (m, 1H), 7.71–7.74 (m, 1H), 8.09–8.13 (m, 1H), 9.94 (br s, 1H); MS (ESI) *m/z* 564 $[M+H]^+$; HRMS (ESI) Calcd for $C_{27}H_{28}ClF_2N_3O_4S$+H: 564.15354. Found: 564.14881.

5.15. *trans*-4-[1-[[3-chloro-4-(6-Chloro-2-benzothiazolyl) aminophenyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy] cyclohexanecarboxylic acid (9d)

Yield 79% (two steps). A pale yellow amorphous solid. ^{1}H NMR (DMSO-d_6) δ 1.16–1.36 (m, 4H), 1.87–2.20 (m, 7H), 3.15–3.87 (m, 7H), 4.08–4.18 and 4.28–4.38 (each m, total 1H), 5.22–5.47 (m, 1H), 7.21–7.23 (m, 1H), 7.29–7.32 (m, 1H), 7.37–7.38 (m, 1H), 7.48–7.51 (m, 1H), 7.92 (s, 1H), 8.05–8.09 (m, 1H); MS (ESI) *m/z* 580 $[M+H]^+$; HRMS (ESI) Calcd for $C_{27}H_{28}Cl_2FN_3O_4S$+H: 580.12398. Found: 580.11988.

5.16. *trans*-4-[1-[[3-Chloro-4-(5-fluoro-2-benzothiazolyl) aminophenyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy] cyclohexanecarboxylic acid (9e)

Yield 80% (two steps). A colorless solid. ^{1}H NMR (DMSO-d_6) δ 1.16–1.38 (m, 4H), 1.78–1.95 (m, 4H), 2.15–2.21 (m, 3H), 3.16–3.88 (m, 7H), 4.08–4.18 and 4.29–4.39 (each m, total 1H), 5.24–5.48 (m, 1H), 6.98–7.02 (m, 1H), 7.23–7.24 (m, 1H), 7.34–7.39 (m, 2H), 7.78–7.82 (m, 1H), 8.01–8.05 (m, 1H), 10.11 (br s, 1H), 12.06 (br s, 1H); MS (ESI) *m/z* 564 $[M+H]^+$; Anal. Calcd for $C_{27}H_{28}ClF_2N_3O_4S{\cdot}H_2O$: C, 55.71; H, 5.19; N, 7.22; S, 5.51. Found: C, 56.00; H, 4.99; N, 6.89; S, 5.38.

5.17. *trans*-4-[1-[[3-chloro-4-(4-Chloro-2-benzothiazolyl) aminophenyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy] cyclohexanecarboxylic acid (9f)

Yield 88% (two steps). A pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.13–1.37 (m, 4H), 1.86–1.95 (m, 4H), 2.08–2.33 (m, 3H), 3.16–3.88 (m, 7H), 4.08–4.18 and 4.30–4.40 (each m, total 1H), 5.23–5.48 (m, 1H), 7.11–7.15 (m, 1H), 7.23–7.26 (m, 1H), 7.38–7.41 (m, 2H), 7.75–7.77 (m, 1H), 8.14–8.18 (m, 1H), 10.23 (br s, 1H), 12.05 (br s, 1H); MS (ESI) *m/z* 580 $[M+H]^+$; HRMS (ESI) Calcd for $C_{27}H_{28}Cl_2FN_3O_4S$+H: 580.12398. Found: 580.12176.

5.18. *trans*-4-[1-[[4-(2-Benzothiazolyl)amino-5-chloro-2-fluorophenyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy] cyclohexanecarboxylic acid (9g)

Yield 62% (two steps). A colorless amorphous solid. ^{1}H NMR (DMSO-d_6) δ 1.16–1.38 (m, 4H), 1.89–2.23 (m, 7H), 3.17–3.93 (m, 7H), 4.08–4.18 and 4.30–4.40 (each m, total 1H), 5.24–5.50 (m, 1H), 7.17–7.21 (m, 1H), 7.32–7.36 (m, 1H), 7.40–7.45 (m, 1H), 7.61–7.63 (m, 1H), 7.82–7.84 (m, 1H), 8.32–8.34 (m, 1H); MS (ESI) *m/z* 564 $[M+H]^+$; HRMS (ESI) Calcd for $C_{27}H_{28}ClF_2N_3O_4S$+H: 564.15354. Found: 564.15126.

5.19. *trans*-4-[1-[[5-Chloro-2-fluoro-4-(5-fluoro-2-benzothiaz olyl)aminophenyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmeth oxy]cyclohexanecarboxylic acid (9h)

Yield 70% (two steps). A pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.18–1.38 (m, 4H), 1.86–1.99 (m, 4H), 2.10–2.21 (m, 3H), 3.17–4.02 (m, 7H), 4.08–4.18 and 4.31–4.41 (each m, total 1H), 5.24–5.50 (m, 1H), 7.03–7.08 (m, 1H), 7.41–7.48 (m, 2H), 7.83–7.87 (m, 1H), 8.25–8.27 (m, 1H); MS (ESI) *m/z* 582 $[M+H]^+$; Anal. Calcd for $C_{27}H_{27}ClF_3N_3O_4S{\cdot}0.5H_2O$: C, 54.87; H, 4.77; N, 7.11; S, 5.43. Found: C, 54.68; H, 4.62; N, 6.85; S, 5.45.

5.20. *trans*-4-[1-[[2-(5-Fluoro-1-methyl-3-indolyl)-7-fluoro-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy] cyclohexanecarboxylic acid (7f)

To a stirred solution of [2-(5-fluoro-1-methylindolyl)-7-fluoro-6-benzoxazolyl]acetic acid (**3f**) (202 mg, 0.59 mmol), methyl *trans*-4-[(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy] cyclohexanecarboxylate (**6**) (153 mg, 0.59 mmol) and EDC·HCl (124 mg, 0.65 mmol) in DMF (15 ml) was added HOBt (16.0 mg, 0.12 mmol), and the resulting mixture was stirred at room temperature overnight. The mixture was poured into water and extracted with EtOAc. The extracts were washed thrice with water, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by thin layer chromatography on silica gel with *n*-hexane–EtOAc (5:1, v/v) to give methyl *trans*-4-[1-[[2-(5-fluoro-1-methylindolyl)-7-fluoro-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylate (295 mg, 86%) as a colorless amorphous solid. ^{1}H NMR ($CDCl_3$) δ 1.20–1.31 (m, 2H), 1.39–1.55 (m, 2H), 1.99–2.55 (series of m, 8H), 3.21–3.40 and 3.52–3.59 (series of m, 2H), 3.64 and 3.67 (s, total 3H), 3.68–4.08 (series of m, 7H, including s, 3H, at δ 3.90), 4.30–4.44 (m, 1H), 5.19–5.40 (m, 1H), 7.08–7.13 (m, 1H), 7.20 (dd, J = 8.8, 15.2 Hz, 1H), 7.32 (dd, J = 4.0, 8.8 Hz, 1H), 7.45 (t, J = 8.0 Hz, 1H), 7.98 (s, 1H), 8.11 (dd, J = 2.4, 9.6 Hz, 1H); MS (ESI) *m/z* 584 $[M+H]^+$.

To a stirred solution of methyl *trans*-4-[1-[[2-(5-fluoro-1-methylindolyl)-7-fluoro-6-benzoxazolyl]acetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]cyclohexanecarboxylate (288 mg, 0.49 mmol) in

THF–MeOH (20 ml, 1:1, v/v) was added aqueous 0.25 N NaOH (9.9 ml, 2.45 mmol), and the resulting mixture was stirred at room temperature overnight. The mixture was poured into aqueous 1 N HCl and extracted with $CHCl_3$–MeOH (10:1, v/v). The extracts were dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica gel with $CHCl_3$–MeOH (10:1, v/v) as eluent to give the title compound (296 mg, quant.) as a colorless amorphous solid. ^{1}H NMR ($CDCl_3$) δ 1.18–1.38 (m, 2H), 1.39–1.58 (m, 2H), 1.96–2.56 (series of m, 7H), 3.21–3.60 (series of m, 2H), 3.66–4.10 (series of m, 8H, including s, 3H, at δ 3.89), 4.30–4.46 (m, 1H), 5.20–5.40 (m, 1H), 7.06–7.13 (m, 1H), 7.16–7.24 (m, 1H), 7.30–7.32 (m, 1H), 7.46 (t, J = 8.0 Hz, 1H), 7.99 (s, 1H), 8.10 (dd, J = 2.0, 9.2 Hz, 1H); MS (ESI) m/z 570 $[M+H]^+$.

5.21. General procedure D: preparation of [2-(1-methyl-3-indolyl)-6-benzoxazolyl]acetic acid (3a)

A mixture of 1-methylindole-3-carboxylic acid (**10**) (1.00 g, 5.71 mmol) and methyl 4-amino-3-hydroxyphenylacetate (**11a**)[12] (1.03 g, 5.71 mmol) in xylene (20 ml) was refluxed in the presence of boric acid (1.05 g, 17.1 mmol) for 24 h, during which time water was removed with Dean–Stark water trap. The precipitate was filtered off and the filtrate was concentrated to dryness. The residue was purified by column chromatography with *n*-hexane–EtOAc (4:1, v/v) as eluent to give methyl [2-(1-methyl-3-indolyl)-6-benzoxazolyl]acetate (0.20 g, 11%) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 3.72 (s, 3H), 3.76 (s, 2H), 3.88 (s, 3H), 7.21 (dd, J = 8.1, 1.5 Hz, 1H), 7.32–7.40 (m, 3H), 7.49 (s, 1H), 7.65 (d, J = 8.1 Hz, 1H), 7.91 (s, 1H), 8.45 (m, 1H); MS (ESI) m/z 321 $[M+H]^+$.

To a solution of methyl [2-(1-methyl-3-indolyl)-6-benzoxazolyl]acetate (200 mg, 0.62 mmol) in THF (6.5 ml) was added 0.25 N NaOH (3.75 ml, 0.93 mmol). The reaction mixture was stirred for 2 h and poured into 1 N HCl (10 ml). The precipitate was collected, washed with water, and dried to give the title compound (189 mg, 99%) as a colorless solid. ^{1}H NMR (DMSO-d_6) δ 3.73 (s, 2H), 3.94 (s, 3H), 7.24 (dd, J = 7.8, 1.5 Hz, 1H), 7.31 (td, J = 7.8, 1.5 Hz, 1H), 7.35 (td, J = 7.8, 1.5 Hz, 1H), 7.60–7.64 (m, 3H), 8.32 (dd, J = 8.1, 1.5 Hz, 1H), 8.34 (s, 1H), 12.37 (br s, 1H); MS (ESI) m/z 307 $[M+H]^+$.

Compound **4a** was prepared according to general procedure D.

5.22. 2-Phenyl-6-benzoxazolylacetic acid (4a)

Yield 11% (two steps). A colorless solid. ^{1}H NMR (DMSO-d_6) δ 3.76 (s, 2H), 7.32 (dd, J = 8.3, 1.1 Hz, 1H), 7.60–7.66 (m, 3H), 7.70 (d, J = 1.1 Hz, 1H), 7.74 (d, J = 8.3 Hz, 1H), 8.18–8.23 (m, 2H), 12.42 (br s, 1H); MS (ESI) m/z 254 $[M+H]^+$.

5.23. General procedure E: preparation of [2-(6-fluoro-1-methyl-3-indolyl)-6-benzoxazolyl]acetic acid (3b)

5.23.1. 6-Fluoro-1-methylindole-3-carbaldehyde (13a)

To a solution of 6-fluoroindole (**12a**) (6.10 g, 45.1 mmol) in DMF (65 ml) was added NaH (60% in oil, 1.45 g, 36.3 mmol) at 0 °C. After being stirred for 30 min at 0 °C, MeI (2.46 ml, 39.6 mmol) was added to the mixture, which was stirred for further 2.5 h and quenched by the addition of saturated aqueous NH_4Cl. The resulting mixture was diluted with water and extracted with EtOAc. The extract was washed brine, dried over $MgSO_4$, and concentrated to dryness. The residue was purified by column chromatography on silica gel with *n*-hexane–EtOAc (5:1, v/v) as eluent to give 6-fluoro-1-methylindole (6.71 g, 99%) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 3.73 (s, 3H), 6.45 (d, J = 3.2 Hz, 1H), 6.86 (ddd, J = 9.8, 8.5, 2.2 Hz, 1H), 6.98 (dd, J = 9.8, 2.2 Hz, 1H), 7.01 (d, J = 3.2 Hz, 1H), 7.51 (dd, J = 8.5, 5.3 Hz, 1H); MS (ESI) m/z 150 $[M+H]^+$.

$POCl_3$ (5.87 ml, 63.0 mmol) was added dropwise to DMF (45 ml) at 0 °C, and the mixture was stirred for 20 min at same temperature. A solution of 6-fluoro-1-methylindole (6.71 g, 45.0 mmol) in DMF (45 ml) was added slowly to the mixture at 0 °C. After being stirred at room temperature for 1.5 h, the reaction mixture was poured into ice, and the resulting mixture was neutralized with 1 N NaOH and extracted with EtOAc. The extract was washed with brine, dried over $MgSO_4$, and concentrated to dryness. The residue was purified by column chromatography on silica gel with EtOAc as eluent to give the title compound (4.18 g, 52%) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3H), 7.03–7.11 (m, 2H), 7.68 (s, 1H), 8.25 (dd, J = 8.8, 5.6 Hz, 1H), 9.97 (s, 1H).

5.23.2. [2-(6-Fluoro-1-methyl-3-indolyl)-6-benzoxazolyl]acetic acid (3b)

A mixture of 6-fluoro-1-methylindole-3-carbaldehyde (**13a**) (500 mg, 2.82 mmol) and methyl 4-amino-3-hydroxyphenylacetate (**11a**) (767 mg, 4.23 mmol) in EtOH (15 ml) was stirred for 22 h, and iodobenzene diacetate (1.09 g, 3.38 mmol) was added portionwise to the mixture. After being stirred for 1 h, the mixture was concentrated, and the residue was purified column chromatography with *n*-hexane–AcOEt (1:1, v/v) as eluent to give methyl [2-(6-fluoro-1-methyl-3-indolyl)-6-benzoxazolyl]acetate (889 mg, 93%) as a brown solid. ^{1}H NMR ($CDCl_3$) δ 3.72 (s, 3H), 3.76 (s, 2H), 3.85 (s, 3H) 7.05–7.12 (m, 2H), 7.22 (dd, J = 8.1, 1.5 Hz, 1H), 7.49 (d, J = 1.5 Hz, 1H), 7.65 (d, J = 8.1 Hz, 1H), 7.89 (s, 1H), 8.38 (dd, J = 8.9, 5.4 Hz, 1H); MS (ESI) m/z 339 $[M+H]^+$.

To a solution of methyl [2-(6-fluoro-1-methyl-3-indolyl)-6-benzoxazolyl]acetate (889 mg, 2.63 mmol) in THF (25 ml) was added 0.25 N NaOH (15.8 ml, 3.95 mmol) at room temperature. After being stirred for 4 h, the reaction mixture was poured into 1 N HCl (30 ml). The precipitate was collected, washed with water, and dried to give the title compound (741 mg, 87%) as a brown solid. ^{1}H NMR (DMSO-d_6) δ 3.71 (s, 2H), 3.94 (s, 3H), 7.18 (td, J = 9.5, 2.5 Hz, 1H), 7.23 (d, J = 7.8 Hz, 1H), 7.59–7.64 (m, 3H), 7.97 (dd, J = 9.5, 2.5 Hz, 1H), 8.39 (s, 1H), 12.35 (br s, 1H); MS (ESI) m/z 325 $[M+H]^+$.

Compound **3c–g**, **4b–d** and **13b–d** were prepared according to general procedure E.

5.24. 5-Fluoro-1-methylindole-3-carbaldehyde (13b)

Quantitative yield (two steps). A colorless solid. ^{1}H NMR ($CDCl_3$) δ 3.88 (s, 3H), 7.09 (m, 1H), 7.29 (d, J = 9.3, 4.2 Hz, 1H), 7.71 (s, 1H), 7.98 (dd, J = 9.3, 2.4 Hz, 1H), 9.96 (s, 1H); MS (ESI) m/z 178 $[M+H]^+$.

5.25. [2-(5-Fluoro-1-methyl-3-indolyl)-6-benzoxazolyl]acetic acid (3c)

Yield 24% (two steps). A brown solid. ^{1}H NMR (DMSO-d_6) δ 3.72 (s, 2H), 3.90 (s, 3H), 7.15 (td, J = 9.8, 2.2 Hz, 1H), 7.23 (dd, J = 8.1, 1.2 Hz, 1H), 7.50 (dd, J = 9.8, 2.2 Hz, 1H), 7.59 (d, J = 1.2 Hz, 1H), 7.62 (d, J = 8.1 Hz, 1H), 8.29 (dd, J = 8.8, 5.4 Hz, 1H), 8.33 (s, 1H), 12.37 (br s, 1H); MS (ESI) m/z 325 $[M+H]^+$.

5.26. 5-Chloro-1-methylindole-3-carbaldehyde (13c)

Yield 80% (two steps). A colorless solid. ^{1}H NMR ($CDCl_3$) δ 3.87 (s, 3H), 7.27 (d, J = 8.5 Hz, 1H), 7.31 (dd, J = 8.5, 1.9 Hz, 1H), 7.69 (s, 1H), 8.30 (d, J = 1.9 Hz, 1H), 9.96 (s, 1H).

5.27. [2-(5-Chloro-1-methyl-3-indolyl)-6-benzoxazolyl]acetic acid (3d)

Yield 9% (two steps). A brown solid. ^{1}H NMR (DMSO-d_6) δ 3.73 (s, 2H), 3.94 (s, 3H), 7.25 (dd, J = 8.1, 1.7 Hz, 1H), 7.36 (dd, 1H,

J = 8.8, 1.7 Hz, 1H), 7.62–7.68 (m, 3H), 8.30 (d, *J* = 1.7 Hz, 1H), 8.41 (s, 1H), 12.38 (br s, 1H); MS (ESI) *m/z* 341 $[M+H]^+$.

5.28. [7-Fluoro-2-(1-methyl-3-indolyl)-6-benzoxazolyl]acetic acid (3e)

Yield 22% (two steps). A dark green solid. ^{1}H NMR (DMSO-d_6) δ 3.79 (s, 2H), 3.95 (s, 3H), 7.26–7.39 (m, 3H), 7.50 (d, *J* = 8.3 Hz, 1H), 7.61–7.66 (m, 1H), 8.27–8.31 (m, 1H), 8.46 (s, 1H); MS (ESI), *m/z* 325 $[M+H]^+$.

5.29. [2-(5-Fluoro-1-methyl-3-indolyl)-7-fluoro-6-benzoxazolyl]acetic acid (3f)

Yield 21% (two steps). A brown solid. ^{1}H NMR (DMSO-d_6) δ 3.78 (s, 2H), 3.95 (s, 3H), 7.19–7.24 (m, 1H), 7.29 (d, *J* = 8.0 Hz, 1H), 7.50 (d, *J* = 8.4 Hz, 1H), 7.66 (dd, *J* = 8.8, 4.4 Hz, 1H), 7.94 (br d, *J* = 9.6 Hz, 1H), 8.50 (br s, 1H), 12.52 (br s, 1H).

5.30. 1-Methyl-1*H*-pyrrolo[2,3-*b*]pyridine-3-carbaldehyde (13d)

Yield 72% (two steps). A yellow solid. ^{1}H NMR ($CDCl_3$) δ 3.98 (s, 3H), 7.28 (dd, *J* = 7.8, 4.7 Hz, 1H), 7.85 (s, 1H), 8.44 (d, *J* = 4.7 Hz, 1H), 8.55 (d, *J* = 7.8 Hz, 1H), 9.97 (s, 1H).

5.31. [2-[1-Methyl-3-(1*H*-pyrrolo[2,3-*b*]pyridinyl)]-6-benzoxazolyl]acetic acid (3g)

Yield 62% (two steps). A brown solid. ^{1}H NMR (DMSO-d_6) δ 3.74 (s, 2H), 3.96 (s, 3H), 7.26 (dd. *J* = 8.1, 1.5 Hz, 1H), 7.36 (dd, *J* = 7.8, 4.7 Hz, 1H), 7.62 (d, *J* = 1.5 Hz, 1H), 7.65 (d, *J* = 8.1 Hz, 1H), 8.45 (dd, *J* = 4.7, 1.5 Hz, 1H), 8.53 (s, 1H), 8.62 (dd, *J* = 7.8, 1.5 Hz, 1H), 12.37 (br s, 1H); MS (ESI) *m/z* 308 $[M+H]^+$.

5.32. [2-(1-Isoquinolinyl)-6-benzoxazolyl]acetic acid (4b)

Yield 29% (two steps). A pale brown solid. ^{1}H NMR (DMSO-d_6) δ 3.82 (s, 2H), 7.41–7.43 (m, 1H), 7.82 (s, 1H), 7.88–7.95 (m, 3H), 8.13–8.17 (m, 2H), 8.76–8.78 (m, 1H), 9.53–9.55 (m, 1H), 12.45 (br s, 1H); MS (ESI), *m/z* 305 $[M+H]^+$.

5.33. [2-(1-Naphthyl)-6-benzoxazolyl]acetic acid (4c)

Yield 38% (two steps). A pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 3.78 (s, 2H), 7.34–7.36 (m, 1H), 7.65–7.78 (m, 4H), 7.84 (d, *J* = 8.1 Hz, 1H), 8.09 (d, *J* = 8.1 Hz, 1H), 8.21–8.23 (m, 1H), 8.43–8.45 (m, 1H), 9.40–9.42 (m, 1H).

5.34. [2-(4-Quinolinyl)-6-benzoxazolyl]acetic acid (4d)

Yield 85% (two steps). A brown solid. ^{1}H NMR (DMSO-d_6) δ 3.82 (s, 2H), 7.41 (dd, *J* = 8.1, 1.0 Hz, 1H), 7.82 (s, 1H), 7.85 (d, *J* = 8.3 Hz, 1H), 7.92 (m, 2H), 8.19 (d, *J* = 7.8 Hz, 1H), 8.31 (d, *J* = 4.4 Hz, 1H), 9.14 (d, *J* = 4.4 Hz, 1H), 9.42 (d, *J* = 8.1 Hz, 1H); MS (ESI) *m/z* 305 $[M+H]^+$.

5.35. 1-Isoquinolinecarbaldehyde (13f)

To a stirred solution of 1-methylisoquinoline (**12g**) (300 mg, 2.10 mmol) in dioxane (20 ml) was added SeO_2 (323 mg, 2.91 mmol), and the reaction mixture was refluxed for 1.5 h under N_2 atmosphere. After cooled to room temperature, the mixture was filtered through a celite pad, and the filtrate was evaporated. The residue was purified by column chromatography on silica gel with *n*-hexane–EtOAc (5:1, v/v) as eluent to give the title compound (252 mg, 77%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 7.73–7.79 (m, 2H), 7.88–7.93 (m, 2H), 8.74–8.76 (m, 1H), 9.30–9.33 (m, 1H), 10.39 (s, 1H); MS (ESI), *m/z* 158 $[M+H]^+$.

5.36. General procedure F: preparation of [3-chloro-4-(6-methyl-2-benzothiazolyl)aminophenyl]acetic acid (5b)

5.36.1. 2-Bromo-6-methylbenzothiazole (15b)

To a stirred suspension of CuBr (1.57 g, 10.9 mmol) in MeCN (25 ml) was added ^{t}BuONO (1.63 ml, 13.7 mmol) and the mixture was stirred at 60 °C for 10 min. Then 2-amino-6-methylbenzothiazole (**14b**) (1.50 g, 9.13 mmol) was added to the mixture, and the reaction mixture was stirred at 60 °C for 1 h. After cooled to room temperature, the mixture was poured into 1 N HCl. The resulting precipitate was dissolved into EtOAc, washed with 1 N HCl, brine, dried over Na_2SO_4, and evaporated. The residue was purified by column chromatography on silica gel with *n*-hexane–EtOAc (7:1, v/v) as eluent to give the title compound (703 mg, 34%) as a brown oil. ^{1}H NMR ($CDCl_3$) δ 2.47 (s, 3H), 7.27–7.29 (m, 1H), 7.58–7.59 (m, 1H), 7.84–7.87 (m, 1H).

5.36.2. [3-Chloro-4-(6-methyl-2-benzothiazolyl)aminophenyl] acetic acid (5b)

A mixture of 2-bromo-6-methylbenzothiazole (**15b**) (703 mg, 3.08 mmol), methyl 4-amino-3-chlorophenylacetate (**16a**) (615 mg, 3.08 mmol), and PPTS (232 mg, 0.92 mmol) in xylene (10 ml) was refluxed for 10 h. After cooled to room temperature, the solvent was evaporated. The residue was purified by column chromatography on silica gel with *n*-hexane–EtOAc (6:1, v/v) as eluent to give methyl [3-chloro-4-(6-methyl-2-benzothiazolyl)aminophenyl]acetate (274 mg, 26%) as a pale yellow oil. ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 3H), 3.59 (s, 2H), 3.71 (s, 3H), 7.17–7.19 (m, 1H), 7.23–7.25 (m, 1H), 7.35 (m, 1H), 7.46 (s, 1H), 7.58 (d, *J* = 8.3 Hz, 1H), 8.35 (d, *J* = 8.3 Hz, 1H); MS (ESI) *m/z* 347 $[M+H]^+$.

To a stirred solution of methyl [3-chloro-4-(6-methyl-2-benzothiazolyl)aminophenyl]acetate (274 mg, 0.79 mmol) in THF (5 ml) was added 0.5 N NaOH (5.0 ml, 2.50 mmol), and the reaction mixture was stirred at room temperature for 15 h. The mixture was poured into ice-1 N HCl. The resulting precipitate was collected with suction, and dried under vacuum to give the title compound (202 mg, 77%) as a pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 3H), 3.60 (s, 2H), 7.10–7.13 (m, 1H), 7.25–7.27 (m, 1H), 7.41–7.42 (m, 2H), 7.59 (s, 1H), 8.13–8.15 (m, 1H).

Compound **5a**, **5c–h** and **15c–g** were prepared according to general procedure F.

5.37. [4-(2-Benzothiazolyl)amino-3-chlorophenyl]acetic acid (5a)

Yield 30% (two steps). A colorless solid. ^{1}H NMR (DMSO-d_6) δ 3.59 (s, 2H), 7.11–7.15 (m, 1H), 7.25–7.31 (m, 2H), 7.42 (d, *J* = 2.0 Hz, 1H), 7.52 (d, *J* = 8.1 Hz, 1H), 7.77 (d, *J* = 8.1 Hz, 1H), 8.11–8.13 (m, 1H); MS (ESI) *m/z* 319 $[M+H]^+$.

5.38. 2-Bromo-6-fluorobenzothiazole (15c)

Yield 40%. A brown solid. ^{1}H NMR ($CDCl_3$) δ 7.19–7.24 (m, 1H), 7.49–7.52 (m, 1H), 7.92–7.96 (m, 1H).

5.39. [3-Chloro-4-(6-fluoro-2-benzothiazolyl)aminophenyl]acetic acid (5c)

Yield 27% (two steps). A pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 3.59 (s, 2H), 7.11–7.15 (m, 1H), 7.25–7.27 (m, 1H), 7.41–7.42 (m, 1H), 7.50–7.53 (m, 1H), 7.70–7.73 (m, 1H), 8.12–8.14 (m, 1H), 9.97 (br s, 1H), 12.42 (br s, 1H); MS (ESI) *m/z* 337 $[M+H]^+$.

5.40. 2-Bromo-6-chlorobenzothiazole (15d)

Yield 69%. A yellow solid. ^{1}H NMR ($CDCl_3$) δ 7.42–7.46 (m, 1H), 7.76–7.90 (m, 2H).

5.41. [3-chloro-4-(6-Chloro-2-benzothiazolyl)aminophenyl] acetic acid (5d)

Yield 23% (two steps). A pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 3.61 (s, 2H), 7.26–7.33 (m, 2H), 7.43–7.44 (m, 1H), 7.50–7.52 (m, 1H), 7.92–7.93 (m, 1H), 8.08–8.10 (m, 1H).

5.42. 2-Bromo-5-fluorobenzothiazole (15e)

Yield 63%. A yellow solid. ^{1}H NMR ($CDCl_3$) δ 7.18–7.23 (m, 1H), 7.64–7.77 (m, 2H).

5.43. [3-Chloro-4-(5-fluoro-2-benzothiazolyl)aminophenyl] acetic acid (5e)

Yield 27% (two steps). A pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 3.61 (s, 2H), 6.98–7.03 (m, 1H), 7.27 (dd, J = 8.3, 2.0 Hz, 1H), 7.34–7.37 (m, 1H), 7.44 (d, J = 2.0 Hz, 1H), 7.78–7.82 (m, 1H), 8.05 (d, J = 8.3 Hz, 1H).

5.44. 2-Bromo-4-chlorobenzothiazole (15f)

Yield 65%. A pale yellow solid. ^{1}H NMR ($CDCl_3$) δ 7.33–7.38 (m, 1H), 7.49–7.53 (m, 1H), 7.67–7.71 (m, 1H).

5.45. [3-chloro-4-(4-Chloro-2-benzothiazolyl)aminophenyl] acetic acid (5f)

Yield 51% (two steps). A pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 3.63 (s, 2H), 7.12–7.16 (m, 1H), 7.29–7.31 (m, 1H), 7.38–7.41 (m, 1H), 7.46–7.47 (m, 1H), 7.76–7.78 (m, 1H), 8.18–8.21 (m, 1H), 10.26 (br s, 1H), 12.45 (br s, 1H).

5.46. 2-Bromobenzothiazole (15g)

Yield 57%. A brown oil. ^{1}H NMR ($CDCl_3$) δ 7.39–7.51 (m, 2H), 7.77–7.82 (m, 1H), 7.94–8.00 (m, 1H).

5.47. [4-(2-Benzothiazolyl)amino-5-chloro-2-fluorophenyl] acetic acid (5g)

Yield 36% (two steps). A pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 3.63 (s, 2H), 7.17–7.21 (m, 1H), 7.32–7.39 (m, 1H), 7.52–7.54 (m, 1H), 7.61–7.63 (m, 1H), 7.82–7.84 (m, 1H), 8.38 (m, 1H), 10.17 (br s, 1H), 12.52 (br s, 1H).

5.48. [5-Chloro-2-fluoro-4-(5-fluoro-2-benzothiazolyl) aminophenyl]acetic acid (5h)

Yield 28% (two steps). A pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 3.64 (s, 2H), 7.03–7.08 (m, 1H), 7.45–7.55 (m, 2H), 7.83–7.86 (m, 1H), 8.28–8.31 (m, 1H), 10.26 (br s, 1H), 12.56 (br s, 1H).

5.49. VLA-4/VCAM-1 binding assay

A human VLA-4-expressing cell line, 4B4, was established at Pharmacopeia Inc., by transfecting both the α4 gene and β1 gene of VLA-4 into CHO-K1 cells. The 4B4 cells were maintained in Ham's F-12 medium (Sigma Corp.) supplemented with 10% (v/v) fetal calf serum (REHATUIN Fetal Bovine Serum, Serologicals Corp.), 100 U/ml penicillin (Invitrogen Corp.), 100 μg/ml streptomycin (Invitrogen Corp.), 2 mM L-glutamine (Invitrogen Corp.) and 1 mg/ml G-418 (Geneticin, Invitrogen Corp.). A Eu-labelling Reagent (PerkinElmer Inc.) was used to label the human VCAM-1/Fc chimera (R&D Systems Inc.). The Eu-labelled protein was purified with a PD-10 column (Amersham Biosciences KK.) and stored at −80 °C until use. All assays were performed in duplicate. In preparation for the assay, the 4B4 cells were suspended at 3×10^5 cells/ml in Ham's F-12 medium. One hundred microliter of the 4B4 cell suspension was placed into each well of a 96-well-culture plate (Costar Inc.). The plates were incubated at 37 °C in a 5% CO_2 atmosphere for 2 days. Prior to the assay, the medium was discarded and each well was washed twice with 300 μl of chilled Wash Buffer (25 mM HEPES, pH 7.5; 150 mM NaCl; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 4 mM $MnCl_2$). Then, 50 μl of compound solution was added to a well, followed by 50 μl of 2 nM of Eu-labelled human VCAM-1/Fc chimera diluted with the Wash Buffer (final concentration: 1 nM). For assays conducted in the presence of human serum albumin, 50 μl of compound at various concentrations and an equal volume of 2 nM Eu-labelled human VCAM-1/Fc chimera in 6% (w/v) human serum albumin (Sigma Corp.) were distributed into each well (final concentration: 1 nM). The plates were incubated for 60 min at room temperature and the wells were washed four times with 300 μl of chilled Wash Buffer. Finally, 100 μl of the enhancement solution (PerkinElmer Inc.) was added to each well. The plates were placed on a shaker for 5 min. Eu fluorescence was then measured using a time-resolved fluorometer (DELFIA Research fluorometer, model 1234–001; PerkinElmer Inc.). The concentration of compound required for 50% inhibition in the assay was determined.

5.50. Estimated serum concentration

Female BALB/c mice (8 weeks old, Charles River Japan, Inc.) were used. Each group consisted of four animals. The mice were orally administered the compound dissolved in 0.5% (w/v) methylcellulose (MC) at a dose of 10 mg/ml/kg. After 15 or 60 min, blood samples were collected via inferior vena cava from the animals under ether anesthesia. The blood samples were stored at room temperature and centrifuged at 2000 rpm for 10 min at 4 °C. The serum samples were subsequently stored in a −20 °C freezer prior to analysis. According to the VLA-4/VCAM-1 binding assay, instead of the compound solution, 50 μl of serum samples at various concentrations were added into each well (final concentration: 0.01–10%). As for the calibration curve, each diluted compound solution was also assayed in the presence of the same concentration of untreated mouse serum.

5.51. Murine bronchial inflammatory model

Female BALB/c AnNCrj mice (8 weeks old, Charles River Japan, Inc.) received an oral administration of cyclophosphamide dissolved in water at a dose of 150 mg/kg (day 0). On day 2 and 14, five-hundred μg protein of *Ascaris suum* extract (LSL Co., Ltd) in 0.2 ml saline containing 4.5 mg aluminum hydroxide was injected intraperitoneally. On day 22, the mice were challenged intratracheally under anesthesia with 300 μg (30 μl) protein of *Ascaris suum* extract. In the negative control group, sensitized mice were challenged with saline instead of the antigen.

5.52. Effect on eosinophil infiltration

Test compounds, which were dissolved in 0.5% MC containing 0.03% Tween 80, were orally administered 15 min before and 8, 24, and 32 h after the antigen challenge at a dose of 5 or 15 mg/kg. Forty-eight hours after antigen challenge, the mice were sacrificed and BALF was collected using tracheal polyethylene cannula with

2 × 0.5 ml Hanks' balanced salt solution. The cells in the BALF were counted in a particle analyzer CDA-500 (Sysmex Corp.). Cytocentrifuged preparations (Cytospin 2; Shandon) were stained with Wright's stain solution (Muto Chemical Co., Ltd) for differential counts, based on standard morphologic criteria. The number of eosinophils was calculated by multiplying the total cell number by the percentage of eosinophils in the cytocentrifuged preparations.

5.53. Pharmacokinetic studies on rats

Male Sprague–Dawley rats [7 weeks old, SLC Japan] were used. The animals were fasted for 16 h prior to drug administration, whereas access to water was provided ad libitum. Each group consisted of three animals. For the oral administration, the test compound was suspended in 0.5% (w/v) MC and administrated in rats at the dose of 1 mg/kg. For the intravenous administration, the test compound was dissolved in saline with 3 equiv NaOH solution and administrated to the jugular vein at the dose of 1 mg/kg. Blood samples (0.5 ml) were collected at 0.5, 1, 2, 4, and 6 h for po, or 0.08, 0.5, 1, 2, and 4 h for iv after the administration. These analytical samples were stored at room temperature, followed by centrifugation at 3000 rpm for 10 min at 4 °C. The plasma fractions were subsequently stored in a −20 °C freezer until analysis. The concentrations of the test compounds were measured by an LC/MS/MS method consisting of an Alliance 2695 HPLC (Waters), Symmetry Shield RP8, 2.1 × 50 mm, 3.5 μm column (Waters), and TSQ-700 (Thermo Electron, Waltham, MA). The mobile phase consisted of 10 mM $HCOONH_4$ in water/methanol; the gradient condition was 90:10 to 10:90 (the plasma concentrations versus the time data were analyzed by non-compartmental approaches using the WinNonlin software program (version 1.13.1 Pharsight, Mountain View, CA)). The pharmacokinetics parameters after oral and intravenous administration were calculated using the non-compartmental method without extrapolation using a validated program developed in-house.

All the animal experiments were conducted with the approval of the Animal Experiment Ethics Committee of Daiichi Pharmaceutical Co., Ltd.

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