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Synthesis and biological evaluation of benzoic acid derivatives as potent, orally active VLA-4 antagonists

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Abstract—A series of benzoic acid derivatives was synthesized as VLA-4 antagonists. Introduction of chlorine or bromine into the 3-position on the central benzene of the diphenylurea portion as in lead compound **2** led to improvement in the pharmacokinetic properties. In particular, **12l** demonstrated an acceptable plasma clearance and bioavailability in mice and rats as well as dogs (mice, $CL = 18.5\ ml/min/kg, F = 28\%$; rats, $CL = 5.2\ ml/min/kg, F = 36\%$; dogs, $CL = 3.6\ ml/min/kg, F = 55\%$). Additionally, **12l** exhibited potent activity with an IC_{50} value of 0.51 nM and efficacy by oral administration at a dosage of 10 mg/kg in a rat pleurisy model.

1. Introduction

VLA-4 (very late antigen 4; $\alpha_4\beta_1$ integrin; CD49d/CD29) is a key cell receptor expressed on most leukocytes.[1] The natural ligands include VCAM-1 (vascular cell adhesion molecule-1) expressed on cytokine-stimulated endothelial cells, the alternatively spliced connecting segment-1 domain of fibronectin on the extracellular matrix,[2,3] and junctional adhesion molecule 2 on endothelial cells.[4] Through the VLA-4/ligands interactions, VLA-4 plays an important role in the process of adhesion, migration, and activation of inflammatory leukocytes at sites of inflammation. *Anti*-VLA-4 antibodies or small molecular VLA-4 antagonists[5] have been reported to inhibit leukocyte infiltration into extravascular tissue and thus prevent tissue damage in inflammatory disease models of asthma,[6] multiple sclerosis,[7] rheumatoid arthritis,[8] and inflammatory bowel disease.[9] It has recently been reported that a humanized monoclonal *anti*-α_4 antibody, natalizumab,[10] (Elan Pharmaceuticals Inc. and Biogen Idec Inc.), demonstrates promising results in patients with multiple sclerosis, Crohn's disease, and rheumatoid arthritis in clinical trials. Therefore, the development of small molecular VLA-4 antagonists with acceptable oral pharmacokinetic profiles is viewed as a reasonable approach to a novel class of therapeutic agents.

We have reported the identification of morpholinylcarboxypiperidinylacetic acid **1** as a potent VLA-4 antagonist with an IC_{50} value of 4.4 nM[11] (Fig. 1). Compound **1** has, however, been found to show rapid plasma clearance and low bioavailability in rats ($CL = 69\ ml/min/kg, F < 1\%$). It has also been found that **1** is poorly permeable (P_{app} values $= 0.02 \times 10^{-6}$ cm/s) in the permeability assay using Madin–Darby canine kidney (MDCK) cell monolayers owing to its low lipophilicity with a $\log D$ value of -0.7. In our efforts to improve its rapid clearance and poor membrane permeability, structural modification of the morpholine-ring and the piperidinylacetic acid portion of **1** has resulted in the pyrolidinylmethoxybenzoic acid derivative **2** (Fig. 1).[12] This compound has potent activity with an IC_{50} value of 1.2 nM and comparatively high lipophilicity with a $\log D$ value of

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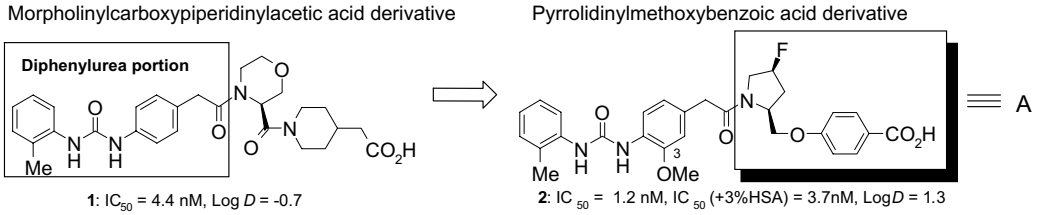


Figure 1. VLA-4 antagonists.

1.3. Thus, we next chose the diphenylurea portion (Fig. 1) for further exploration.

So far, regarding the SAR of the diphenylurea portion, Biogen research group has reported that the presence of a methyl group at the 2-position in the terminal benzene for valine derivatives[6] and a methoxy group at the 3-position in the central benzene for β-alanine derivatives enhances inhibitory activity.[13] Moreover, GlaxoSmithKline research group has very recently reported that the methoxy group at the 3-position in the central benzene, which seems to form an intra-molecular hydrogen bond with acidic urea functionality, contributes to improved pharmacokinetics.[14] From these results, we decided to investigate the effects of substituents at the same positions on the activity and pharmacokinetic properties as a first examination of modifications of the diphenylurea portion. At this point, we selected some substituents on the basis of their molecular refraction index (MR)[15] and lipophilicity (π value).[15] In addition, since we have found [(4*S*)-fluoro-(2*S*)-pyrrolidinyl]methoxybenzoic acid (**A**, Fig. 1) to be a beneficial motif for maintaining potent activity,[12b] the motif was not altered in this study.

In this paper, we wish to report the synthesis and SARs for a variety of substituents in the diphenylurea portion of benzoic acid derivatives as well as the PK profiles for representative compounds in rodents and dogs. Furthermore, we also present the result of the evaluation of the best compound in rat pleurisy model.

2. Chemistry

Benzoic acid derivatives **12a–q** were synthesized according to the General Procedure shown in Scheme 1. Thus, treatment of compounds **3–9** with commercially available isocyanate [2-(R2)PhNCO] and hydrolysis provided 4-ureidophenylacetic acids **10a–q**, which were condensed with methyl 4-[(4*S*)-fluoro-(2S)-pyrrolidinylmethoxy]benzoate (**11**) using EDC and HOBt followed by basic hydrolysis to afford **12a–q**.

Compounds **3–9** in Scheme 1 were prepared as shown in Schemes 2–6.

Commercially available benzoic acids **13a** and **13b** were converted to diazoketone **14a** and **14b** via the corresponding acid chloride, followed by the Arndt-Eistert reaction,[16] to give *tert*-butyl phenylacetate **15a** and **15b**. Subsequently, the nitro groups of **15a** and **15b** were reduced by hydrogenation to afford anilines **3** and **4** (Scheme 2).

Anilines **8** and **9** were prepared from commercially available phenylacetic acids **16a** and **16b** by reported procedure[17,18] (Scheme 3).

3-Bromophenylacetate **18b** was coupled with trimethylsilylacetylene by using the Sonogashira reaction[19] to give **19**, followed by removal of trimethylsilyl group to give **20**. The C–C triple bond and nitro group of **20** were reduced at the same time by hydrogenation to afford aniline **5** (Scheme 4).

Ethyl 4-nitrophenylacetate (**21**) was converted to **23** according to the reported procedure,[20] which was then subjected to hydrogenation to afford aniline **6** (Scheme 5).

Coupling of compound **24** with *tert*-butyl ethyl malonate in the presence of NaH gave **25**, which was successively hydrolyzed and decarboxylated in TFA condition to give **26**. Subsequently, the nitro group of **26** was reduced with $SnCl_2$ to afford aniline **7** (Scheme 6).

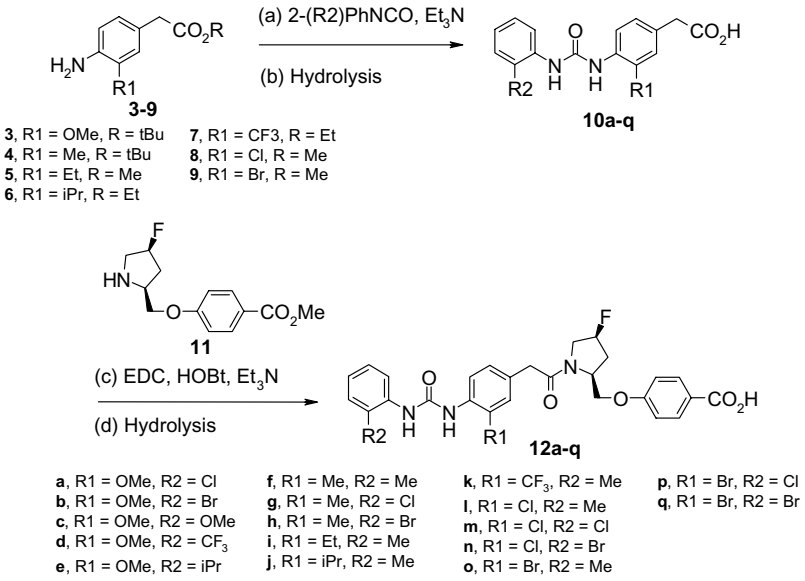
3. Results and discussion

3.1. *In vitro* activity

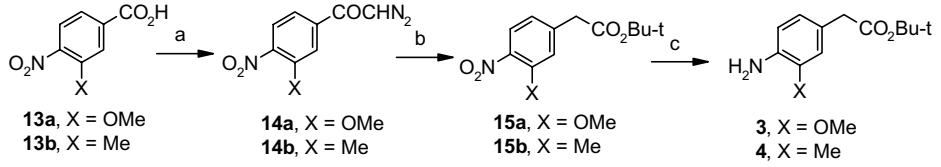
Benzoic acid derivatives were evaluated for their VLA-4 inhibitory activities in the receptor binding assay, with or without the addition of 3% human serum albumin (HSA). These results are summarized in Tables 1 and 2.

First, to investigate how replacement of the methyl group with other substituents (R2) at the 2-position on the terminal benzene in compound **2** affects the activity, we introduced several substituents (Cl, Br, MeO, and CF_3) with MR of 5.02–8.88, which are about the same size as the methyl group (MR = 5.65), and an isopropyl group with a larger size (MR = 14.96) into the position (Table 1).

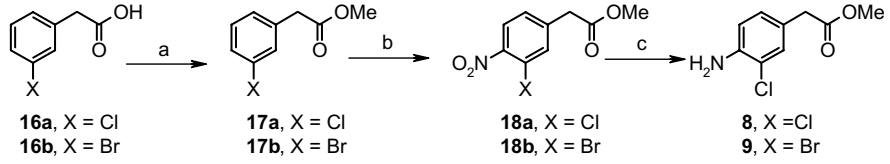
As a result, it was found that chlorine (**12a**, MR = 6.03) and bromine (**12b**, MR = 8.88) maintained the potency (**12a**, IC_{50} = 0.87 nM; **12b**, IC_{50} = 1.6 nM) in comparison with **2** (IC_{50}= 1.2 nM). In contrast, a methoxy group (**12c**, MR = 7.87) and a trifluoromethyl group (**12d**, MR = 5.02) reduced the potency by 1/5 and 1/96, respectively (**12c**, IC_{50} = 9.2 nM; **12d**,



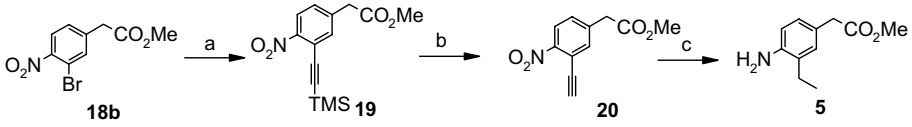
Scheme 1.



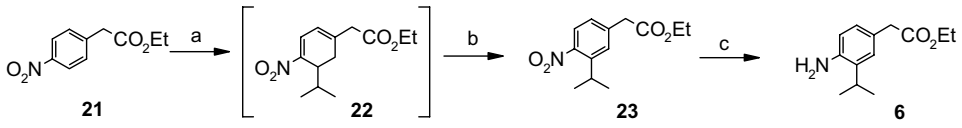
Scheme 2. Reagents: (a) 1-$SOCl_2$, toluene, cat. DMF; 2-$TMSCHN_2$, Et_3N, THF; (b) $PhCO_2Ag$, Et_3N, *tert*-BuOH/toluene; (c) H_2, 5% Pd/C, MeOH.



Scheme 3. Reagents: (a) MeOH, concd H_2SO_4, $ClCH_2CH_2Cl$; (b) concd HNO_3, concd H_2SO_4; (c) Fe, NaOAc, HOAc, MeOH, H_2O.

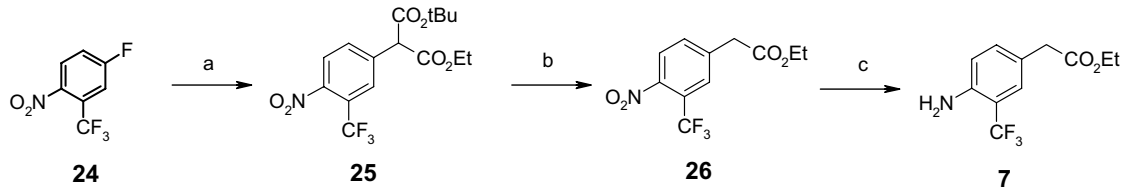


Scheme 4. Reagents: (a) TMS–acetylene, $Pd(Ph_3P)_4$, CuI, TEA, THF; (b) TBAF, THF; (c) H_2, 5% Pd/C, H_2, EtOH.



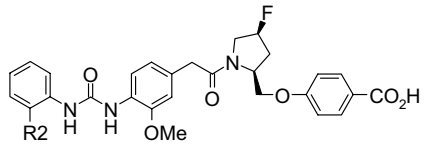
Scheme 5. Reagents: (a) isopropyl magnesium chloride, THF; (b) DDQ, THF; (c) H_2, 5% Pd/C, H_2, EtOH.

IC_{50} = 106 nM). In addition, the isopropyl group (**12e**) significantly reduced the potency by about 1/700-fold (IC_{50} = 769 nM), which was in agreement with the results of piperidone derivatives reported by Glaxo-SmithKline research group.[14] From these results, it was found that the size (MR) of the substituent at this position was a critical factor to maintain the activity; however, substituents containing a polar atom, such as a methoxy group, caused a negative effect on the activity.



Scheme 6. Reagents: (a) *tert*-butyl ethyl malonate, NaH, DMF; (b) TFA, DCM; (c) $SnCl_2$, EtOH, reflux.

Table 1. Inhibitory activity of VLA-4 antagonists



Compound	R2	IC_{50} (nM)	IC_{50} (+3% HSA) (nM)	Ratio = IC_{50} (+3% HSA)/IC_{50}
2	Me	1.2	3.7	3.4
12a	Cl	0.87	6.2	7.1
12b	Br	1.6	16	10.0
12c	MeO	9.2	268	29.1
12d	CF_3	106	>1000	Nd
12e	^{i}Pr	769	>1000	Nd

Nd, not determined; Nt, not tested.

We also evaluated the activities in the presence of 3% human serum albumin (HSA) to estimate in vivo effectiveness.

As a result, all the compounds **12a**–**e** showed a decreased activity by 1/7–1/29 compared with those without the addition of HSA, indicating that these compounds were more likely to be affected by HSA than **2**. The decrease of the activity is probably due to the high protein binding of these compounds [**2**, protein binding (PB) = 95%; **12b**, PB = 98%].

We next investigated the effect of R1 substituents at the 3-position of the central benzene on the activity, where we retained the methyl group, chlorine, and bromine showing high potency as R2 substituents (Table 2).

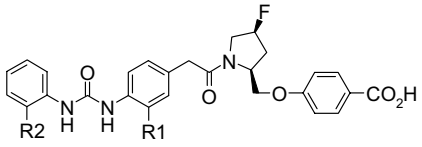
In the case of R2 = Me, replacement of the methoxy group in **2** with chlorine and bromine exhibited improved activity (**12l**, R2 = Cl, IC_{50} = 0.51 nM; **12o**, R2 = Br, IC_{50} = 0.78 nM), and the replacement by a methyl group slightly decreased the activity (**12f**, IC_{50} = 2.3 nM).

However, ethyl and isopropyl groups reduced the activities by 1/14 and 1/196 in accordance with the increase of MR (**12i**, R2 = ethyl, MR = 10.3, IC_{50} = 15 nM; **12j**, R2 = isopropyl, MR = 14.96, IC_{50} = 216 nM). In addition, the introduction of a trifluoromethyl group reduced the activity by 1/14 (**12k**, R2 = CF_3, MR = 5.02, IC_{50} = 15 nM).

In the case of R2 = Cl, **12m** (R1 = Cl) and **12p** (R1 = Br) showed potent activity (IC_{50} = 1.3 nM, each); however, **12q** (R1 = Me) was 12-fold less potent (IC_{50} = 13 nM) than **2**. Similar results were observed for compounds with R2 = Br (**12n**, R1 = Cl, R2 = Br, IC_{50} = 1.7 nM; **12q**, R1 = Br, R2 = Br, IC_{50} = 1.4 nM).

As observed in the variations of R2, all of these compounds were found to show decreased affinity against

Table 2. Inhibitory activity of VLA-4 antagonists



Compound	R2	R1	IC_{50} (nM)	IC_{50} (+3% HSA) (nM)	Ratio = IC_{50} (+3% HSA)/IC_{50}
2	Me	MeO	1.2	3.7	3.4
12f	Me	Me	2.3	49	21.3
12i	Me	Et	15	282	18.8
12j	Me	^{i}Pr	216	>1000	Nd
12k	Me	CF3	15	486	32.4
12l	Me	Cl	0.51	7.2	14.1
12o	Me	Br	0.78	8.4	10.8
12g	Cl	Me	13	416	32.0
12m	Cl	Cl	1.3	81	62.3
12p	Cl	Br	1.3	51	39.2
12h	Br	Me	9.8	622	63.5
12n	Br	Cl	1.7	137	80.6
12q	Br	Br	1.4	95	67.9

Nd, not determined; Nt, not tested.

VLA-4 in the presence of HSA, likely due to their high protein binding (**12l**, PB = 98%). However, since **12l** (R1 = Cl, R2 = Me) and **12o** (R1 = Br, R2 = Me) had extremely high affinity against VLA-4, these compounds maintained single digit nM activities even in the presence of HSA [**12l** and **12o**, IC_{50} (+HSA) = 7.2 and 8.4 nM].

3.2. Membrane permeability and bio-assay

For the twelve compounds showing potent activity with IC_{50} values less than 10 nM, we evaluated their membrane permeability with MDCK cell monolayers and determined their distribution coefficients [log *D*, *n*-octanol–PBS (pH 7.4)] (Table 3).

As a result, it was reasonably concluded that the introduction of halogen atom (Cl or Br) into R1 and/or R2 (**12a–b**, and **12g–q**) increased the lipophilicity and enhanced membrane permeability. Compounds **12c** (R1 = R2 = OMe) and **12f** (R1 = R2 = Me), however, displayed poor membrane permeability.

Furthermore, to assess oral availability of these compounds, we estimated the serum concentration by measuring the binding activity in the serum at 15 min post-oral administration at 10 and/or 50 mg/kg in mice (Table 3).

Compounds **12a–c** (R1 = OMe), **12f–h** (R1 = Me), and **12l–q** (R1 = Cl or Br) showed the estimated concentrations of 100–261 ng/ml, 368–693 ng/ml, and 600–3659 ng/ml, respectively. Among them, **12l** displayed the highest serum concentration in spite of its relatively low membrane permeability. Considering that **12b**, **12h**, **12l**, and **12o** had about the same log *D* values ranging from 1.61 to 1.76, the lipophilicity of substituent R1 (OMe, $\pi = -0.02$; Me, $\pi = 0.56$; Cl, $\pi = 0.71$; Br, $\pi = 0.86$) and/or the inductive effect, rather than the lipophilicity of the molecule, might be critical for oral absorption for this series of compounds.

3.3. Pharmacokinetics evaluation

To further clarify the influence of substituent R1 of central benzene ring on oral absorption, we evaluated pharmacokinetic profiles of the three selected compounds **12b** (R1 = OMe), **12h** (R1 = Me), and **12l** (R1 = Cl) in mice and rats (Table 4).

As a result, **12l** exhibited moderate plasma clearance (CL) of 18.5 ml/min/kg in mice, compared with those of **12h** (CL = 31.7 ml/min/kg) and **12b** (CL = 104.5 ml/min/kg), indicating that substituent R1 improved plasma clearance in the order of chlorine, methyl group, and methoxy group. Additionally, **12l**, **12h**, and **12b** showed *F* values of 28%, 21%, and 16%, respectively. Furthermore, similar results were observed in rats (**12l**, CL = 5.2 ml/min/kg, $F = 36\%$, **12h**, CL = 11.3 ml/min/kg, $F = 34\%$; **12b**, CL = 26.5 ml/min/kg, $F = 11\%$). Moreover, **12l** was found to show better pharmacokinetic profiles in dogs (CL = 3.6 ml/min/kg; $F = 55\%$) than rodents (Table 4).

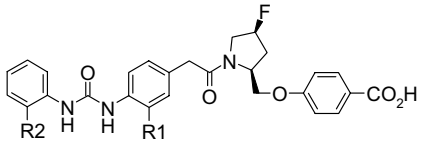
3.4. *In vivo* evaluation

On the basis of the activity and pharmacokinetic properties in rats, we evaluated **12l** in the compound 48/80-inducing rat pleurisy models.[21] As a result, it was found that **12l** dose-dependently inhibited the accumulation of eosinophils in the pleural cavity by oral administration at 10–30 mg/kg (bid) (Fig. 2).

4. Conclusion

We have discovered that the introduction of halogen (Cl and Br) into the 3-position on the central benzene ring significantly improves the pharmacokinetic

Table 3. Plasma concentration and distribution coefficient of VLA-4 antagonists



Compound	R2	R1	log *D* (pH 7.4)	MDCK P_{app} ($\times 10^{-6}$ cm/s)	Serum concentration (ng/ml)
2	Me	MeO	1.31	0.70	22[b]
12a	Cl	MeO	1.87	1.70	142[b]
12b	Br	MeO	1.76	2.65	261[a]/239[b]
12c	MeO	MeO	1.31	0.44	100[a]
12f	Me	Me	1.09	0.25	368[a]
12g	Cl	Me	1.69	3.00	472[a]
12h	Br	Me	1.64	3.00	693[a]
12l	Me	Cl	1.61	2.68	3659[a]
12m	Cl	Cl	2.27	5.50	2523[a]
12n	Br	Cl	2.34	5.25	2162[a]
12o	Me	Br	1.68	1.92	>600[a]
12p	Cl	Br	2.17	4.49	1881[a]
12q	Br	Br	2.28	3.40	3514[a]

[a] Administered at 10 mg/kg and serum concentration measured after 15 min.
[b] Administered at 50 mg/kg and serum concentration measured after 30 min.

Table 4. Pharmacokinetic properties of selected VLA-4 antagonists in mice, rats, and dogs

Compound	Mouse [a] (10 mg/kg)						Rat[b] (10 mg/kg)						Dog[h] (1 mg/kg)					
	F (%)	MRT (h)	AUC (po/iv) (ngh/ml)	Cmax (po) (ngh/ml)	CL (ml/min/kg)	V_{dss} (l/kg)	*F* (%)	MRT (h)	AUC (po/iv) (ngh/ml)	C_{max} (po) (ngh/ml)	CL (ml/min/kg)	V_{dss} (l/kg)	*F* (%)	MRT (h)	AUC (po/iv) (ng h/ml)	C_{max} (po) (ng h/ml)	CL (ml/min/kg)	V_{dss} (l/kg)
12b	16	1.6	261[c]/1595	289[c] (15 min)	104.5	1.25	14	1.1	1867[f]/6450	925[f] (15 min)	26.5	0.98	Nt	Nt	Nt	Nt	Nt	Nt
12h	21	2.3	1095[d]/5259	391[d] (15 min)	31.7	0.67	34	2.0	5231[g]/15610	6701[g] (15 min)	11.3	0.29	Nt	Nt	Nt	Nt	Nt	Nt
12l	28	1.2	2540[e]/8895	2251[e] (15 min)	18.5	0.33	36	1.7	11454[g]/31915	12976[g] (15 min)	5.2	0.24	55	1.6	3117[h]/5684	2374 (15 min)	3.6	0.14

Nt, not tested.
[a,b] AUC and Cmax were normalized for 10 mg/kg dosing.
[c] Calculated from 10 mg/kg iv and 50 mg/kg po dosing.
[d] Calculated from 10 mg/kg iv and 20 mg/kg po dosing.
[e] Calculated from 10 mg/kg iv and po dosing.
[f] Calculated from 10 mg/kg iv and 20 mg/kg po dosing.
[g] Calculated from 10 mg/kg iv and po dosing.
[h] Calculated from 1 mg/kg iv and po dosing.

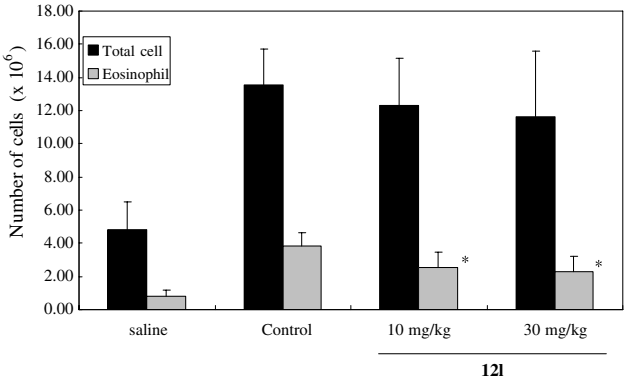


Figure 2. Effect of compound **12l** on pleural eosinophils accumulation in rats induced by compound **48/80**. Compound **12l** was orally administered to rats twice at −0.25 and 8 h (10 mg/kg × 2 or 30 mg/kg × 2, respectively). $*p < 0.05$: Significant difference versus control (Dunnett's test).

properties. Notably, **12l** (R1 = Cl, R2 = Me) demonstrated an acceptable plasma clearance and bioavailability in mice and rats as well as dogs (mice, CL = 28.5 ml/min/kg, $F = 28\%$; rats, CL = 5.3 ml/min/kg, $F = 36\%$; dog, CL = 3.6 ml/min/kg, $F = 55\%$). We have also found that these substitutions maintain the potent inhibitory activity against VLA-4. Compound **12l** exhibited single digit nanomolar IC_{50} values for VLA-4 even in the presence of HSA and demonstrated efficacy in a rat pleurisy model. Further synthetic modification of the diphenylurea portion will be presented in forthcoming publications.

5. Experimental

5.1. General

Melting points were determined on a YANACO MP-J3 and are uncorrected. Column chromatography was performed with Merck silica gel 60 (particle size 0.060–0.200 or 0.040–0.063). Flash column chromatography was performed with Biotage Si and YAMAZEN Hi-Flash packed columns. Thin-layer chromatography (TLC) was performed on Merck pre-coated TLC glass sheets with silica gel 60 F_{254}. ^{1}H NMR spectra were recorded on a JEOL JNM-EX-400 spectrometer, and chemical shifts are given in ppm (δ) from tetramethylsilane as the internal standard. IR spectra were recorded on a HITACHI 270-30 spectrometer. ESI mass spectra were recorded on a SCIEX API-150EX spectrometer; FAB mass spectra were recorded on a JEOL JMS-HX110 spectrometer. HR-FAB mass spectra were recorded on a JEOL JMS-700 spectrometer.

5.2. General procedure A: preparation of 4-[*N*′-(2-chlorophenyl)ureido]-3-methoxyphenylacetic acid (10a)

To a stirred solution of *tert*-butyl 4-amino-3-methoxyphenylacetate (**3**) (29.9 g, 126 mmol) in THF (200 ml) were added 2-chlorophenyl isocyanate (19.4 g,

126 mmol) and a catalytic amount of Et_3N at room temperature. After 12 h of stirring, the mixture was poured into water (300 ml), and the resulting precipitate was collected by filtration, followed by drying in vacuo, to give *tert*-butyl 4-[*N*′-(2-chlorophenyl)ureido]-3-methoxyphenylacetate (46.7 g, 94%) as a pale yellow crystalline powder.

To a stirred solution of *tert*-butyl 4-[*N*′-(2-chlorophenyl)ureido]-3-methoxyphenylacetate (46.7 g, 120 mmol) in CH_2Cl_2 (100 ml) was added TFA (100 ml) at room temperature. After 12 h of stirring, the mixture was concentrated and poured into water. The resulting precipitate was collected by filtration. The solid was recrystallized from $CHCl_3$–MeOH to give the compound **10a** (30.5 g, 76%) as a pale yellow crystalline powder. Mp: 177–180 °C; ^{1}H NMR (DMSO-d_6) δ 3.51 (2H, s), 3.87 (3H, s), 6.79 (1H, dd, J = 8.3, 1.5 Hz), 6.94 (1H, d, J = 1.5 Hz), 7.02 (1H, t, J = 7.3 Hz), 7.28 (1H, t, J = 8.3 Hz), 7.44 (1H, d, J = 7.8 Hz), 7.98 (1H, d, J = 8.3 Hz), 8.09 (1H, dd, J = 8.3, 1.5 Hz), 8.90 (1H, s), 8.95 (1H, s), 12.27 (1H, s); MS (FAB), *m*/*z* 335 (M^++1); Anal. Calcd for $C_{16}H_{15}ClN_2O_4$: C, 57.41; H, 4.52; N, 8.37; Cl, 10.59. Found: C, 57.41; H, 4.47; N, 8.28; Cl, 10.69.

Compounds **10b–h** were prepared according to general procedure A.

5.3. 4-[*N*′-(2-Bromophenyl)ureido]-3-methoxyphenylacetic acid (10b)

Yield 61% (two steps). Pale yellow powder recrystallized from MeOH–$CHCl_3$–*n*-hexane. Mp: 187–191 °C; ^{1}H NMR (DMSO-d_6) δ 3.51 (2H, s), 3.87 (3H, s), 6.77–6.81 (1H, m), 6.93–7.02 (2H, m), 7.30–7.35 (1H, m), 7.59–7.64 (1H, m), 7.94–7.98 (2H, m), 8.75 (1H, s), 8.95 (1H, s), 12.31 (1H, s); MS (FAB), *m*/*z* 381 (M^++2); Anal. Calcd for $C_{16}H_{15}BrN_2O_4$: C, 50.68; H, 3.99; Br, 21.07; N, 7.39. Found: C, 50.59; H, 4.04; Br, 21.20; N, 7.25.

5.4. 3-Methoxy-4-[*N*′-(2-methoxyphenyl)ureido]phenylacetic acid (10c)

Yield 74% (two steps). Colorless powder (1.40 g, 82%). Mp: 182–185 °C; ^{1}H NMR (CD_3OD) δ 3.55 (2H, s), 3.88 (3H, s), 3.89 (3H, s), 6.80–6.99 (5H, m), 7.94 (1H, d, J = 8.4 Hz), 8.00 (1H, d, J = 7.2 Hz); MS (ESI) *m*/*z* , 330 (M^+).

5.5. 3-Methoxy-4-[*N*′-(2-trifluoromethylphenyl)ureido]phenylacetic acid (10d)

Yield 49% (two steps). Colorless crystalline powder recrystallized from MeOH–$CHCl_3$–IPE. Mp: 218–220 °C; ^{1}H NMR (DMSO-d_6) δ 3.51 (2H, s), 3.87 (3H, s), 6.76–6.79 (1H, m), 6.93–6.94 (1H, m), 7.27–7.30 (1H, m), 7.61–7.69 (2H, m), 7.82–7.84 (1H, m), 7.97–7.99 (1H, m), 8.71 (1H, s), 8.89 (1H, s), 12.30 (1H, s); Anal. Calcd for $C_{17}H_{15}F_3N_2O_4$: C, 55.44; H, 4.11; N, 7.61; F, 15.47. Found: C, 55.30; H, 4.08; N, 7.63; F, 15.13.

5.6. 4-[*N*′-(2-Isopropylphenyl)ureido]-3-methoxyphenylacetic acid (10e)

Yield 74% (two steps). Colorless crystalline material recrystallized from EtOH–*n*-hexane. Mp: 144–147 °C; ^{1}H NMR ($CDCl_3$) δ 1.17 (6H, d, J = 7.2 Hz), 3.16–3.23 (1H, m), 3.49 (2H, s), 3.86 (3H, s), 6.74–6.71 (6H, m), 8.00–8.03 (1H, m), 8.48 (2H, d, J = 4.0 Hz); MS (ESI) *m*/*z* , 343 (M^++H).

5.7. 4-[*N*′-2-(Methylphenyl)ureido]-3-methylphenylacetic acid (10f)

Yield 92% (two steps). Pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 2.24 (3H, s), 2.26 (3H, s), 3.47 (2H, s), 6.84–7.27 (5H, m), 7.70 (1H, d, J = 8.1 Hz), 7.80 (1H, d, J = 8.1 Hz), 8.15–8.25 (2H, m); MS (ESI), *m*/*z* 297 (M^++1).

5.8. 4-[*N*′-(2-Chlorophenyl)ureido]-3-methylphenylacetic acid (10g)

Yield 93% (two steps). Yellow powder. Mp: 243–245 °C (dec); ^{1}H NMR ($CDCl_3$) δ 2.24 (3H, s), 3.47 (2H, s), 6.99–7.08 (3H, m), 7.28 (1H, t, J = 7.6 Hz), 7.44 (1H, dt, J = 8.0, 2.4 Hz), 7.66 (1H, dd, J = 8.3, 1.9 Hz), 8.13 (1H, dd, J = 6.1, 1.7 Hz), 8.61 (2H, d, J = 6.3 Hz); MS (ESI), *m*/*z* 319 (M^++1), 321 (M^++3).

5.9. 4-[*N*′-(2-Bromophenyl)ureido]-3-methylphenylacetic acid (10h)

Yield 90% (two steps). Pale yellow powder. Mp: 245–248 °C (dec); ^{1}H NMR ($CDCl_3$) δ 2.24 (3H, s), 3.48 (2H, s), 6.96 (1H, dt, J = 7.3, 1.5 Hz), 7.02 (1H, d, J = 8.3 Hz), 7.07 (1H, s), 7.32 (1H, t, J = 8.1 Hz), 7.59–7.66 (2H, m), 8.44 (1H, s), 8.62 (1H, s); MS (ESI), *m*/*z* 363 (M^++1), 365 (M^++3); Anal. Calcd for $C_{16}H_{15}BrN_2O_3 \cdot 0.75H_2O$: C, 51.01; H, 4.41; Br, 21.72; N, 7.44. Found: C, 50.84; H, 4.02; Br, 21.72; N, 7.18.

5.10. General procedure B: preparation of 3-chloro-4-[*N*′-(2-methylphenyl)ureido]phenylacetic acid (10l)

To a mixture of methyl 4-amino-3-chlorophenylacetate (**8**) (1.00 g, 5.01 mmol) and 2-methylphenyl isocyanate (0.60 ml, 5.01 mmol) in THF (20 ml) was added Et_3N (0.14 ml, 1.00 mmol) at room temperature. After 1 day of stirring, 2-methylphenyl isocyanate (0.60 ml, 5.01 mmol) was re-added to the reaction mixture and stirred for 17 h. The reaction mixture was concentrated in vacuo. The residue was triturated by the addition of *n*-hexane to give methyl 3-chloro-4-[*N*′-(2-methylphenyl)ureido]phenylacetate (1.23 g, 74%) as a colorless powder.

To a stirred solution of methyl 3-chloro-4-[*N*′-(2-methylphenyl)ureido]phenylacetate (1.23 g, 3.70 mmol) in THF (30 ml) was added 0.25 M NaOH (30 ml). After stirring at room temperature for 14 h, the reaction mixture was concentrated in vacuo. The residue was triturated by the addition of 1 M HCl. The resulting precipitate was collected and dried under a reduced pressure to give the compound **10l** (1.22 g, 100%) as a color-

less powder. ^{1}H NMR (DMSO-d_6) δ 2.26 (3H, s), 3.40 (2H, s), 6.95 (1H, t, J = 7.3 Hz), 7.11 (2H, d, J = 7.6 Hz), 7.16 (1H, d, J = 7.3 Hz), 7.32 (1H, s), 7.76 (1H, d, J = 8.0 Hz), 7.94 (1H, dd, J = 9.3, 1.0 Hz), 8.72 (2H, s); MS (ESI), m/z 319 (M^++1), 321 (M^++3).

Compounds **10i–q** were prepared according to general procedure B.

5.11. 3-Ethyl-4-[*N*′-(2-methylphenyl)ureido]phenylacetic acid (10i)

Yield 67% (two steps). Colorless crystalline solid. ^{1}H NMR (DMSO-d_6) δ 1.17 (3H, t, J = 7.6 Hz), 2.26 (3H, s), 2.61 (2H, q, J = 7.6 Hz), 3.49 (2H, s), 6.91–7.17 (6H, m), 7.63 (1H, d, J = 8.3 Hz), 7.78 (1H, d, J = 8.0 Hz), 8.24 (1H, d, J = 5.4 Hz).

5.12. 3-Isopropyl-4-[*N*′-(2-methylphenyl)ureido]phenylacetic acid (10j)

Yield 98% (two steps). Colorless crystalline solid. ^{1}H NMR (DMSO-d_6) δ 1.19 (6H, d, J = 6.8 Hz), 2.26 (3H, s), 3.18–3.22 (1H, m), 3.52 (2H, s), 6.90–7.17 (6H, m), 7.48–7.52 (1H, m), 7.79–7.83 (1H, m), 8.25 and 8.36 (total 1H, each s, amide isomers).

5.13. 4-[*N*′-(2-Methylphenyl)ureido]-3-trifluoromethylphenylacetic acid (10k)

Yield 40% (two steps). Colorless solid recrystallized with $CHCl_3$–n-hexane. ^{1}H NMR ($CDCl_3$) δ 2.30 (3H, s), 3.66 (2H, s), 7.00–7.04 (1H, m), 7.16–7.20 (2H, m), 7.53–7.57 (3H, m), 7.78–7.82 (1H, m); MS (ESI) m/z 353 (M^++1).

5.14. 3-Chloro-4-[*N*′-(2-chlorophenyl)ureido]phenylacetic acid (10m)

Yield 65% (two steps). Colorless powder. ^{1}H NMR (DMSO-d_6) δ 3.52 (2H, s), 7.03–7.07 (1H, m), 7.17 (1H, d, J = 8.5 Hz), 7.30 (1H, d, J = 7.6 Hz), 7.37 (1H, s), 7.46 (1H, dd, J = 8.0, 1.5 Hz), 7.95 (1H, dd, J = 8.3, 1.2 Hz), 8.07 (1H, d, J = 8.3 Hz), 9.00 (2H, d, J = 8.0 Hz); MS (FAB), m/z 339 (M^++1), 341 (M^++3), 343 (M^++5).

5.15. 4-[*N*′-(2-Bromophenyl)ureido]-3-chlorophenylacetic acid (10n)

Yield 54% (two steps). Colorless powder. ^{1}H NMR (DMSO-d_6) δ 3.56 (2H, s), 6.98–7.02 (1H, m), 7.17 (1H, dd, J = 9.0, 1.7 Hz), 7.32–7.40 (2H, m), 7.62 (1H, dd, J = 8.0, 1.2 Hz), 7.95 (2H, m), 8.83 (1H, s), 9.01 (1H, s), 12.41 (1H, br); MS (FAB), m/z 385 (M^++2), 386 (M^++4), 388 (M^++6).

5.16. 3-Bromo-4-[*N*′-(2-methylphenyl)ureido]phenylacetic acid (10o)

Yield 72% (two steps). Colorless powder. ^{1}H NMR (DMSO-d_6) δ 2.26 (3H, s), 3.32 (2H, s), 6.91–6.95 (2H, m), 7.10–7.17 (4H, m), 7.76 (2H, d, J = 8.1 Hz), 8.52 (1H, s); MS (ESI), m/z 385 (M^++Na), 387 (M^++2+Na).

5.17. 3-Bromo-4-[*N*′-(2-chlorophenyl)ureido]phenylacetic acid (10p)

Yield 70% (two steps). Colorless powder. ^{1}H NMR (DMSO-d_6) δ 3.56 (2H, s), 7.03–7.07 (1H, m), 7.21 (1H, dd, J = 8.6, 1.7 Hz), 7.29 (1H, t, J = 7.8 Hz), 7.46 (1H, d, J = 8.1 Hz), 7.53 (1H, d, J = 1.7 Hz), 7.83 (1H, d, J = 8.3 Hz), 8.06 (1H, d, J = 7.6 Hz), 8.86 (1H, s), 8.89 (1H, s), 12.40 (1H, s); MS (ESI), m/z 382 (M^++1), 384 (M^++3).

5.18. 3-Bromo-4-[*N*′-(2-bromophenyl)ureido]phenylacetic acid (10q)

Yield 69% (two steps). Colorless powder. ^{1}H NMR (DMSO-d_6) δ 3.56 (2H, s), 6.99 (1H, dt, J = 7.8, 1.5 Hz), 7.21 (1H, dd, J = 8.3, 1.7 Hz), 7.33 (1H, dt, J = 7.1, 1.5 Hz), 7.53 (1H, d, J = 1.7 Hz), 7.62 (1H, dd, J = 8.1, 1.5 Hz), 7.82 (1H, d, J = 8.3 Hz), 7.93 (1H, dd, J = 8.1, 1.5 Hz), 8.82 (1H, s), 8.86 (1H, s), 12.39 (1H, s); MS (ESI), m/z 428 (M^++1), 430 (M^++3).

5.19. General procedure C: preparation of 4-[1-[3-chloro-4-[*N*′-(2-methylphenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12l)

A mixture of 3-chloro-4-[*N*′-(2-methylphenyl)ureido]phenylacetic acid (**10l**) (319 mg, 1.00 mmol), methyl 4-[(4*S*)-fluoro-(2*S*)-pyrrolidinyl]methoxybenzoate (**11**) (253 mg, 1.00 mmol), EDC·HCl (288 mg, 1.50 mmol), HOBT (203 mg, 1.50 mmol), and Et_3N (0.70 ml, 5.00 mmol) in DMF (4 ml) was stirred at room temperature for 15 h. The mixture was poured into ice water and extracted with EtOAc. The combined extracts were washed with ice water and brine. After drying over Na_2SO_4, the extracts were concentrated in vacuo. The residue was purified on TLC [$CHCl_3$–acetone (5/1, v/v)] to give methyl 4-[1-[3-chloro-4-[*N*′-(2-methylphenyl) ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinyl]methoxybenzoate (480 mg, 87%) as a colorless amorphous solid. ^{1}H NMR ($CDCl_3$) δ 2.10–2.60 (2H, m), 2.29 (3H, s), 3.56 (1H, d, J = 6.8 Hz), 3.71–3.84 (1H, m), 3.87 and 3.89 (total 3H, each s, amide isomers), 3.91–4.20 (3H, m), 4.49–4.60 (2H, m), 5.32 (1H, dt, J = 53.0, 4.2 Hz), 6.80 (1H, br), 6.89 and 6.95 (total 2H, each d, each J = 8.8 Hz, amide isomers), 7.09–7.26 (6H, m), 7.50 (1H, d, J = 7.3 Hz), 7.94 and 8.00 (total 2H, each d, each J = 8.8 Hz, amide isomers), 8.10 and 8.15 (total 1H, each d, J = 8.1 and 8.3 Hz, respectively, amide isomers); FAB-MS, m/z 554 (M^++1), 556 (M^++3).

To a solution of methyl 4-[1-[3-chloro-4-[*N*′-(2-methylphenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinyl]methoxybenzoate (480 mg, 0.866 mmol) in THF (30 ml) was added 0.25 N NaOH (30 ml). After 2 days of stirring at room temperature, the mixture was concentrated under a reduced pressure and acidified with 1 N HCl. The precipitates were collected, washed with water, and dried under a reduced pressure to give **12 l** (374 mg, 80%) as a colorless solid. IR (KBr) 3354, 3060, 2976, 1709, 1604, 1244 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.27 (3H, s), 2.31 (2H, s), 3.66 (2H, d, J = 7.2 Hz), 3.71–4.67 (5H, m), 5.32–5.53 (1H, m), 6.97 (1H, t, J = 7.3 Hz),

7.04–7.22 (5H, m), 7.32 and 7.35 (total 1H, each d, each $J = 1.7$ Hz, amide isomers), 7.77 (1H, d, $J = 7.6$ Hz), 7.87 and 7.90 (total 2H, each d, $J = 8.8$ and 9.0 Hz, respectively, amide isomers), 8.01 and 8.03 (total 1H, each d, $J = 7.5$ and 8.5 Hz, respectively, amide isomers), 8.57 and 8.59 (total 1H, each s, amide isomers), 8.63 and 8.65 (total 1H, each s, amide isomers), 12.63 (1H, s); MS (ESI), *m/z* 540 (M^++1), 542 (M^++3). Anal. Calcd for $C_{28}H_{27}ClFN_3O_5 \cdot 0.5H_2O$: C, 61.26; H, 5.14; N, 7.65; Cl, 6.46; F, 3.46. Found: C, 61.16; H, 5.27; N, 7.25; Cl, 6.47; F, 3.39.

Compounds **12a**–**q** were prepared according to general procedure C.

5.20. 4-[1-[4-[*N*′-(2-Chlorophenyl)ureido]-3-methoxyphenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12a)

Yield 24% (two steps), as a pale yellow amorphous solid. IR (KBr) 3338, 3059, 2960, 1705, 1685, 1628, 1533, 1466, 1421 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 2.24–2.50 (2H, m), 3.48–4.65 (10H, m), 5.30–5.50 (1H, m), 6.75–7.08 (5H, m), 7.29 (1H, t, $J = 7.3$ Hz), 7.43–7.45 (1H, m), 7.89–7.98 (2H, m), 7.99 (1H, d, $J = 8.3$ Hz), 8.09 (1H, d, $J = 7.1$ Hz), 8.90–8.96 (2H, m); MS (FAB) *m/z* , 556 (M^++1); Anal. Calcd for $C_{28}H_{27}ClFN_3O_6 \cdot 0.25H_2O$: C, 60.00; H, 4.95; N, 7.50. Found: C, 59.67; H, 5.08; N, 7.10.

5.21. 4-[1-[4-[*N*′-(2-Bromophenyl)ureido]-3-methoxyphenylacetyl]-(4*S*)-fluoro-(2*S*)- pyrrolidinylmethoxy]benzoic acid (12b)

Yield 67% (two steps), as a colorless amorphous solid. IR (KBr) 3332, 2956, 1705, 1687, 1604, 1581, 1529, 1464, 1435, 1298 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 2.24–2.31 (2H, m), 3.21–4.63 (10H, m), 5.31–5.51 (1H, m), 6.74–7.10 (5H, m), 7.32 (1H, t, $J = 7.8$ Hz), 7.60 (2H, d, $J = 7.8$ Hz), 7.87–7.99 (4H, m), 8.74–8.75 (1H, m), 8.92–8.94 (1H, m); MS (FAB) *m/z* , 601 (M^++1); Anal. Calcd for $C_{28}H_{27}BrFN_3O_6 \cdot 2H_2O$: C, 52.84; H, 4.91; N, 6.60. Found: C, 52.38; H, 4.62; N, 5.99.

5.22. 4-[(4*S*)-Fluoro-1-[4-[*N*′-(2-methoxyphenyl)ureido]-3-methoxyphenylacetyl]-(2*S*)- pyrrolidinylmethoxy]benzoic acid (12c)

Yield 59% (two steps). Colorless amorphous solid. IR (ATR) 3336, 2937, 2839, 1682, 1628, 1599, 1525, 1458, 1417, 1333, 1250, 1232 cm^{-1}; ^1H NMR (CD_3OD) δ 2.14–2.48 (2H, m), 3.69–4.20 (5H, m), 3.88 (3H, s), 3.89 (3H, s), 4.46–4.57 (2H, m), 5.27–5.41 (1H, m), 6.79–7.04 (7H, m), 7.90–8.02 (4H, m); MS (ESI) *m/z* , 552 (M^++H); Anal. Calcd for $C_{29}H_{30}FN_3O_7 \cdot 1.5H_2O$: C, 60.20; H, 5.75; N, 7.26; F, 3.28. Found: C, 60.03; H, 5.40; N, 7.27; F, 3.14.

5.23. 4-[(4*S*)-Fluoro-1-[3-methoxy-4-[*N*′-(2-trifluoromethylphenyl)ureido]phenylacetyl]-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12d)

Yield 42% (two steps). Colorless crystalline powder. Mp: 129–132 °C; IR (KBr) 3354, 3072, 2972, 2939, 2893, 1709, 1685, 1628, 1604 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.23–2.29 (2H, m), 3.54–4.38 (8H, m), 4.40–4.61 (2H, m), 5.30–5.49 (1H, m), 6.72–6.91 (2H, m), 7.02–7.08 (2H, m), 7.25–7.29 (1H, m), 7.59–7.67 (2H, m), 7.81–7.99 (4H, m), 8.69–8.70 (1H, m), 8.87–8.90 (1H, m), 12.67 (1H, s); FAB, *m/z* 590 (M^++1); HR-MS (FAB) calcd for $C_{29}H_{27}F_4N_3O_6$: 590.1914. Found: 590.1898.

5.24. 4-[(4*S*)-Fluoro-1-[4-[*N*′-(2-isopropylphenyl)ureido]-3-methoxyphenylacetyl]-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12e)

Yield 89% (two steps). Colorless amorphous solid. IR (ATR) 3338, 3060. 2964. 2870, 1685, 1657, 1603, 1530, 1448, 1417 cm^{-1}; ^1H NMR (CD_3OD) δ 1.23 (6H, d, $J = 6.8$ Hz), 2.15–2.49 (2H, m), 3.19–3.25 (1H, m), 3.62–4.76 (7H, m), 3.87 and 3.93 (total 3H, each s, amide isomers), 5.23–5.40 (1H, m), 6.77–8.01 (11H, m); MS (ESI) *m/z* , 564 (M^++H); $C_{31}H_{34}FN_3O_6 \cdot 1.25H_2O$: C, 63.52; H, 6.28; N, 7.17; F, 3.24. Found: C, 63.46; H, 6.02; N, 7.11; F, 3.06.

5.25. 4-[(4*S*)-Fluoro-1-[3-methyl-4-[*N*′-(2-methylphenyl)ureido]phenylacetyl]-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12f)

Yield 65% (two steps). Colorless amorphous solid. IR (KBr) 3356, 2974, 1604, 1537, 1454, 1252 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.24 (3H, s), 2.26 (3H, s), 3.60 (2H, d, $J = 3.7$ Hz), 3.65–4.65 (8H, m), 5.31–5.50 (1H, m), 6.92–7.18 (7H, m), 7.67–7.92 (4H, m), 8.22–8.32 (2H, m); MS (FAB), *m/z* 520 (M^++1); Anal. Calcd for $C_{29}H_{30}FN_3O_5 \cdot H_2O$: C, 64.79; H, 6.00; F, 3.53; N, 7.82. Found: C, 64.71; H, 5.90; F, 3.24; N, 7.51.

5.26. 4-[1-[4-[*N*′-(2-Chlorophenyl)ureido]-3-methylphenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12g)

Yield 42% (two steps). Colorless amorphous solid. IR (KBr) 3346, 2976, 1709, 1685, 1604, 1533, 1439 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.20 and 2.24 (total 3H, each s, amide isomers), 2.28–2.32 (1H, m), 3.60 (2H, s), 3.71–4.62 (6H, m), 5.30–5.50 (1H, m), 7.01–7.09 (5H, m), 7.28 (1H, t, $J = 7.8$ Hz), 7.44 (1H, d, $J = 8.1$ Hz), 7.66 (1H, t, $J = 8.1$ Hz), 7.87 (2H, d, $J = 7.1$ Hz), 8.13 (1H, d, $J = 7.9$ Hz), 8.62 (2H, d, $J = 6.1$ Hz); MS (FAB), *m/z* 540 (M^++1), 542 (M^++3); Anal. Calcd for $C_{28}H_{27}ClFN_3O_7 \cdot 0.5H_2O$: C, 61.26; H, 5.14; N, 7.65. Found: C, 61.22; H, 5.30; N, 7.33.

5.27. 4-[1-[4-[*N*′-(2-Bromophenyl)ureido]-3-methylphenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12h)

Yield 49% (two steps). Colorless amorphous solid. IR (KBr) 3325, 2972, 1709, 1604, 1529, 1252 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.25 (3H, s), 2.29–2.33 (1H, m), 3.17 (1H, s), 3.60 (2H, d, $J = 4.7$ Hz), 3.83–4.67 (5H, m), 5.31–5.51 (1H, m), 6.97 (1H, t, $J = 7.3$ Hz), 7.02–7.09 (5H, m), 7.33 (1H, t, $J = 8.0$ Hz), 7.61 (1H, d,

J = 7.8 Hz), 7.64 (1H, d, J = 8.3 Hz), 7.87 (2H, d, J = 8.3 Hz), 7.90 (1H, d, J = 8.8 Hz), 8.44–8.65 (2H, m); MS (ESI), m/z 585 (M^++1), 587 (M^++3); Anal. Calcd for $C_{28}H_{27}BrFN_3O_5 \cdot 0.5H_2O$: C, 56.67; H, 4.76; Br, 13.46; F, 3.20; N, 7.08. Found: C, 56.91; H, 4.93; Br, 13.23; F, 3.15; N, 6.88.

5.28. 4-[1-[3-Ethyl-4-[*N*′-(2-methylphenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12i)

Yield 70% (two steps). Colorless solid. IR (KBr) 3353, 2967, 1604, 1536, 1249, 1166 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.10–1.19 (3H, m), 2.15–2.32 (5H, m), 2.55–2.65 (2H, m), 3.55–4.20 (5H, m), 4.32–4.65 (2H, m), 5.28–5.52 (1H, m), 6.90–7.20 (7H, m), 7.60–7.95 (4H, m), 8.17–8.20 (2H, m); MS (FAB) m/z 533 $(M+H)^+$; Anal. calcd for $C_{30}H_{32}N_3O_5F \cdot 0.5\ H_2O$: C, 66.41; H, 6.13; N, 7.74. Found: C, 66.14; H, 6.11; N, 7.63.

5.29. 4-[1-[3-Isopropyl-4-[*N*′-(2-methylphenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12j)

Yield 91% (two steps). Colorless solid. IR (KBr) 3372, 2964, 1604, 1533, 1249, 1166, 1112 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.08–1.21 (6H, m), 2.18–2.40 (5H, m), 3.10–3.25 (1H, m), 3.45–4.21 (5H, m), 4.35–4.68 (2H, m), 5.30–5.55 (1H, m), 6.90–7.17 (7H, m), 7.47–7.52 (1H, m), 7.80–7.90 (3H, m), 8.12–8.23 (2H, m); MS (FAB) m/z 547 $(M+H)^+$; Anal. calcd for $C_{31}H_{34}N_3O_5F \cdot 0.5H_2O$: C, 66.89; H, 6.34; N, 7.55. Found: C, 66.57; H, 6.30; N, 7.39.

5.30. 4-[1-[4-[*N*′-(2-Methylphenyl)ureido]-3-trifluoromethylphenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12k)

Yield 65% (two steps). Colorless solid. Mp: 155–160 °C; IR (KBr) 3354, 3300, 3060, 2958, 2875, 1709, 1687, 1641, 1604, 1541 cm^{-1}; ^{1}H NMR (CD_3OD) δ 2.10–2.50 (5H, m), 3.60–4.30 (5H, m), 4.45–4.90 (2H, m), 5.27–5.40 (1H, m), 6.95–7.05 (3H, m), 7.12–7.21 (2H, m), 7.42–7.64 (3H, m), 7.80–7.99 (3H, m); MS (ESI) m/z , 574 (M^++1); Anal. Calcd for $C_{29}H_{27}F_4N_3O_5 \cdot 0.25H_2O$: C, 60.26; H, 4.80; N, 7.27. Found: C, 60.20; H, 5.12; N, 7.07.

5.31. 4-[1-[3-Chloro-4-[*N*′-(2-chlorophenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12m)

Yield 80% (two steps). Colorless solid. IR (KBr) 3348, 3072, 2954, 1703, 1604,1529, 1439 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.25–2.42 (2H, m), 3.67 (2H, d, J = 8.3 Hz), 3.81–4.68 (5H, m), 5.39 and 5.46 (total 1H, each d, J = 54.0 and 54.4 Hz, respectively, amide isomers), 7.04–7.10 (3H, m), 7.18 (1H, d, J = 8.3 Hz), 7.31 (1H, t, J = 8.3 Hz), 7.33 and 7.37 (total 1H, each s, amide isomers), 7.47 (1H, d, J = 8.1 Hz), 7.88 (2H, dd, J = 9.0, 3.2 Hz), 7.98 (1H, dd, J = 8.5, 3.0 Hz), 8.09 (1H, d, J = 8.3 Hz), 8.99 (1H, d, J = 2.9 Hz), 9.02 (1H, s), 12.64 (1H, s); MS (ESI), m/z 560 (M^++1), 562 (M^++3), 564 (M^++5); Anal. Calcd for $C_{27}H_{24}Cl_2FN_3O_5 \cdot 0.25H_2O$: C, 57.41; H, 4.37; N, 7.44; Cl, 12.55; F, 3.36. Found: C, 57.72; H, 4.47; N, 7.14; Cl, 12.44; F, 3.44.

5.32. 4-[1-[4-[*N*′-(2-Bromophenyl)ureido]-3-chlorophenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12n)

Yield 9% (two steps). Colorless amorphous solid. IR (KBr) 3329, 3060, 2976, 1712, 1526, 1435 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.28–2.33 (2H, m), 3.48–4.68 (7H, m), 5.32–5.53 (1H, m), 6.99–7.19 (4H, m), 7.36 (1H, s), 7.63 (1H, dd, J = 6.7, 1.2 Hz), 7.86–8.18 (4H, m), 8.83 (1H, s), 9.02 (1H, s), 12.67 (1H, br); MS (ESI), m/z 604 (M^++1), 606 (M^++3), 608 (M^++5); Anal. Calcd for $C_{27}H_{24}BrClFN_3O_5 \cdot 0.5H_2O$: C, 52.83; H, 4.10; N, 6.85; Cl, 5.78; F, 3.09. Found: C, 53.243; H, 4.32; N, 6.43; Cl, 6.01; F, 3.07.

5.33. 4-[1-[3-Bromo-4-[*N*′-(2-methylphenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12o)

Yield 85% (two steps). Colorless solid. IR (KBr) 3313, 3060, 2976, 1687, 1604, 1525, 1244 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.27 (3H, s), 2.29 (2H, m), 3.66 (2H, d, J = 8.1 Hz), 3.72–4.68 (5H, m), 5.31–5.53 (1H, m), 6.92–6.99 (1H, m), 7.04 and 7.07 (total 2H, each d, J = 9.3 and 8.3 Hz, respectively, amide isomers), 7.11–7.21 (3H, m), 7.48 and 7.51 (total 1H, each s, amide isomers), 7.75 and 7.79 (total 1H, each d, each J = 8.1 Hz, amide isomers), 7.86–7.92 (3H, m), 8.45 and 8.47 (total 1H, each s, amide isomers), 8.59 (1H, s), 12.64 (1H, s); MS (FAB), m/z 584 (M^++1), 586 (M^++3); Anal. Calcd for $C_{28}H_{27}BrFN_3O_5$: C, 57.54; H, 4.66; N, 7.19; Br, 13.67; F, 3.25. Found: C, 57.93; H, 4.97; N, 7.04; Br, 13.35; F, 2.89.

5.34. 4-[1-[3-Bromo-4-[*N*′-(2-chlorophenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12p)

Yield 86% (two steps). Pale yellow solid. IR (KBr) 3317, 3072, 1709, 1685, 1604, 1529, 1290 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.24–2.50 (2H, m), 3.67 (2H, d, J = 8.3 Hz), 3.73–4.68 (5H, m), 5.31–5.52 (1H, m), 7.03–7.09 (3H, m), 7.22 (1H, dt, J = 8.3, 1.7 Hz), 7.30 (1H, d, J = 7.3 Hz), 7.46 (1H, dd, J = 8.0, 1.4 Hz), 7.49 and 7.52 (total 1H, each d, J = 1.7 and 2.0 Hz, respectively, amide isomers), 7.80–7.91 (3H, m), 8.07 (1H, dd, J = 8.3, 1.2 Hz), 8.85 and 8.86 (total 1H, each s, amide isomers), 8.96 and 8.97 (total 1H, each s, amide isomers), 12.62 (1H, s); MS (FAB), m/z 605 (M^++1), 607 (M^++3), 609 (M^++3), 626 (M^++1+Na); Anal. Calcd for $C_{27}H_{24}BrClFN_3O_5 \cdot 0.75H_2O$: C, 52.44; H, 4.16; N, 6.80; F, 3.07. Found: C, 52.63; H, 4.12; N, 6.62; F, 2.97.

5.35. 4-[1-[3-Bromo-4-[*N*′-(2-bromophenyl)ureido]phenylacetyl]-(4*S*)-fluoro-(2*S*)-pyrrolidinylmethoxy]benzoic acid (12q)

Yield 74% (two steps). Colorless amorphous solid. IR (KBr) 3450, 3313, 3070, 1709, 1684, 1525, 1435 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.25–2.50 (2H, m), 3.67 (2H, d, J = 8.3 Hz), 3.73–4.68 (5H, m), 5.31–5.53 (1H, m), 6.98–7.08 (3H, m), 7.21 (1H, d, J = 8.2 Hz), 7.34 (1H, t, J = 8.8 Hz), 7.50 and 7.53 (total 1H, each s, amide iso-

mers), 7.62 (1H, d, J = 8.0 Hz), 7.80–7.96 (4H, m), 8.82 (1H, s), 8.85 and 8.86 (total 1H, each s, amide isomers), 12.63 (1H, s); MS (FAB), m/z 650 (M^++1), 652 (M^++3), 654 (M^++3), 672 (M^++Na); Anal. Calcd for $C_{27}H_{24}Br_2FN_3O_5 \cdot H_2O$: C, 48.60; H, 3.93; N, 6.30; F, 2.85. Found: C, 48.96; H, 3.98; N, 5.92; F, 2.77.

5.36. General procedure D: preparation of *tert*-butyl 4-amino-3-methoxyphenylacetate (3)

5.36.1. *tert*-Butyl 3-methoxy-4-nitrophenylacetate (15a). To a stirred suspension of **13a** (19.9 g, 0.101 mol) in toluene (150 ml) were added $SOCl_2$ (14 ml, 0.191 mol) and a catalytic amount of DMF at room temperature. The mixture was refluxed for 3 h with stirring. After cooling to room temperature, the reaction mixture was concentrated under reduced pressure. The resulting residue was dissolved in THF (250 ml), and then Et_3N (23 ml, 0.165 mol) and 2.0 M $TMSCHN_2$ in hexane solution (83 ml, 0.165 mol) were added at 0 °C. After 12 h of stirring, the mixture was poured into sat. $NaHCO_3$ solution and extracted with EtOAc. The combined extracts were washed with brine, dried over $MgSO_4$, and evaporated to give crude **14a** as a yellow solid.

To a refluxed solution of Et_3N (42 ml) and $PhCO_2Ag$ (6.97 g, 30 mmol) in *tert*-butanol–toluene (1/1, v/v, 400 ml) was added dropwise a solution of crude **14a** in *tert*-butanol (300 ml) with stirring. After 1 h of refluxing, the reaction mixture was cooled to room temperature. Activated carbon powder was added to the reaction mixture, which was then filtered through Celite. The filtrate was diluted with EtOAc and washed with brine. After drying over $MgSO_4$, the resulting solution was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with *n*-hexane–EtOAc (20/1, v/v) as an eluent to afford compound **15a** (17.4 g, two steps, 65%) as a brown oil. ^{1}H NMR ($CDCl_3$) δ 1.45 (9H, s), 3.58 (2H, s), 3.97 (3H, s), 6.93 (1H, dd, J = 8.3, 2.2 Hz), 7.03 (1H, d, J = 2.0 Hz), 7.83 (1H, d, J = 8.3 Hz).

5.36.2. *tert*-Butyl 4-amino-3-methoxyphenylacetate (3). A suspension of **15a** (17.4 g, 65.1 mmol) and 5% Pd/C (3.5 g) in EtOH–HOAc (1/1, v/v, 200 ml) was hydrogenated for 1 day at room temperature. After the catalyst was removed by filtration, the filtrate was concentrated. The residue was purified by column chromatography on silica gel with *n*-hexane–EtOAc (4/1, v/v) as an eluent to afford compound **3** (13.8 g, 89%) as a brown oil. ^{1}H NMR ($CDCl_3$) δ 1.43 (9H, s), 3.41 (2H, s), 3.85 (3H, s), 6.64–6.73 (3H, m); MS (ESI), m/z 238 (M^++1).

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

5.37. Preparation of *tert*-butyl 4-amino-3-methylphenylacetate (4)

5.37.1. *tert*-Butyl 3-methyl-4-nitrophenylacetate (15b). Yield 55% (three steps). Yellow oil. ^{1}H NMR ($CDCl_3$) δ 1.45 (9H, s), 2.61 (3H, s), 3.57 (2H, s), 7.22–7.27 (2H, m), 7.95 (1H, d, J = 8.8 Hz).

5.37.2. *tert*-Butyl 4-amino-3-methylphenylacetate (4). Yield 82%. Brown solid. ^{1}H NMR ($CDCl_3$) δ 1.43 (9H, s), 2.15 (3H, s), 3.39 (2H, s), 3.47–3.64 (2H, m), 6.62 (1H, d, J = 7.8 Hz), 6.89–6.96 (2H, m); MS (ESI), m/z 222 (M^++1).

5.38. General procedure E: preparation of methyl 4-amino-3-chlorophenylacetate (8)

5.38.1. Methyl 3-chlorophenylacetate (17a). To a stirred solution of 3-chlorophenylacetic acid (**16a**) (21.76 g, 127.6 mmol) in dichloroethane (100 ml) were added MeOH (15.6 ml, 383 mmol) and H_2SO_4 (1 ml) at room temperature. After 20 min of stirring, the mixture was heated at 80 °C for 2 h. The reaction mixture was poured into ice water and extracted with $CHCl_3$. The combined extracts were washed with aq $NaHCO_3$ and brine. After drying over Na_2SO_4, the extract was concentrated in vacuo to give the compound **17a** (25.4 g, 100%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 3.60 (2H, s), 3.70 (3H, s), 7.15–7.26 (4H, m).

5.38.2. Methyl 3-chloro-4-nitrophenylacetate (18a). To a stirred mixture of methyl 3-chlorophenylacetate (**17a**) (25.4 g, 128 mmol) in H_2SO_4 (44 ml) was added concd HNO_3 (5.5 ml, 138 mmol) at 0 °C. The reaction mixture was gradually raised to room temperature for 4 h. The reaction mixture was poured into ice water and extracted with EtOAc. The combined extracts were washed with aq $NaHCO_3$ and brine. After drying over Na_2SO_4, the extracts were concentrated in vacuo. The residue was purified by column chromatography with *n*-hexane–EtOAc (40/1, v/v) as an eluent to give the compound **18a** (11.4 g, 36%) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 3.69 (2H, s), 3.74 (3H, s), 7.33 (1H, dd, J = 8.3, 1.5 Hz), 7.49 (1H, d, J = 1.5 Hz), 7.87 (1H, d, J = 8.3 Hz).

5.38.3. Methyl 4-amino-3-chlorophenylacetate (8). A mixture of methyl 3-chloro-4-nitrophenylacetate (**18a**) (10.9 g, 47.5 mmol), reduced iron powder (8.58 g, 153.6 mmol), $NaOAc \cdot 3H_2O$ (6.05 g, 44.5 mmol), and HOAc (17.6 ml) in MeOH–H_2O (100/400 ml) was heated at 110 °C for 1 h. After cooling to room temperature, the reaction mixture was filtered through Celite and the filtered cake was washed with MeOH. The filtrate was evaporated and extracted with EtOAc. The combined extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography with $CHCl_3$–EtOAc (10/1, v/v) as an eluent to give the compound **8** (4.58 g, 48%) as a red oil. ^{1}H NMR ($CDCl_3$) δ 3.49 (1H, s), 3.68 (3H, s), 4.01 (2H, br), 6.70 (1H, d, J = 7.4 Hz), 6.96 (1H, dd, J = 8.1, 2.0 Hz), 7.17 (1H, d, J = 2.0 Hz).

Compound **9** was prepared according to general procedure E.

5.39. Preparation of methyl 4-amino-3-bromophenylacetate (9)

5.39.1. Methyl 3-bromophenylacetate (17b). Yield 99%. Colorless oil. ^{1}H NMR ($CDCl_3$) δ 3.60 (2H, s), 3.71 (3H, d, J = 1.0 Hz), 7.18–7.44 (4H, m).

5.39.2. Methyl 3-bromo-4-nitrophenylacetate (18b). Yield 29%. Yellow oil. ^{1}H NMR ($CDCl_3$) δ 3.68 (2H, s), 3.73 (3H, s), 7.38 (1H, dd, J = 8.3, 1.2 Hz), 7.67 (1H, d, J = 1.3 Hz), 7.83 (1H, d, J = 8.3 Hz).

5.39.3. Methyl 4-amino-3-bromophenylacetate (9). Yield 69%. Brown oil. ^{1}H NMR ($CDCl_3$) δ 3.48 (2H, s), 3.68 (3H, s), 4.05 (2H, br), 6.69 (1H, d, J = 8.3 Hz), 7.00 (1H, dd, J = 8.1, 2.0 Hz), 7.32 (1H, d, J = 2.0 Hz).

5.40. Preparation of methyl 4-amino-3-ethylphenylacetate (5)

5.40.1. Methyl 3-nitro-4-trimethylsilylethynylphenylacetate (19). To a stirred solution of methyl 3-bromo-4-nitrophenylacetate (**18b**) (5.0 g, 18.2 mmol), trimethylsilylacetylene (3.9 ml, 27.4 mmol), CuI (693 mg, 3.7 mmol), and TEA (15 ml, 109 mmol) in THF (40 ml) was added a solution of $Pd(Ph_3P)_4$ (1.0 g, 0.9 mmol) in THF (20 ml) at room temperature. After stirring at room temperature for 18 h, the reaction mixture was concentrated in vacuo. The reaction mixture was poured into water and extracted with EtOAc. The combined extracts were washed with sat. $NaHCO_3$, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica gel with *n*-hexane–EtOAc (5/1, v/v) as an eluent to give the compound **19** (4.5 g, 85%) as a dark yellow oil. ^{1}H NMR ($CDCl_3$) δ 0.25–0.29 (9H, m), 3.68 (2H, s), 3.73 (3H, s), 7.37 (1H, dd, J = 1.9, 8.5 Hz), 7.57 (1H, d, J = 1.9 Hz), 7.99 (1H, d, J = 8.5 Hz).

5.40.2. Methyl 3-ethynyl-4-nitrophenylacetate (20). To a stirred solution of methyl 3-nitro-4-trimethylsilylethynylphenylacetate (**19**) (4.5 g, 15.5 mmol) in THF (30 ml) was added 1.0 M TBAF in THF solution (18.5 ml, 18.5 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with water and extracted with EtOAc. The combined extracts were washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography with *n*-hexane–EtOAc (1/2, v/v) as an eluent to give the compound **20** (1.5 g, 44%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 3.52 (1H, s), 3.70 (2H, s), 3.73 (3H, s), 7.42 (1H, dd, J = 1.9, 8.3 Hz), 7.61 (1H, d, J = 1.9 Hz), 8.03 (1H, d, J = 8.3 Hz).

5.40.3. Methyl 4-amino-3-ethylphenylacetate (5). A suspension of methyl 3-ethynyl-4-nitrophenylacetate (**20**) (1.5 g, 6.8 mmol) and 5% Pd/C (1.5 g) in EtOH (15.0 ml) was hydrogenated at room temperature for 18 h. The catalyst was filtered off, and the filtrate was concentrated in vacuo. The residue was purified by column chromatography with *n*-hexane–EtOAc (3/1, v/v) as an eluent to give the compound **5** (1.0 g, 77%) as a pale yellow oil. ^{1}H NMR ($CDCl_3$) δ 1.24 (3H, t, J = 7.6 Hz), 2.50 (2H, t, J = 7.6 Hz), 3.51 (2H, s), 3.59 (2H, s), 3.67 (3H, s), 6.63 (1H, d, J = 7.8 Hz), 6.93–6.97 (2H, m).

5.41. Preparation of ethyl 4-amino-3-isopropylphenylacetate (6)

5.41.1. Ethyl 3-isopropyl-4-nitrophenylacetate (23). To a stirred solution of ethyl 4-nitrophenylacetate (**21**) (4.18 g, 20.0 mmol) in THF (100 ml) was added 2.0 M isopropylmagnesium chloride in diethyl ether solution (12 ml, 24 ml) at −15 °C and stirred for 1.5 h. DDQ (5.9 g, 26 mmol) was added to the reaction mixture at the same temperature. After 4 h of stirring at room temperature, the reaction mixture was poured into water and extracted with CH_2Cl_2. The combined extracts were washed with water, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography on silica gel with *n*-hexane–EtOAc (7/1, v/v) as an eluent to give the compound **23** (1.6 g, 32%) as a yellow oil. ^{1}H NMR ($CDCl_3$) δ 1.24–1.30 (9H, m), 3.42–3.46 (1H, m), 3.67 (2H, s), 4.15–4.20 (2H, m), 7.23 (1H, d, J = 8.3 Hz), 7.37 (1H, s), 7.68 (1H, d, J = 8.3 Hz).

5.41.2. Ethyl 4-amino-3-isopropylphenylacetate (6). A mixture of ethyl 4-nitro-3-isopropylphenylacetate (**23**) (1.6 g, 6.4 mmol) and 5% Pd/C (0.8 g) in EtOH (20 ml) was hydrogenated at room temperature for 3 h. The catalyst was filtered off, and the filtrate was concentrated in vacuo. The residue was purified by column chromatography on silica gel with *n*-hexane–EtOAc (3/1, v/v) as an eluent to give the compound **6** (1.4 g, quant.) as a pale yellow oil. ^{1}H NMR ($CDCl_3$) δ 1.22–1.27 (9H, m), 2.84–2.89 (1H, m), 3.49 (2H, s), 3.61 (2H, s), 4.09–4.15 (2H, m), 6.62 (1H, d, J = 8.0 Hz), 6.93 (1H, dd, J = 1.9, 8.0 Hz), 7.03 (1H, d, J = 1.9 Hz).

5.42. Preparation of ethyl 4-amino-3-trifluoromethylphenylacetate (7)

5.42.1. Ethyl 4-nitro-3-trifluoromethylphenylacetate (26). To a stirred solution of butyl ethyl malonate (4.53 ml, 23.9 mmol) in DMF (200 ml) was added NaH (60% in oil, 2.87 g, 71.7 mmol) at room temperature. After 20 min, 4-fluoro-3-trifluoromethylnitrobenzene (**24**) (5 g, 23.9 mmol) in DMF (50 ml) was added dropwise via a dropping funnel. After 3 h of stirring, the reaction mixture was poured into ice water and extracted with EtOAc. The combined extracts were washed with brine, dried over $MgSO_4$, filtered, and concentrated to give *tert*-butyl 2-ethoxycarbonyl-4-nitro-3-trifluoromethylphenylacetate (**25**).

To a solution of *tert*-butyl 2-ethoxycarbonyl-4-nitro-3-trifluoromethylphenylacetate (**25**) in CH_2Cl_2 (20 ml) was added TFA (20 ml) at room temperature. The reaction mixture was refluxed for 18 h. The reaction mixture was evaporated in vacuo and then co-evaporated with toluene. The residue was purified by middle pressure chromatography system (YAMAZEN YFLC-5404-FC, ∅ 50 mm × 300 mm, 15 ml/min) with linear gradient *n*-hexane–EtOAc (10/0 to 1/1, v/v) as an eluent to afford the compound **26** (6.6 g, 94%) as a yellow oil. ^{1}H NMR (CD_3OD) δ 1.30 (3H, t, J = 7.3 Hz), 3.79 (2H, s), 4.22 (2H, q, J = 7.3 Hz), 7.68 (1H, dd, J = 8.3, 1.6 Hz), 7.76 (1H, s), 7.87 (1H, d, J = 8.3 Hz); MS (ESI) m/z , 277 (M^+).

5.42.2. Ethyl 4-amino-3-trifluoromethylphenylacetate (7). To a stirred solution of ethyl 3-trifluoromethyl-4-nitrophenylacetate (**26**) (6.6 g, 23.8 mmol) in EtOH

(125 ml) was added $SnCl_2$ (16 g, 71.4 mmol) at room temperature. Stirring was continued for 18 h at reflux. The reaction mixture was evaporated in vacuo. The residue was dissolved in $CHCl_3$ and 4 M NaOH at 0 °C, and then extracted with $CHCl_3$. The combined extracts were washed with sat. $NaHCO_3$ and brine, and then dried over $MgSO_4$. After removal of the solvent, the residue was purified by middle pressure chromatography system (YAMAZEN YFLC-5404-FC, Φ 50 mm × 300 mm, 15 ml/min) with linear gradient *n*-hexane–EtOAc (9/1–4/1, v/v) as an eluent to afford the compound **7** (4.37 g, 74%) as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.28 (3H, t, J = 8.8 Hz), 3.52 (2H, s), 4.14 (2H, q, J = 8.8 Hz), 6.70 (1H, d, J = 8.3 Hz), 7.22 (1H, d, J = 8.8 Hz), 7.32 (1H, s); MS (ESI) m/z 248 (M^++1).

5.43. VLA-4/VCAM-1 binding assay

A human VLA-4-expressing cell line, 4B4, was established at Pharmacopeia, 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) supplemented with 10% (v/v) fetal calf serum (REHATUIN Fetal Bovine Serum, Serologicals Corporation), 100 U/ml penicillin (Invitrogen Corporation), 100 μg/ml streptomycin (Invitrogen Corporation), 2 mM L-glutamine (Invitrogen Corporation), and 1 mg/ml G-418 (Geneticin, Invitrogen Corporation). An Eu-labeling Reagent (Perkin-Elmer Inc.) was used to label the human VCAM-1/Fc chimeric antibody (R&D Systems Inc.). The Eu-labeled protein was purified with a PD-10 column (Amersham Biosciences KK.) and stored at −80 °C until used. 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 microliters of the 4B4 cell suspension was distributed into each well of a 96-well culture plate (Costar). The plates were incubated at 37 °C in a 95% air/5% CO_2 atmosphere humidified incubator (Themo Forma, model 3120; Forma Scientific) 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 solutions was added to a well, followed by 50 μl of 2 nM Eu-labeled 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-labeled human VCAM-1/Fc Chimera in 3% (w/v) human serum albumin (Sigma) were distributed into each well (final concentration: 1 nM). The plates were incubated for 60 min at room temperature, and the wells were washed 4 times with 300 μl of chilled Wash Buffer. Finally, 100 μl of the enhancement solution (Perkin-Elmer 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; Perkin-Elmer Inc.). The concentration of compound required for 50% inhibition in the assay was determined.

5.44. Bio-assay

Female BALB/c mice (18–23 g, 8 weeks of age, Charles River Japan, Inc.) were used. Each group consisted of 4 animals. Mice were orally administered the compound dissolved in 0.5% (w/v) carboxymethylcellulose (CMC) at a dose of 10 mg/1 ml/kg (or 50 mg/1 ml/kg). After 15 min (or 30 min), blood samples were collected via inferior vena cava from the animals under ether anesthesia. Blood samples were stored at room temperature and centrifuged at 2000 rpm for 10 min at 4 °C. Serums 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 plasma samples at various concentrations was distributed into each well (final concentration: 0.01–10%). As for the metage line, each diluted compound solution was also evaluated.

5.45. Distribution coefficient

The distribution coefficients ($\log D$) were determined by the shake-flask method.[22] Four hundred μM of compound solution of each compound in a 2 ml *n*-octanol–2 ml PBS solution was placed on a shaker for 30 min. After centrifuging each solution separately at 3000 rpm for 10 min, an LC/MS method was used to assay each layer. The LC/MS system consisted of an 1100 Series LC/MSD (Agilent) and an X Terra® MSC18 3.5μm, 3.0 × 30 mm column (Waters). The mobile phase was a 10 mM ammonium acetate buffer (pH 4.5)/0.05% (v/v) acetic acid mixture in acetonitrile; the gradient condition (95/5–10/90). Analyst software program (version 1.4, Applied Bio. Systems) was used to calculate the $\log D$.

5.46. Madin–Darby canine kidney cell permeability

The cell permeability of selected compounds was determined with Madin–Darby canine kidney (MDCK, American Type Culture Collection) cells. MDCK cells were maintained in Minimum Essential Medium (GIBCO) containing 10% (v/v) fetal bovine serum (Bioproducts Inc.), a penicillin–streptomycin mixture (GIBCO), and L-glutamine. For the transport assay, cells were seeded into HTS 24-well transwells (Costar) at 3×10^5 cells/ml and grown for 6 days after seeding to allow the formation of a cell monolayer. Transport Buffer was prepared using $NaHCO_3$ (final 0.35 g/l), D-glucose (final 3.5 g/l), HEPES (Sigma; final 10 mM), $CaCl_2$ (final 0.14 g/l), and $MgSO_4$ (final 0.098 g/l) in 10× Hanks' Balanced Salt Solution (GIBCO) and adjusted to pH 6.0 or 7.4 with 1 M HCl or 1 M NaOH. For each test compound, a dosing solution containing one of the compounds at a concentration of 10 μM in Transport Buffer (pH 6.0) (100 μl) was added to the apical (A) side of a monolayer. A blank solution containing 4% (w/v) BSA in Transport Buffer (pH 7.4) (600 μl), which was re-adjusted to pH 7.4, was added to the basolateral (B) side of the monolayer. Metoprolol was used as a positive control. After 1 h of incubation at 37 °C, aliquots of the apical and basal solutions were separately analyzed on an LC/MS/MS system comprising an Alliance 2790 HPLC (Waters), Atlantis dC18, 2.1 mm ID

×20 mm L, 3 μm particle size column (Waters), and TSQ7000 mass spectrometer (ThermoQuest). The mobile phase consisted of a 10 mM HCO_2NH_4–acetonitrile step gradient 100/0–80/20–100/0. The concentrations of each compound in the apical and the basolateral solutions were determined from a peak area versus concentration standard curve. For each compound, Eq. 1 was used to calculate an apparent permeability coefficient (P_{app}) from the LC/MS/MS-determined concentration in the basolateral compartment (C_b, μM) and the initial 10 μM concentration in the donor compartment. In the following equation, 3600 s is the total time for the measurement of compound flux, and 0.33 cm^2 is the area of the transwell filter.

$$P_{app}(10^{-6}\text{cm/s}) = (C_b \times 600\ \mu\text{l})/(10\ \mu\text{M} \times 3600\ \text{s} \times 0.33\ \text{cm}^2) \quad (1)$$

5.47. Protein binding

Protein binding was determined by the ultra filtration method. One μM of compound dissolved in DMSO and 1458 μl of human serum solution (CONSERA, lyophilized Human serum, Nissui Pharmaceutical) were distributed into a Deep well plate (Waters) and mixed by pipetting. Three hundred microliters of the mixed solution was distributed into each well of a 96-well filter plate (MultiScreen® Filter Plate with Ultracel, Millipore) and was centrifuged at 3000 rpm for 60 min at 37 °C. The filtrate (50 μl) was collected and subjected to LC/MS analysis. The LC/MS system consisted of Alliance 2795 (Waters), ZQ (Waters), and an X Terra® MS C_{18} 3.5 μm, 3.0 × 30 mm column (Waters). The mobile phase was a 10 mM ammonium format buffer–MeOH; the gradient condition, 80/20–10/90 (v/v). Protein binding was analyzed using QuantLynx (Waters).

5.48. Pharmacokinetic studies on mice

Female BALB/c mice (8–10 weeks of age, Charles River Japan, Inc.) were used. Each group consisted of 3–4 animals. Mice were orally or intravenously administered the test compound at a dose of 5 mg/5 ml/kg dissolved in 0.5% (w/v) MC aqueous solution and in saline for the oral and intravenous doses, respectively. Blood samples were collected at 0.25, 0.5, 1, 2, and 6 h after the administration. Blood samples were stored on ice, followed by centrifugation at 2000 rpm (1500*g*)for 10 min at 4 °C. Plasma fractions were subsequently stored in a −20 °C freezer until analyzed. Concentrations of test compounds were determined by an LC/MS/MS method, comprising 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 90/10–10/90. Plasma concentrations versus time data were analyzed by non-compartmental approaches using WinNonlin software program (version 1.13.1 Pharsight, Mountain View, CA).

5.49. Pharmacokinetic studies on rats

Male Sprague–Dawley rats [Crj: CD(SD) IGS, 6 weeks of age, Charles River Laboratories] were used. Animals were fasted for 18 h prior to dosing. Each group consisted of 3–4 animals. Rats were orally administered the test compound at the doses of 10 mg/5 ml/kg dissolved in 0.5% (w/v) MC aqueous solution or 20 mg/ml/kg dissolved in 20% (w/v) PEG aqueous solution. Rats were intravenously administered the test compound at a dose of either 10 mg/5 ml/kg or 10 mg/ml/kg dissolved in saline. Blood samples (0.4 ml) were collected at 0.08, (or 0.25 for po), 0.5, 1, 2, and 6 h after the administration. These analytical samples were prepared and analyzed according to the Pharmacokinetic Studies on mice.

5.50. Pharmacokinetic studies on dogs

Male Beagle dogs (10–12 kg, Gokitabreeding and Nihonnousannkou) were used. Animals were fasted for 18 h prior to dosing. Each group consisted of 2 animals. Dogs were orally administered the test compound dissolved in 0.5% (w/v) MC at a dose of 2 mg/5 ml/kg or intravenously dissolved in saline at a dose of 2 mg/5 ml/kg. Blood samples (1 ml) were collected after 0.08, (or 0.25 for po), 0.5, 1, 2, 4, 8, and 24 h. After the 4-h sampling, animals were provided food. These analytical samples were prepared and analyzed according to the Pharmacokinetic Studies on mice.

5.51. Evaluation of compound 48/80-induced rat pleurisy models (*in vivo* evaluation)

Male Sprague–Dawley rats (Slc: SD, 8 weeks of age, Nihon SLC) were used. Each group consisted of 7 animals. Rats received were orally administered the test compound dissolved in 0.5% (w/v) methylcellulose (MC) at a dose of 30 mg/5 ml/kg or 10 mg/5 ml/kg. After 15 min, rats were injected intercostally with compound 48/80 (Sigma, Lot No. 79H4070, 50 μg/0.1 ml/cavity). In the negative control group, rats were injected with saline instead of compound 48/80. The test compound was re-orally administered 8 h later at the same dosage. Sixteen hours later, the rats were euthanized by cutting the abdominal aorta while the animals were under ether anesthesia. The plural cavity was opened and rinsed with 2 × 2.5 ml Hanks' balanced salt solution supplemented with 0.2% EDTA. The pleural exudate was sampled and measured (with the volume of the rinse fluid subtracted). The cells were counted in a particle analyzer CDA-500 (Sysmex). Cytocentrifuged preparations (Cytospin 2; Shandon) were stained with Wright's stain solution (Muto Chemicals) for differential counts, based on standard morphological criteria. The eosinophil and total cell numbers of all groups were analyzed by Dunnett's method.

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