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Synthesis and biological evaluation of new fluorine substituted derivatives as angiotensin II receptor antagonists with anti-hypertension and anti-tumor effects

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ABSTRACT

The synthesis and pharmaceutical activity of new potent non-tetrazole angiotensin II (Ang II) receptor antagonists were described. These compounds were fluorine substituted derivatives of Losartan, Valsartan and Irbesartan with carboxylic acid group as replacements to the known potent tetrazole moiety at the 2′-biphenyl position. Their activities were evaluated by Ang II receptor binding assay as well as by in vivo assay. All of the synthesized compounds showed nanomolar affinity for the AT_1 receptor subtype. The vivo biological evaluation showed that compounds **1a**, **2** and **4** produced a dose-dependent antihypertensive effect both in spontaneously hypertensive rats (SHR) and renal hypertensive rats (RHR). Compound **4** especially showed an efficient and long-lasting effect in reducing blood pressure which can last more than 24 h at dose of 10 mg/kg in SHR, which was much better than control Losartan and Valsartan. Compound **4** can also inhibit the prostate cancer in vitro and in vivo. So compound **4** was selected for in-depth investigation as potent, novel and long-lasting non-tetrazole anti-hypertension and anti-tumor drug candidate.

1. Introduction

Hypertension is recognized as one of the leading risk factors for human morbidity and mortality. On a worldwide basis hypertension has been ranked third as a cause of disability.[1] Angiotensin II (Ang II) is a vasoconstrictive peptide hormone formed within the renin–angiotensin system (RAS) which plays an important role in regulating cardiovascular homeostasis.[2–4] Research efforts for the control of hypertension have focused on competing Ang II binding to AT_1 receptors. Ang II receptor antagonists have been proved to lower blood pressure effectively,[5] to treat congestive heart failure and reduce the cardiac and vascular remodeling associated with cardiovascular disease.[6] They are better tolerated than other classes of drugs.[7,8]

A number of Ang II type 1 (AT_1) receptor antagonists, namely the angiotensin II type 1 receptor blockers (ARBs) with different acidic moiety have been investigated by David,[9] and other scientists. It was reported that tetrazole-containing compounds had the greatest binding affinities and oral activities. Most of ARBs have acidic group tetrazole ring, such as Valsartan, Losartan, Candesartan and Ibersartan. However the tetrazole group had some disadvantages in chemical synthesis and biometabolism. For example, the synthesis of tetrazole derivatives could be dangerous due to the use of toxic and explosive azide compounds such as sodium azide or trialkytin azide. In this report we designed and synthesized a series of new compounds with carboxylic acid moiety to replace tetrazolium.

Frequently, the introduction of fluorine atoms or fluorine-containing substituents into a drug molecule decreases toxicity and increases stability of the compound, the biological activity being unchanged or even enhanced. Besides in the past decades, much progress has been achieved in the development of organo-fluorine chemistry, including the synthesis of new fluorinating agents, discovery of new reactions and development of new technologies. As a result, fluorine containing-organic compounds of various classes became more available.[10] Considering pK_a for o-, m- and p-fluorobenzoic were 3.47, 3.86 and 4.13, respectively, while the pK_a for benzoic acid was 4.2, fluorine atoms was introduced as electron withdrawing moiety to the biphenyl part to enhance the ionization of carboxylic acid into negative charge for better binding with the positive charge of AT_1. It could be suggested that compounds with fluorine substituent on the biphenyl group would exhibited better antihypertensive activity.

Although the RAS has a crucial role as vasopressor system that maintains blood pressure, fluid homeostasis and electrolyte balance, it is now recognized to have much broader functions in the

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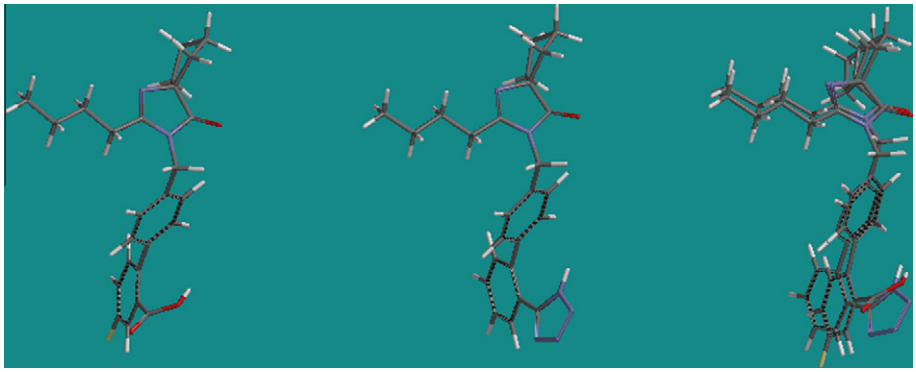


Figure 1A. Energy-minimized conformation of compound **3** (179.8151 kJ/mol), Irbesartan (219.8643 kJ/mol) and their overlay conformation.

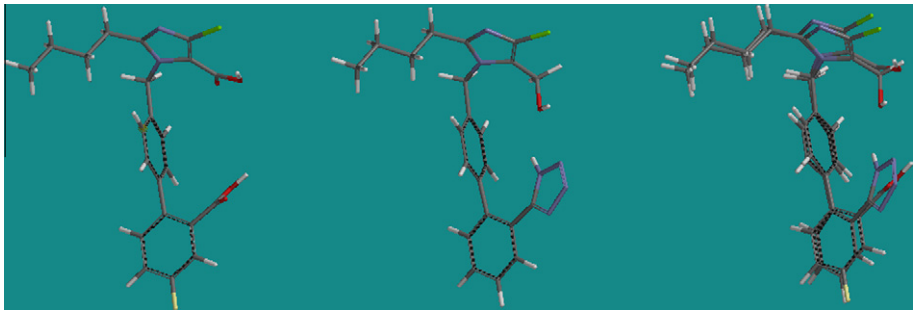


Figure 1B. Energy-minimized conformation of Losartan (60.0774 kJ/mol), compound **1a** (−29.0096 kJ/mol) and their overlay conformation.

body with important actions on growth factors and cell growth. Tissue-based or local RAS have been identified in the testis, epididymis, ovary uterus, brain, heart, peripheral blood vessels and kidney. In recent years, all components of the classical RAS have been reported in the prostate[11] and some other tumors. Some evidence suggests that Ang II directly stimulates cell growth via the AT_1 receptor and that its blockade inhibits tumor growth. AT_1 receptor blockade can reduce tumor volume, vascular density, mitotic index and cell proliferation. These results suggest a possibility that AT_1 receptor blockades are potential therapeutic drugs for the treatment of prostate cancer and other prostate diseases.

In this study, six compounds **1a**, **1b**,**1c, 2, 3** and **4** were designed and synthesized. The dominant conformations of compound **1a**, **3**, Irbesartan and Losartan were shown as Figure 1A and B using computer software Spartan 8. We found that the minimal energy conformations of the compounds **1a** and **3** were lower than Irbesartan and Losartan. The conformation of compoud **3** fits perfectly to Irbesartan and compound **1a** fits perfectly to Losartan. These compounds were tested for their affinity for the AT_1 receptor as measured by their ability to displace [^{125}I] Ang II from its specific binding sites in the rat vascular smooth muscle cells (VSMC). The anti-hypertension effects of them were evaluated in spontaneously hypertensive rats (SHR) in vivo after oral administration. Selected compounds were further tested in vivo in renal hypertensive rats (RHR) in vivo. Moreover, compound **4** were selected to evaluate the anti-proliferative activity in vitro and antitumor activity in vivo in prostate cancer.

2. Results and discussion

2.1. Chemistry

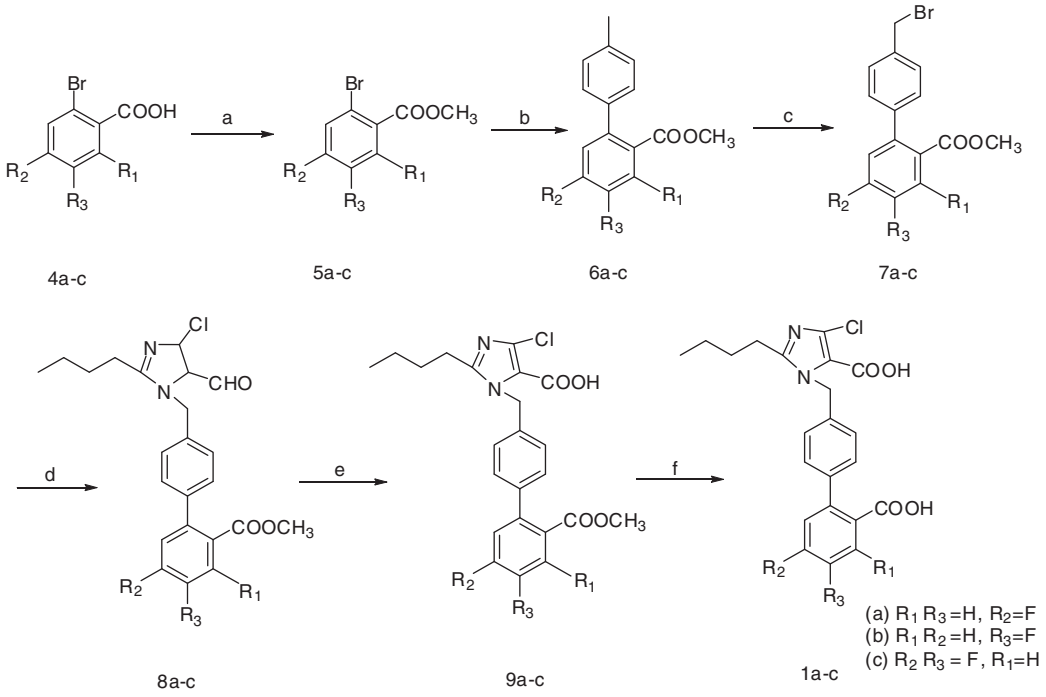
The synthetic route described in Scheme 1 was employed to synthesize the target compounds **1a–c**. Compounds **4a–c** as starting materials were esterified with CH_3OH catalyzed by H_2SO_4 to give **5a–c**. Compounds **6a–c** were obtained through the Suzuki coupling reaction of **5a–c** with *p*-tolylboronic acid catalyzed by PPh_3 and Pd $(OAc)_2$.

Compounds **6a–c** were brominated with NBS and AIBN to produce compounds **7a–c**, and then reacted with 2-butyl-4-chloro-1*H*-imidazole-5-carboxaldehyde in the presence of K_2CO_3 to give compounds **8a–c**. Compounds **8a–c** were oxidated with NaClO to produce compounds **9a–c**. Compounds were obtained through the hydrolysis of **9a–c** with sodium hydroxide solution.

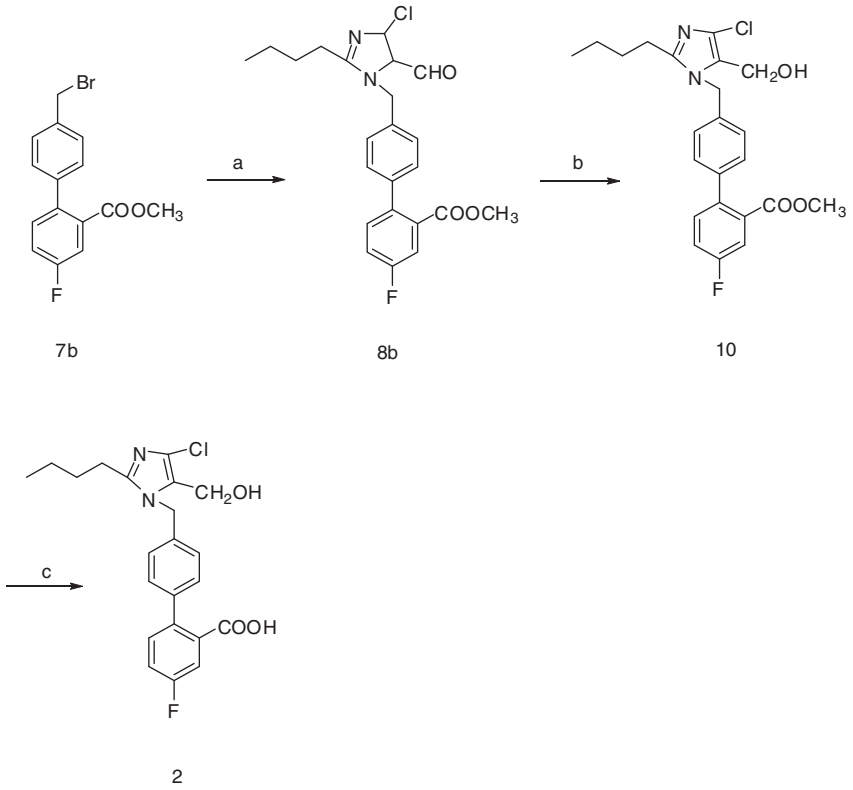
Compound **2** was obtained from **8b** which was reduced by $NaBH_4$ and then hydrolyzed with sodium hydroxide solution in Scheme 2.

Scheme 3 was employed to synthesize compound **3**. Methyl 4-fluoro-4′-methylbiphenyl-2-carboxylate **6b** was obtained as the method in Scheme 1. Compound **6b** was hydrolyzed to give 4-fluoro-4′-methylbiphenyl-2-carboxylic acid **11** which was reacted with oxalylchloride and *t*-C_4H_9OK to give tert-butyl 4-fluoro-4′-methylbiphenyl-2-carboxylate **12** in 44.2% yield. Compound **12** was brominated to produce tert-butyl 4′-(bromomethyl)-4-fluorobiphenyl-2-carboxylate **13**. Tert-butyl-4′-((2-butyl-4-oxo-1,3-iazaspiro[4.4]non-1-en-3-yl)methyl)-4-fluorobiphenyl-2-carboxylate **14** was obtained from **13** and 2-butyl-4-spirocyclopentane-2-imidazolin-5-one hydrochloride in the presence of NaH. Compound **3** was obtained through the hydrolysis of **14** with TFA in CH_2Cl_2.

Valsartan derivative **4** was synthesized in Scheme 4. A mixture of **7b**, L-Valine methyl ester hydrochloride and DIPEA was refluxed to produce (*S*)-methyl 4-fluoro-4′-((1-methoxy-3-methyl-1-oxobutan-2-ylamino)-methyl)-biphenyl-2-carboxylate **15** which was acylated with *n*-butyryl chloride under the presence of DIPEA to give (*S*)-methyl 4-fluoro-4′-((*N*-(1-methoxy-3-methyl-1-oxobutan-2-yl)butyramido)methyl)biphenyl-2-carboxylate **16**. The compound **3** was obtained through the hydrolysis of **16** with TFA.



Scheme 1. Preparation of compounds **1a–c**.

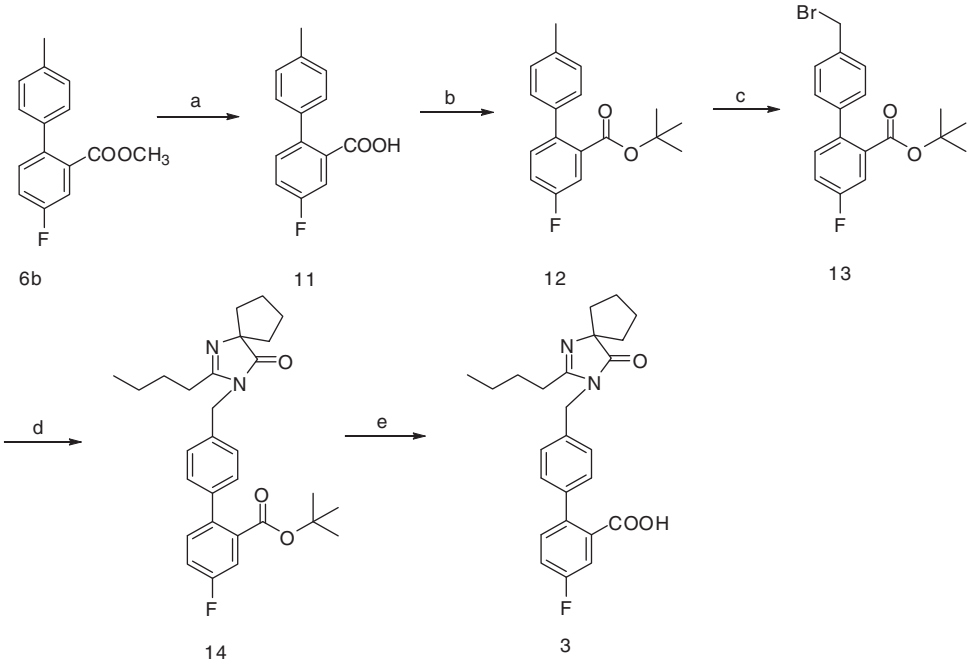


Scheme 2. Preparation of compound **2**.

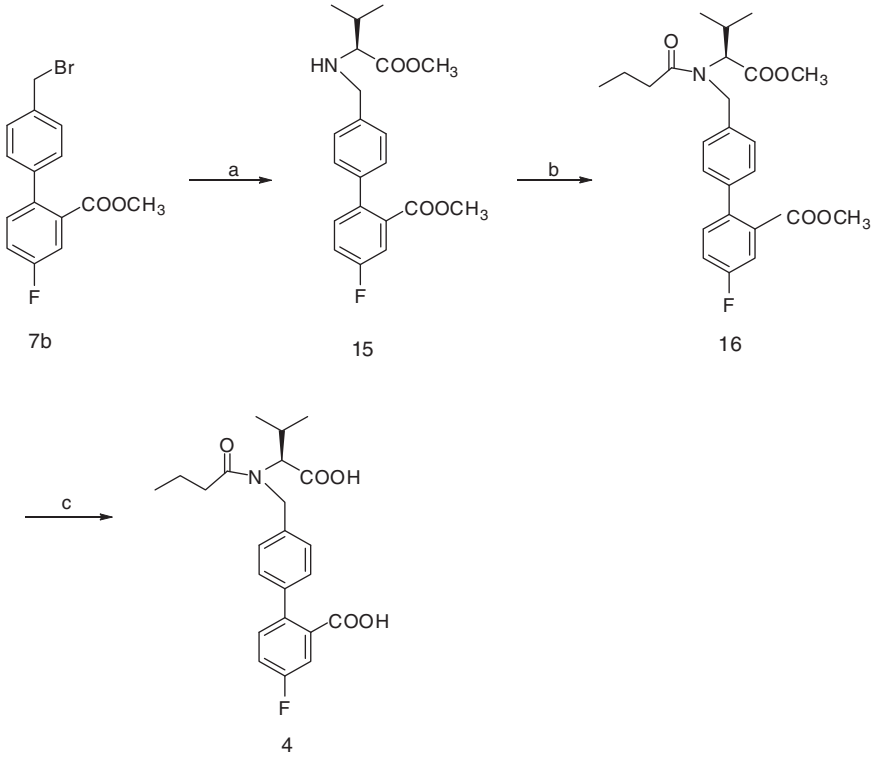
2.2. Biological evaluation

2.2.1. Radioligand binding assay

Angiotensin II interacted with a single population of binding sites in the rat vascular smooth muscle cells (VSMC) AT_1 receptors. Radioligand binding assay showed that all of these compounds have nanomolar affinity for the AT_1 receptor subtype (Table 1). The specific binding of ^{125}I-Ang II was inhibited in a concentration-dependent manner by compounds **1a**, **1b**, **1c**, **2**, **3**, **4** and Irbesartan, Losartan, Valsartan as exemplified by compounds **1a**



Scheme 3. Preparation of compound **3**.



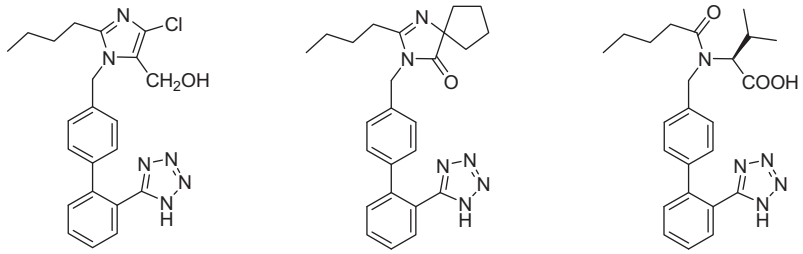
Scheme 4. Preparation of compound **4**.

and **4** in Figure 2. The IC_{50} values of compound **4**, Losartan, Irbesartan and Valsartan were 0.58 ± 0.10, 1.64 ± 0.22, 3.11 ± 0.24 and 2.64 ± 0.75 nM, respectively. The K_i values of compound **4**, Losartan, Irbesartan and Valsartan were 0.42 ± 0.54, 1.41 ± 0.12, 2.75 ± 0.97 and 2.08 ± 0.17, respectively. These results suggested that compound **4** exhibited more affinity to AT_1 receptors than Losartan, Irbesartan and Valsartan because their IC_{50} and K_i values were significantly lower compared with the positive control groups (P <0.05). So compound **4** might had better antihypertensive activity in vivo.

2.2.2. Antihypertensive effects in rats

In spontaneously hypertensive rats (SHR), the effects of compounds **1a**, **1b, 1c, 2**, **3** and **4** (5,10 mg/kg) and Losartan (10 mg/

Table 1
IC_{50} and K_i values of tested compounds



Losartan Irbesartan Valsartan

Compound	R_1	R_2	R_3	mp(°C)	Formula	IC_{50} ± SEM (nM)	K_i (nM)
1a	H	H	F	208–211	$C_{22}H_{20}ClFN_2O_4$	13.57 ± 3.90	9.17 ± 2.30
1b	H	F	H	196–199	$C_{22}H_{20}ClFN_2O_4$	26.12 ± 0.15	17.65 ± 0.15
1c	H	F	F	218–221	$C_{22}H_{19}ClF_2N_2O_4$	13.48 ± 0.03	9.11 ± 0.11
2	H	F	H	206–209	$C_{22}H_{22}ClFN_2O_3$	4.11 ± 1.20	3.78 ± 1.20
3				214–217	$C_{25}H_{27}FN_2O_3$	9.98 ± 0.03	6.75 ± 2.60
4				215–218	$C_{23}H_{26}FNO_5$	0.584 ± 0.10	0.42 ± 0.54
Losartan						1.64 ± 0.22	1.41 ± 0.12
Irbesartan						3.11 ± 0.24	2.75 ± 0.97
Valsartan						2.64 ± 0.75	2.08 ± 0.17

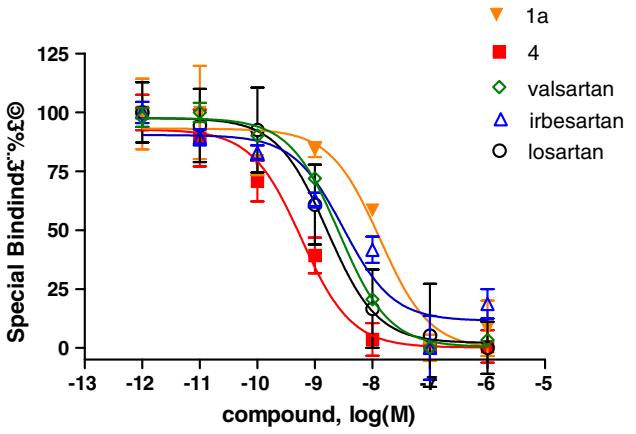


Figure 2. Inhibitory effects of compounds **1a**, **4**, Losartan, Irbesartan and Valsartan (10^{-6}–10^{-12} M) on the specific binding of ^{125}I-Ang II to AT_1 receptors in VSMCs.

kg), Valsartan (10 mg/kg) on the mean arterial pressure (MAP) in vivo after oral administration were shown in Figure 3A. The results indicated that under the dosage of 5 mg/kg or 10 mg/kg, compound **1b**, **1c** and **3** could not decreased blood pressure significantly compared with the negative control group. Compounds **1a**, **2** and **4** could cause significant decrease on MAP in a dose dependent manner. The maximal response of compound **1a** (5,10 mg/kg) was observed at 3 h after dosing, it lowered 23 mm Hg and 32 mm Hg of MAP, respectively, and the significant (p <0.05) antihypertensive effect of it was sustained for at least 8 h. Compound **2** (5,10 mg/kg) lowered MAP to 18, 25 mm Hg, respectively. The maximal reduction and the time of duration are almost the same as compound **1a**. The antihypertensive effects of compounds **1a** and **2** at 10 mg/kg were almost equal to Losartan and Valsartan at the same dose.

When compound **4** at 5 mg/kg and 10 mg/kg was administered orally, the MAP was lowered 27 mmHg and 42 mmHg, respectively. The maximal response was obtained at 3–5 h after dosing. With 10 mg/kg, the reductive effect of compound **4** was observed even at 24 h after drug administration, which was much better than Losartan and Valsartan whose antihypertensive effects maintained for 7 and 9 h, respectively, and the maximal reduction was 27, 28 mm Hg, respectively at 10 mg/kg.

All these compounds did not influence heart rate of the rats.

Because compounds **1a**, **2** and **4** had significant antihypertensive effects in SHR, renal hypertensive rats (RHR) was used to test these three compounds at dose 5, 10 mg/kg compared with Losartan at 10 mg/kg and Valsartan at 10 mg/kg. In RHR, we observed almost the same effects as in SHR (Fig. 3B). Compound **4** at 5 mg/kg showed the equal effect to that of Losartan and Valsartan at 10 mg/kg. And at dose of 10 mg/kg, compound **4** had much better and longer antihypertensive effect. Compounds **1a** and **2** at 10 mg/kg showed the similar effect to that of Losartan and Valsartan at the same dose.

These results in SHR and in RHR showed that compound **4** was superior to Losartan and Valsartan, which were in accord with the data of the former receptor binding assay.

2.2.3. Antiproliferative activity in LNCap cells

Compound **4** was selected to examine the activity on carcinoma of prostate respecting its favorable antihypertensive activity. The effect of compound **4** on cell viability was assessed by MTT assay. Treatment of LNCaP cells with Ang II at the concentration of 1×10^{-4} M resulted in an increase in the viability of cells. A significant reduction in the viability as compared to DMSO-treated controls was observed after 24 h of treatment with Losartan and compound **4**. Increase in treatment times to 48, 72 and 96 h further reduced the viability of cells (Fig. 4). However, Losartan and compound **4** could not influence the growth of LNCaP cells without treatment with Ang II.

2.2.4. Antitumor activity in nude mice

Based on the in vitro results, we further tested the antitumor activity of compound **4** in vivo, LNCap cells were established as xenografts in nude mice (n = 10). When the tumors reached about 5 mm in diameter, the animals were given compound **4** at 5.0 or 10 mg/kg/day. Losartan at 5.0 or 10 mg/kg/day were used as positive control group. The negative control group was received water containing sodium hypochlorite (10 ppm).[12] As shown in Figure 5, at 4 weeks, control group had developed large tumor of

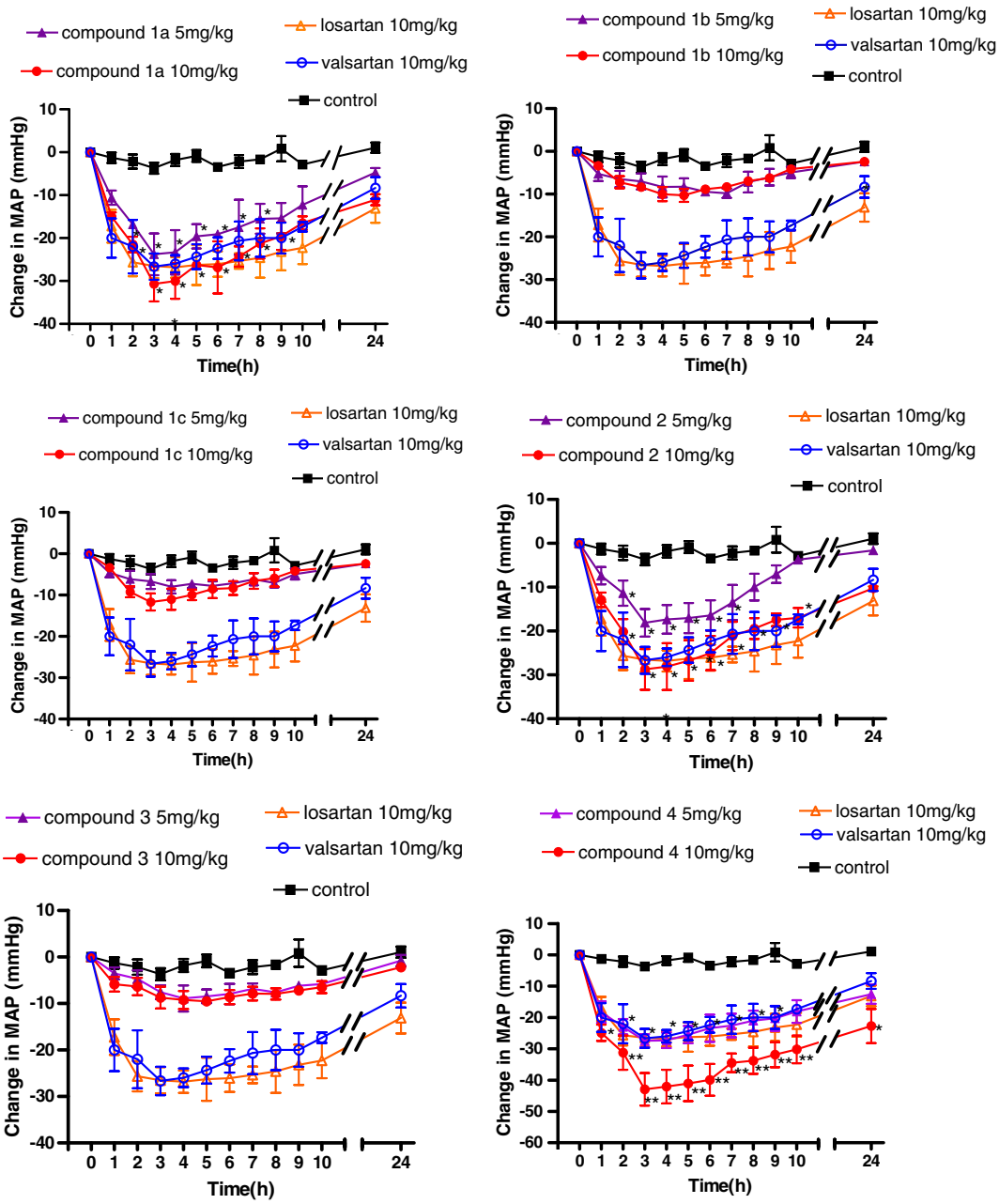


Figure 3A. Effects of compounds **1a**, **1b**, **1c**, **2**, **3**, **4**, Losartan and Valsartan on arterial pressure (MAP) in spontaneously hypertensive rats. *,**Significant difference from the control, p <0.05 and p <0.01, respectively.

28.34 ± 2.05 relative volume compared with those at 0 week. Mice treated with compound **4** at 5.0 or 10 mg/kg/day was showed more inhibition of tumor relative volume at 4 weeks by 17.44 ± 1.78 and 12.39 ± 1.23, respectively, compared with tumor relative volume at 4 weeks by 21.44 ± 2.182 and 15.75 ± 1.89, respectively, treated with Losartan at 5.0 or 10 mg/kg/day.

3. Conclusions

In this paper the tetrazole moiety of Ang II type 1 receptor antagonists has been successfully replaced by carboxylic acid group at the 2′-biphenyl position with fluorine substitution at 4′ or 5′-biphenyl position of Losartan, Valsartan and Irbesartan as a novel class of angiotensin II receptor antagonists

Radioligand binding assay showed that all of these compounds have nanomolar affinity for the AT_1 receptor subtype. The IC_{50} values and K_i values showed that compound **4** had the strongest inhibitory ability against Ang II. In anti-hypertensive assays in vivo, compounds **1a, 2** and **4** showed efficient and long effect in decreasing blood pressure in spontaneously hypertensive rats and renal hypertensive rats. These results were corresponding to the data in radioligand binding assay. Both in spontaneously hypertensive rats and in renal hypertensive rats, compound **4** showed an efficient and long-lasting effect in reducing blood pressure, it could last more than 24 h which was much better than Losartan and Valsartan. The anti-prostate cancer test showed that compound **4** could inhibit the prostate cancer in vitro and in vivo.

This led to the discovery of compound **4** as a potent, non-tetrazole AT_1 receptor antagonist. This orally active compound produced a marked and long lasting decrease in blood pressure in SHR models and RHR models, which was proved to be superior to Losartan and Valsartan. It has also anti-prostate cancer effects. On the

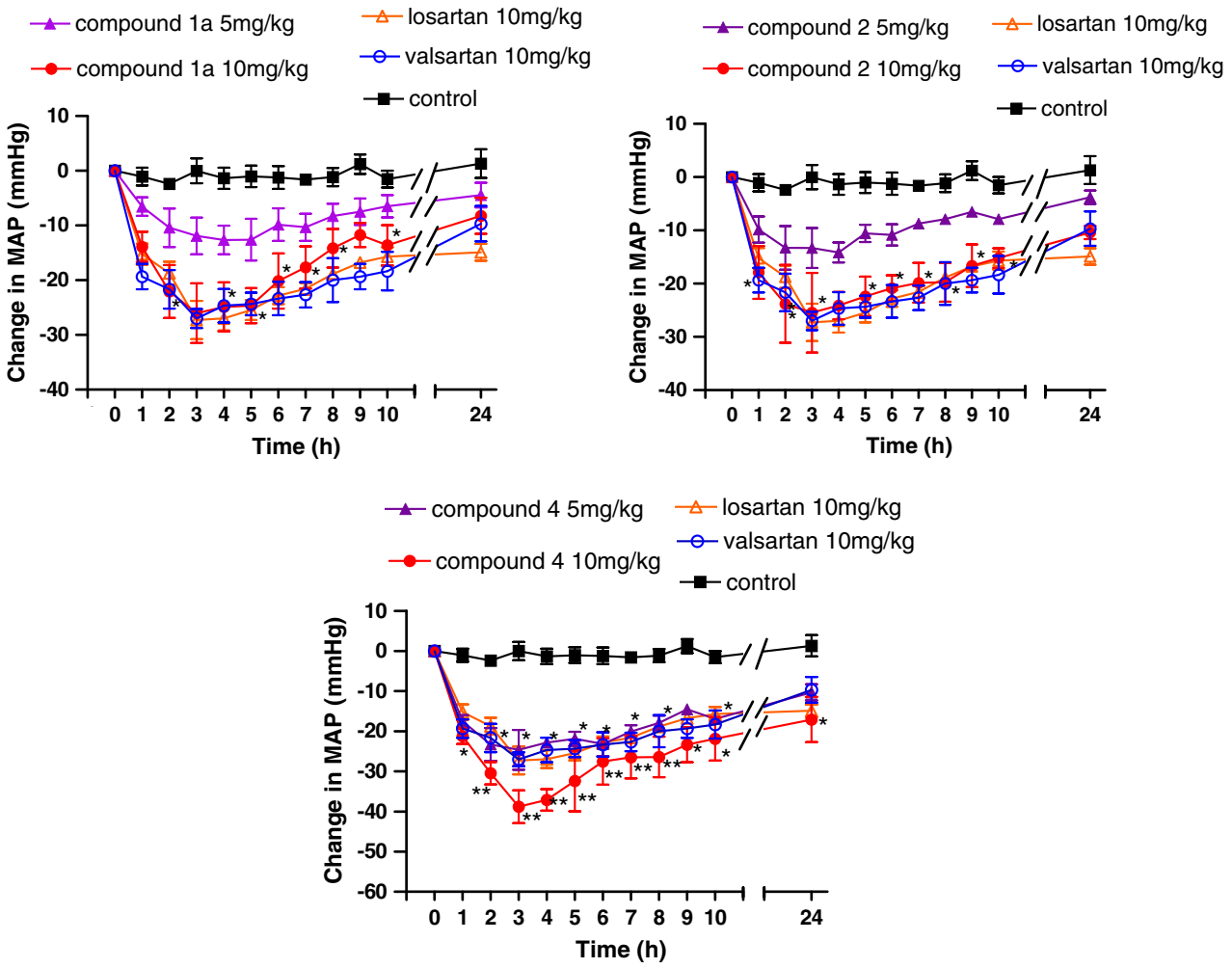


Figure 3B. Effects of compounds **1a**, **2**, **4**, Losartan and Valsartan on arterial pressure (MAP) in renal hypertensive rats. *,**Significant difference from the control, p <0.05 and p <0.01, respectively.

basis of this profile, compound **4** was chose as novel anti-hypertension and anti-tumor drug candidates and deserved for further investigation.

4. Experimental section

4.1. Chemistry

All chemicals used were of reagent grade. Yields refer to purified products and are not optimized. ^{1}H NMR spectra were measured on a Bruker 400 MHz spectrometer. ESI-MS spectra were recorded on a Micromass triple quadrupole mass spectrometer. Column chromatography was performed using silic gel H (300–400). All melting points were measured using an Eletrothermal 9200 apparatus and are uncorrected. Solvents were dried according to standard procedures. Solutions were dried over $MgSO_4$ before evaporation under reduced pressure.

4.1.1. Methyl 2-bromo-4-fluorobenzoate (5a)

To a solution of 2-bromo-4-fluorobenzoic acid (2.17 g, 10.00 mmol) in methanol (10 mL) was added sulfuric acid (1.63 mL, 30 mmol) , the reaction mixture was refluxed for 4 h. The solvent was removed in vacuo. The mixture was diluted with diethyl ether, and washed water (50 mL × 3) and brine (50 mL × 3), dried over $MgSO_4$. The solvent was removed under reduced pressure. The residue was purified by flash column chromatography with petroleum ether–ethyl acetate (80/1) as eluant to afford colorless liquid. Yield: 88.3%. This compound was prepared according to the general procedure reported by Baker.[13] The spectral data were consistent with that reported in the literature.

4.1.2. Methyl 2-bromo-5-fluorobenzoate (5b)

Compound **5b** was prepared according to the procedure of **5a**. Yield: 84.7%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.74–6.92(3H, m, Ph-H), 3.80(3H, s, Ph-COOCH_3). ESI-MS (m/z): 234.0 $[M+1]^+$.

4.1.3. Methyl 2-bromo-4, 5-difluorobenzoate (5c)

Compound **5c** was prepared as the procedure of **5a**. Yield: 80.3%. ESI-MS (m/z): 252.0 $[M+1]^+$.

4.1.4. Methyl 5-fluoro-4′-methylbiphenyl-2-carboxylate (6a)

To a solution of **5a (2.00 g, 8.58 mmol),** Palladium acetate (0.008 g, 0.04 mmol), PPh_3 (0.47 g, 1.69 mmol) and p-tolylboronic acid (10 mL) was added 6 mL of 2 M K_2CO_3. The mixture was refluxed under nitrogen for 5 h, then diluted with ethyl acetate, and washed with water (50 mL × 3) and brine (50 mL × 3). The organic layer was dried over $MgSO_4$. The solvent was removed under reduced pressure. The residue was purified by flash column chromatography with petroleum ether–ethyl acetate (100/1) as eluant. The product was obtained as a yellow oil. Yield: 82.6%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.53(1H, dd, J_1 = 8.4 Hz, J_2 = 2.8 Hz, Ph-H), 7.35(1H,dd, J_1 = 8.4 Hz, J_2 = 5.6 Hz, Ph-H), 7.25–7.16(5H, m, Ph-H),

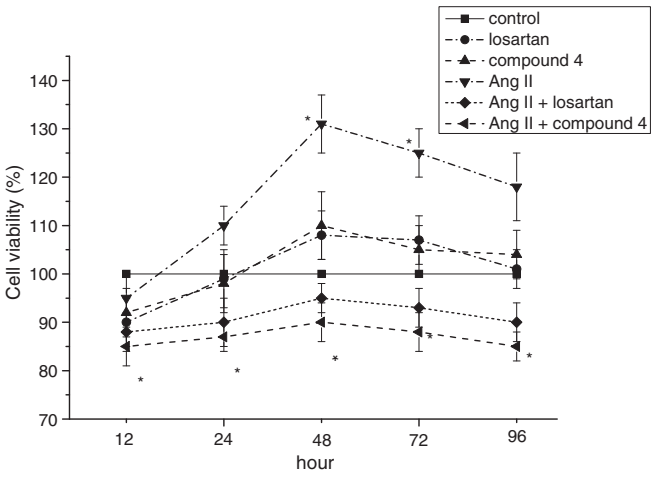


Figure 4. Cell viability effect of losartan and compound **4** in prostate carcinoma cell lines (LNCaP) and normal prostate cells measured by MTT. *Significant difference from the control, P <0.05.

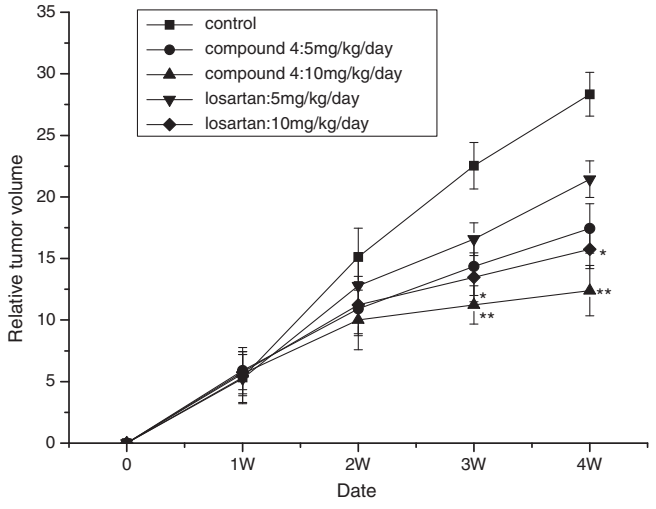


Figure 5. Antitumor activity of compound **4** and Losartan (5,10 mg/kg/day) in nude mice, tumor growth of LNCap xenografts was measured at indicated times. *,**Significant difference from the control, p <0.05 and p <0.01, respectively.

3.69(3H, s, Ph-COOCH_3)2.41(3H, s, Ph-CH_3). ESI-MS (m/z): 245.2 $[M+1]^+$.

4.1.5. Methyl 4-fluoro-4′-methylbiphenyl-2-carboxylate (6b)

Compound **6b** was prepared according to the procedure of **6a**. Yield: 82.6%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.86(1H, m, Ph-H), 7.21(4H, m, Ph-H), 7.13–7.08 (2H, m, Ph-H), 3.70(3H, s, Ph-COOCH_3), 3.43(3H, s, Ph-CH_3). ESI-MS (m/z): 245.2 $[M+1]^+$.

4.1.6. Methyl 4,5-difluoro-4′-methylbiphenyl-2-carboxylate (6c)

Compound **6a** was prepared according to the procedure of **6a**. Yield: 82.6%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.74(1H, dd, J_1 = 10.8 Hz, J_2 = 2.8 Hz, Ph-H), 7.28–7.18(5H, m, Ph-H), 3.73(3H, s, Ph-COOCH_3), 2.46(3H, s, Ph-CH_3). ESI-MS (m/z): 263.2 $[M+1]^+$.

4.1.7. Methyl 4′-(bromomethyl)-5-fluorobiphenyl-2-carboxylate (7a)

A solution of **6a (1.57 g, 6.43 mmol),** NBS 1.26 g, 7.08 mmol), AIBN (0.16 g, 0.97 mmol) and cyclohexane(30 mL) was refluxed for 3 h. The mixture was diluted with dichloromethane (30 mL), and washed with brine (30 mL × 3). The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography with petroleum ether–ethyl acetate (100/1) as eluant to afford a yellow oil. Yield: 67.8%. ^{1}H NMR($CDCl_3$, 400 MHz) δ: 7.53(1H, dd, J_1 = 8.4 Hz, J_2 = 2.8 Hz, Ph-H),7.44(2H, m, Ph-H), 7.32–7.15(4H, m, Ph-H), 4.56(2H, s, Ph-CH_2–Br), 3.69(3H, s, Ph-COOCH_3); ^{13}C NMR($CDCl_3$, 400 MHz) δ: 169.74, 165.26, 162.79, 142.88, 140.45, 139.27, 134.82, 134.61, 131.23, 128.66, 120.87, 120.68, 119.11, 54.54, 35.62. ESI-MS (m/z): 323.1 $[M+1]^+$.

4.1.8. Methyl 4′-(bromomethyl)-4-fluorobiphenyl-2-carboxylate (7b)

Compound **7b** was prepared as the procedure of **7a**. Yield: 67.8%. ^{1}H NMR($CDCl_3$, 400 MHz) δ: 7.92(1H, m, J = 6.0 Hz, Ph-H), 7.44(2H, d, J = 2.0 Hz, Ph-H), 7.27(2H, d, J = 2.0 Hz, Ph-H), 7.16–7.03(2H, m, Ph-H), 4.56(2H, s, Ph-CH_2–Br), 3.67(3H, s, Ph-COOCH_3). ESI-MS (m/z): 323.1 $[M+1]^+$.

4.1.9. Methyl 4′-(bromomethyl)-4,5-difluorobiphenyl-2-carboxylate (7c)

Compound **7c** was prepared according to the procedure of **7a**. Yield: 87.9%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.75(1H, dd, J_1 = 10.0 Hz, J_2 = 8.4 Hz, Ph-H), 7.44(2H, d, J = 2.0 Hz, Ph-H), 7.25(2H, dd, J_1 = 5.4 Hz, J_2 = 1.6 Hz, Ph-H), 7.16(2H, dd, J_1 = 10.4 Hz, J_2 = 7.6 Hz, Ph-H), 4.54(2H, s, Ph-CH_2–Br), 3.67(3H, s, Ph-COOCH_3). ESI-MS (m/z): 341.1 $[M+H]^+$.

4.1.10. Methyl 4′-((2-butyl-4-chloro-5-formyl-4,5-dihydro-1*H*-imidazol-1-yl)methyl)-5-fluorobiphenyl-2-carboxylate (8a)

To a solution of **7a** (2.262 g, 7 mmol), 2-butyl-4-chloro-1*H*-imidazole-5-carboxaldehyde (1.437 g, 7.7 mmol) and 25 mL of acetonitrile was added potassium carbonate (1.449 g, 10.5 mmol). The mixture was stirred at 50 °C under nitrogen for 6 h. The mixture was diluted with ethylacetate. The organic layer was washed with water (30 mL × 3) and brine (30 mL × 3), dried over $MgSO_4$. After filtration, the solvent was removed under reduced pressure. The residue was purified by flash column chromatography with petroleum ether–ethyl acetate (3/1) as eluant to afford a white solid. Yield: 54.5%. ^{1}H NMR($CDCl_3$, 400 MHz) δ: 9.94(1H, s, C-C*H*O),7.58(1H, dd, J_1 = 9.2 Hz, J_2 = 2.8 Hz, Ph-H), 7.32∼7.22(4H, m, Ph-H), 7.08(2H, d, J = 8.4 Hz, Ph-H), 5.23(2H, s, Ph-CH_2–N), 3.67(3H, s, Ph-COOCH_3), 2.67(2H, t, J = 8.0 Hz, CH_2CH$_2$CH$_2$CH$_3$), 1.71(2H, m, J = 8.0 Hz, CH$_2$CH_2CH$_2$CH$_3$), 1.37(2H, m, J = 8.0 Hz, CH$_2$CH$_2$CH_2CH$_3$),0.90(3H, t, J = 8.0 Hz, CH$_2$CH$_2$CH$_2$CH_3); ^{13}C NMR ($CDCl_3$, 400 MHz) δ: 182.97, 169.50, 165.32,162.85, 152.70, 143.07, 140.18, 136.69, 135.71, 134.85, 131.63, 128.31, 127.27, 120.87, 119.36, 54.50, 49.22, 32.01, 31.53, 29.86, 24.69, 15.99, 12.31; ESI-MS(m/z): 431.2 $[M+1]^+$.

4.1.11. Methyl4′-((2-butyl-4-chloro-5-formyl-4,5-dihydro-1*H*-imidazol-1-yl)methyl)-4-fluorobiphenyl-2-carboxylate (8b)

Compound **8b** was prepared as the procedure of **8a**. Yield: 34.1%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.92(1H, s, C-C*H*O), 7.91(1H, dd, J_1 = 8.4 Hz, J_2 = 5.6 Hz, Ph-H), 7.28(2H, t, J = 8.0 Hz, Ph-H), 7.11(3H, dd, J_1 = 11.2 Hz, J_2 = 2.4 Hz, Ph-H, Ph-H),7.00(1H, dd, J_1 = 9.2 Hz, J_2 = 2.4 Hz, Ph-H), 5.23(2H, s, Ph-CH_2–N), 3.64(3H, s, Ph-COOCH_3), 2.67(2H, t, J = 7.6 Hz, CH_2CH$_2$CH$_2$CH$_3$), 1.71(2H, m, J = 7.6 Hz, CH$_2$CH_2CH$_2$CH$_3$), 1.37(2H, m, J = 7.6 Hz, CH$_2$CH$_2$CH_2CH$_3$), 0.90(3H, t, J = 7.6 Hz, CH$_2$CH$_2$ CH$_2$CH_3). ESI-MS(m/z): 431.2 $[M+1]^+$.

4.1.12. Methyl 4′-((2-butyl-4-chloro-5-formyl-4,5-dihydro-1*H*-imidazol-1-yl)methyl)-4,5-difluorobiphenyl-2-carboxylate (8c)

Compound **8c** was prepared according to the procedure of **8a**. Yield: 43.2%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.90(1H, s, C-C*H*O), 7.72(1H, dd, J_1 = 10.4 Hz, J_2 = 8.0 Hz, Ph-H), 7.25(2H, t, J = 7.6 Hz, Ph-H), 7.12–7.06(3H, m, Ph-H), 5.23(2H, s, Ph-CH_2–N), 3.64(3H, s,

Ph-COOCH_3), 2.65(2H,t, J = 7.6 Hz, CH_2CH$_2$CH$_2$CH$_3$), 1.68(2H, m, J = 7.6 Hz, CH$_2$CH_2CH$_2$CH$_3$), 1.35(2H, m, J = 7.6 Hz, CH$_2$CH$_2$CH_2CH$_3$), 0.88(3H, t, J = 7.6 Hz, CH$_2$CH$_2$CH$_2$CH_3); ESI-MS(m/z): 449.2 [M+1]$^+$.

4.1.13. 2-Butyl-4-chloro-1-((5′-fluoro-2′-(methoxycarbonyl) biphenyl-4-yl)methyl)-1*H*-imidazole-5-carboxylic acid (9a)

To a solution of **8a** (0.368 g, 0.86 mmol) and *n*-butanol (10 mL) was added dropwise to a solution of sodium hypochlorite (0.670 g, 7.37 mmol), sodium dihydrogen phosphate (0.677 g, 5.65 mol) and distilled water (10 mL). The mixture was stirred for 10 h, and diluted with ethyl acetate. The organic layer was washed with water (30 mL × 3) and brine (30 mL × 3), dried over $MgSO_4$. The solvent was removed under reduced pressure. After recrystallization, a white solid was obtained. mp: 203–205 °C. Yield: 98.5%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.57(1H, dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz, Ph-H), 7.32–7.22(4H, m, Ph-H), 7.08(2H, d, J = 8.0 Hz, Ph-H), 5.23(2H, s, Ph-CH_2-N), 3.66(3H, s, Ph-COOCH_3), 2.73(2H, t, J = 8.0 Hz, CH_2CH$_2$CH$_2$CH$_3$), 1.65(2H, m, J = 8.0 Hz, CH$_2$CH_2CH$_2$CH$_3$), 1.36(2H, m, J = 8.0 Hz, CH$_2$CH$_2$CH_2CH$_3$), 0.86(3H, t, J = 8.0 Hz, CH$_2$CH$_2$CH$_2$CH_3); ^{13}C NMR($CDCl_3$, 400 MHz) δ : 182.11, 169.57, 165.30, 162.83, 151.39, 144.12, 142.97, 140.23, 135.88, 134.81, 134.42, 131.57, 128.29, 120.95, 120.43, 119.43,54.52, 49.56, 31.82, 29.63, 24.63, 15.99, 12.42; ESI-MS(m/z): 445.0 [M+1]$^+$.

4.1.14. 2-Butyl-4-chloro-1-((4′-fluoro-2′-(methoxycarbonyl) biphenyl-4-yl)methyl)-1*H*-imidazole-5-carboxylic acid (9b)

Compound **9b** was prepared according to the procedure of **9a**. mp: 194–196 °C. Yield: 96.0%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.91(1H, dd, J_1 = 8.4 Hz, J_2 = 5.6 Hz, Ph-H), 7.28(2H, t, J = 8.0 Hz,Ph-H), 7.11(3H, dd, J_1 = 11.2 Hz, J_2 = 2.4 Hz, Ph-H), 7.00(1H, dd, J_1 = 9.2 Hz, J_2 = 2.4 Hz, Ph-H),5.23(2H, s, Ph-CH_2–N), 3.64(3H, s, Ph-COOCH_3), 2.67(2H, t, J = 7.6 Hz, CH_2CH$_2$CH$_2$CH$_3$), 1.71(2H, m, J = 7.6 Hz, CH$_2$CH_2CH$_2$CH$_3$), 1.37(2H, m, J = 7.6 Hz, CH$_2$CH$_2$CH_2CH$_3$), 0.90(3H, t, J = 7.6 Hz, CH$_2$CH$_2$CH$_2$CH_3). ESI-MS(m/z): 445.0 [M+1]$^+$.

4.1.15. 2-Butyl-4-chloro-1-((4′,5′-difluoro-2′-(methoxycarbonyl) biphenyl-4-yl)methyl)-1*H*-imidazole-5-carboxylic acid (9c)

Compound **9c** was prepared according to the procedure of **9a**. mp: 208–211 °C. Yield: 98.2%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.75(dd, 1H, J_1 = 10.4 Hz, J_2 = 8.0 Hz, Ph-H), 7.27(2H, t, J = 8.0 Hz, Ph-H), 7.15∼7.07(3H, m, Ph-H), 5.23(2H, s, Ph-CH_2–N), 3.66(3H, s, Ph-COOCH_3), 2.71(2H,t, J = 7.2 Hz, CH_2CH$_2$CH$_2$CH$_3$), 1.67(2H, m, J = 7.2 Hz, CH$_2$CH_2CH$_2$CH$_3$), 1.36(2H, m, J = 7.2 Hz, CH$_2$CH$_2$CH_2CH$_3$), 0.91(3H, t, J = 7.2 Hz, CH$_2$CH$_2$CH$_2$CH_3); ESI-MS(m/z): 463.0 [M+1]$^+$.

4.1.16. 2-Butyl-1-((2′-carboxy-5′-fluorobiphenyl-4-yl)methyl)-4-chloro-1*H*-imidazole-5-carboxylic acid (1a)

A solution of **9a** (49 mg, 0.11 mmol) and 10 mL of 2 M NaOH in methanol (10 mL) was refluxed for 5 h. The mixture was diluted with ethyl acetate, and washed with water (50 mL × 3) and brine(50 mL × 3), dried over $MgSO_4$. The solvent was removed under reduced pressure. The residue was recrystallized with petroleum ether and ethyl acetate to give a white solid. mp: 208–211 °C. Yield: 95.5%. Anal. Calcd for $C_{22}H_{20}ClFN_2O_4$: C, 61.33; H, 4.68; N, 6.50. Found: C, 61.35; H, 4.70; N, 6.47. ^{1}H NMR (DMSO, 400 MHz) δ: 7.49(1H, dd, J_1 = 9.2 Hz, J_2 = 1.6 Hz, Ph-H), 7.45–7.36(2H, m, Ph-H), 7.32(2H, d, J = 8.0 Hz, Ph-H), 7.05(2H, d, J = 12.8 Hz, Ph-H), 5.30(2H, s, Ph-CH_2-N), 2.63(2H, t, J = 7.6 Hz, CH_2CH$_2$CH$_2$CH$_3$), 1.55(2H, m, J = 7.6 Hz, CH$_2$CH_2CH$_2$CH$_3$), 1.28(2H, m, J = 7.6 Hz, CH$_2$CH$_2$CH_2CH$_3$), 0.82(3H, t, J = 7.6 Hz, CH$_2$CH$_2$CH$_2$CH_3); ^{13}C NMR (DMSO, 400 MHz) δ: 170.92, 165.44, 164.99, 162.55, 151.21, 142.10, 139.52, 137.52, 136.93, 135.45, 131.67, 129.37, 128.76, 125.19, 120.41, 118.63, 118.40,49.14, 31.47, 29.25, 24.41, 16.35; ESI-MS(m/z): 431.1 [M+1]$^+$.

4.1.17. 2-Butyl-1-((2′-carboxy-4′-fluorobiphenyl-4-yl)methyl)-4-chloro-1*H*-imidazole-5-carboxylic acid (1b)

Compound **1b** was prepared as the procedure of **1a**. mp: 196–199 °C Yield: 95.3%. Anal. Calcd for $C_{22}H_{20}ClFN_2O_4$: C, 61.33; H, 4.68; N, 6.50. Found: C, 61.30; H, 4.69; N, 6.52. ^{1}H NMR (DMSO, 400 MHz,) δ: 12.68(s, 1H, COO*H*), 7.81(1H, dd, J_1 = 8.8 Hz, J_2 = 6.0 Hz, Ph-H), 7.34(2H, d, J = 8.0 Hz, Ph-H), 7.30–7.26(1H, m, Ph-H), 7.19(1H, dd, J_1 = 9.6 Hz, J_2 = 2.4 Hz, Ph-H), 7.09(2H, d, J = 8.0 Hz, Ph-H), 5.31(2H, s, Ph-CH_2-N), 2.62(2H, t, J = 7.6 Hz, CH_2CH$_2$CH$_2$CH$_3$), 1.55(2H, m, J = 7.6 Hz, CH$_2$CH_2CH$_2$CH$_3$), 1.28(2H, m, J = 7.6 Hz, CH$_2$CH$_2$CH_2CH$_3$), 0.82(3H, t, J = 7.6 Hz, CH$_2$CH$_2$CH$_2$CH$_3$); ^{13}C NMR (DMSO, 400 MHz) δ: 170.93, 165.43, 164.98, 162.54, 151.20, 142.10, 139.54, 137.52, 136.96, 133.44, 131.67, 129.37, 128.75, 125.19, 120.61, 118.63, 49.13, 42.87, 31.46, 39.25, 24.40, 16.35; ESI-MS(m/z): 431.1 [M+1]$^+$.

4.1.18. 2-Butyl-1-((2′-carboxy-4′,5′-difluorobiphenyl-4-yl) methyl)-4-chloro-1*H*-imidazole-5-carboxylic acid (1c)

Compound **1c** was prepared according to procedure of **1a**. Yield: 95.3%. mp: 218–221 °C. Anal. Calcd for $C_{22}H_{19}ClF_2N_2O_4$: C, 58.87; H, 4.27; N, 6.24. Found: C, 58.88; H, 4.29; N, 6.25. ^{1}H NMR (DMSO, 400 MHz) δ: 12.81(1H, s, COO*H*), 7.78(1H, dd, J_1 = 10.8 Hz, J_2 = 8.0 Hz, Ph-H), 7.45(1H, dd, J_1 = 11.2 Hz, J_2 = 7.6 Hz, Ph-H), 7.32(2H, d, J = 8.0 Hz, Ph-H), 7.07(2H, d, J =8.0 Hz, Ph-H), 5.30(2H, s, Ph-CH_2-N), 2.61(2H, t, J = 7.6 Hz, CH_2CH$_2$CH$_2$CH$_3$), 1.55(2H, m, J = 7.6 Hz, CH$_2$CH_2CH$_2$CH$_3$), 1.27(2H, m, J = 7.6 Hz, CH$_2$CH$_2$CH_2CH$_3$), 0.82(3H, t, J = 7.6 Hz, CH$_2$CH$_2$CH$_2$CH_3); ESI-MS(m/z): 449.1 [M+1]$^+$.

4.1.19. Methyl 4′-((2-butyl-4-chloro-5-(hydroxymethyl)-1*H*–imidazol-1-yl)methyl)–4-fluorobiphenyl–2-carboxylate (10)

To a solution of **8b** (661 mg, 1.54 mmol) in methanol (30 mL) was added $NaBH_4$ at 0 °C. The mixture was stirred at room temperature for 1 h. The reaction mixture was poured into ice water and the organic layers were washed with brine (30 mL × 3), dried with $MgSO_4$ and evaporated. The residue was recrystallized with ethyl acetate-petroleum ether to give a white solid. Yield: 94.6%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.55(1H, dd, J_1 = 9.2 Hz, J_2 = 2.8 Hz, Ph-H), 7.32–7.21(4H, m, Ph-H), 7.08(2H, d, J = 8.0 Hz, Ph-H), 5.14(2H, s, Ph-CH_2-N), 4.62(2H, s,CH_2OH), 3.65(3H, s,–OCH_3), 2.63(2H, t, J = 7.6 Hz,CH_2CH$_2$CH$_2$CH$_3$), 1.63(2H, m, J = 7.6 Hz, CH$_2$CH_2CH$_2$CH$_3$), 1.33(2H,m, J = 7.6 Hz, CH$_2$CH$_2$CH_2CH$_3$), 0.88(3H, t, J = 7.6 Hz, CH$_2$CH$_2$CH$_2$CH_3); ^{13}C NMR (400 MHz,$CDCl_3$) δ: 169.67, 150.58, 142.50, 140.36, 140.33, 137.50, 137.09, 134.86, 134.80, 134.49, 134.42, 131.35, 128.34, 120.88, 120.67, 119.11, 161.57, 58.49, 49.17, 32.08, 29.82, 24.72, 16.04; ESI-MS(m/z): 431.3 [M+H]$^+$.

4.1.20. 4′-((2-Butyl-4-chloro-5-hydroxy-1*H*-imidazol-1-yl)methyl)-4-fluorobiphenyl-2-carboxylic acid (2)

A solution of **10** (998 mg, 2.24 mmol) and 15 mL of 2 M NaOH in methanol (30 mL) was refluxed for 6 h. The solvent was removed in vacuo. The mixture was extracted with ethylacetate. The organic layer was washed with water (30 mL × 3) and brine (30 mL×3), dried over $MgSO_4$. The solvent was removed under reduced pressure. The crude product was recrystallized with petroleum ether and ethyl acetate to give a white solid. mp: 206-209 °C. Yield: 41.6%. Anal. Calcd for $C_{22}H_{22}ClFN_2O_3$: C, 63.39; H, 5.32; N, 6.72. Found: C, 63.36; H, 5.33; N, 6.74. ^{1}H NMR ($CDCl_3$, 400 MHz,) δ: 13.40(s, 2H, COO*H*), 7.53(1H, dd, J_1 = 12.76 Hz, J_2 = 7.88 Hz,Ph-H), 7.39(2H, d, J = 8.04 Hz, Ph-H), 7.31(1H, t, J = 8.96 Hz, Ph-H), 7.23(1H, d, J = 7.68 Hz, Ph-H), 7.07(d, J = 8.04 Hz, Ph-H), 5.62(2H, s, Ph-CH_2), 2.60(2H, t, J = 7.52 Hz, CH_2CH$_2$CH$_2$CH$_3$), 1.52(2H, m, J = 7.64 Hz, CH$_2$CH_2CH$_2$CH$_3$), 1.25(2H, m, J = 7.64 Hz, CH$_2$CH$_2$CH_2 CH$_3$), 0.80(3H, t, J = 7.64 Hz, CH$_2$CH$_2$CH$_2$CH_3); ^{13}C NMR (DMSO, 400 MHz) δ: 170.93, 165.43, 162.54, 151.21, 142.10, 139.54, 137.52, 136.89, 135.45, 131.67, 129.37, 125.19, 120.61, 120.41,

118.63, 49.13, 42.66, 41.83, 31.47, 29.25, 24.41, 16.35; ESI-MS(m/z): 417.1 $[M+H]^+$.

4.1.21. 4-Fluoro-4′-methylbiphenyl-2-carboxylic acid (11)

A solution of **6a** (2.687 g, 11 mmol) and 16 mL of 2 M NaOH in methanol (30 mL) was refluxed for 6 h. The solvent was removed in vacuo. The residue was added with H_2O and the aqueous layer was washed with diethyl ether and acidified with 1 M HCl to pH = 3. The resulting precipitate was collected by filtration and dissolved in EtOAc and dried with $MgSO_4$. The solvent was removed under reduced pressure and the residue was recrystallised with EtOAc–petroleum ether. The product was obtained as white solid. mp: 154–157 °C. Yield: 66.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.72–7.67 (3H, m, Ph-H), 7.38–7.22(4H, m, Ph-H), 2.42(3H, s, Ph-CH_3); ESI-MS (m/z): 231.0 $[M+1]^+$.

4.1.22. Tert-butyl 4-fluoro-4′-methylbiphenyl-2-carboxylate (12)

To a solution of **11** (1.884 g, 8.19 mmol) in dichloromethane was added oxalyl chloride (0.86 mL,10 mmol) at 0 °C. After addition, the reaction mixture was warmed to 25 °C and stirred for 3 h. The excess oxalyl chloride was removed under reduced pressure. Then potassium tert-butanolate (0.917 g, 8.19 mmol) was added into the above residue in THF at 0 °C in portions. The reaction mixture was stirred at room temperature for 1 h. The mixture was poured into ice water and extracted with EtOAc .The combined organic layers were washed with brine (10 mL × 3), dried with $MgSO_4$. After filtration, the solvent was removed under reduced pressure. The residue was purified by flash column chromatography with petroleum ether-ethyl acetate (125/1) as eluant. The product was obtained as colourless oil. Yield: 44.2%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.49(1H, dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz, Ph-H), 7.32(1H, dd, J_1 = 8.4 Hz, J_2 = 3.2 Hz, Ph-H), 7.16∼7.27 (5H, m, Ph-H), 2.46(3H, s, Ph-CH_3), 1.32(9H, s, Ph-COOC$_4H_9$); ESI-MS(m/z): 287.3 $[M+1]^+$

4.1.23. Tert-butyl 4′-(bromomethyl)-4-fluorobiphenyl-2-carboxylate (13)

Compound **13** was prepared according to the procedure of **7a**. Yield: 65.7%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.52(1H, dd, J_1 = 9.2 Hz, J_2 = 2.8 Hz, Ph-H),7.43(2H, d, J = 4.4 Hz, Ph-H), 7.31–7.22(3H, m,Ph-H), 7.22–7.15(1H, m, Ph-H), 4.57(2H, s, Ph-CH_2–Br), 1.25(3H, s, Ph-COOC$_4H_9$); ESI-MS(m/z): 365.2 $[M+1]^+$.

4.1.24. Tert-butyl4′-((2-butyl-4-oxo-1,3-diazaspiro[4.4]non-1-en-3-yl)methyl)-4-fluorobiphenyl-2-carboxylate (14)

A solution of 2-butyl-4-spirocyclopentane-2-imidazolin-5-one hydrochloride (0.580 g, 2.63 mmol) and sodium hydride (0.250 g, 6.25 mmol) in THF (30 mL) under nitrogen was stirred for 0.5 h at 50 °C. After cooling, a solution of **13** (0.804 g, 2.2 mmol) in the THF (10 mL) was added dropwise. The resulting mixture was stirred at 50 °C for 3 h. The mixture was poured into 30 mL of ice-water and extracted with EtOAc. The organic layers were washed with brine (10 mL × 3), dried with $MgSO_4$. After filtration, the solvent was removed under reduced pressure. The residue was purified by flash column chromatography with petroleum ether–ethyl acetate (3/1) as eluant. A white solid 330 mg was obtained. Yield: 30.6%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.47(1H, dd, J_1 = 9.2 Hz, J_2 = 2.8 Hz, Ph-H), 7.27∼7.14 (6H, m, Ph-H), 4.72(2H, s, Ph-CH_2–Br), 2.35(2H, t, J = 7.6 Hz, N═CCH_2CH$_2$CH$_2$CH$_3$), 2.05–1.92(8H, m, CH$_2$CH$_2$CH$_2$CH$_2$), 1.61(2H, m, J = 7.6 Hz, N═CCH$_2$CH_2CH$_2$CH$_3$), 1.35(2H, m, J = 7.6 Hz, N═CCH$_2$CH$_2$CH_2CH$_3$), 1.25(3H, s, Ph-COOC$_4H_9$), 0.89(3H, t, J = 7.6 Hz, N═CCH$_2$CH$_2$ CH$_2$CH_3); ESI-MS(m/z): 479.2 $[M+1]^+$.

4.1.25. 4′-((2-Butyl-4-oxo-1,3-diazaspiro[4.4]non-1-en-3-yl)methyl)-4-fluorobiphenyl-2-carboxylic acid (3)

A solution of **14**(0.3 g, 0.627 mmol), TFA (2 mL) and dichloromethane (2 mL) was stirred for 2 h at 25 °C. The solvent was removed in vacuo. The residue was triturated with diethyl ether and filtered to afford a white powder. mp: 214–217 °C. Yield: 99.3%. Anal. Calcd for $C_{25}H_{27}FN_2O_3$: C, 71.07; H, 6.44; N, 6.63. Found: C, 71.24; H, 6.46; N, 6.60. ^{1}H NMR (DMSO, 400 MHz) δ: 9.08(1H, s, Ph-COO*H*), 7.66(1H, dd, J_1 = 8.8 Hz, J_2 = 2.8 Hz, Ph-H), 7.33–7.23(6H, m, Ph-H), 4.81(2H, s, Ph-CH_2–Br), 2.67(2H, t, J = 7.6 Hz, N═CCH_2CH$_2$CH$_2$CH$_3$), 2.15–1.99(6H, m, CH$_2$CH$_2$CH$_2$CH$_2$), 1.56(2H, m, J = 7.6 Hz, N═CCH$_2$CH_2CH$_2$CH$_3$), 1.29(2H, m, J = 7.6 Hz, N═CCH$_2$CH$_2$CH_2CH$_3$), 0.86(3H, t, J = 7.6 Hz, N═CCH$_2$CH$_2$CH$_2$CH_3); ^{13}C NMR (DMSO, 400 MHz) δ: 170.91, 170.89, 164.99, 162.55, 161.23, 142.10, 139.59, 139.56, 137.47, 136.92, 136.85, 135.38, 131.63, 129.33, 120.66, 120.45, 118.67, 118.44, 76.09, 45.82, 30.08, 29.12, 28.08, 24.24, 16.21; ESI-MS(m/z): 423.1 $[M+1]^+$.

4.1.26. (*S*)-Methyl 4-fluoro-4′-((1-methoxy-3-methyl-1-oxobutan-2-ylamino)-methyl)-biphenyl-2-carboxylate (15)

A solution of **7b** (1.844 g, 11 mmol), L-Valine methyl ester hydrochloride (2.9 g, 10 mmol), DIPEA (1.939 g, 15 mmol) and dichloromethane (40 mL) was refluxed for 10 h under nitrogen. The solvent was removed in vacuo. The residue was diluted with H_2O and extracted with ethyl acetate. The organic layer was washed with water (10 mL×3) and brine (10 mL × 3), dried over $MgSO_4$. The solvent was removed under reduced pressure. The residue was purified by flash column chromatography with petroleum ether–ethyl acetate (25/1) as eluant. Yield: 53.5%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.53(1H, dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz, Ph-H), 7.39–7.33(3H, m, Ph-H), 7.25–7.21(3H, m, Ph-H), 3.89(1H, d, J = 13.2 Hz, Ph-CH_2–N), 3.85(1H, d, J_2 = 13.2 Hz, Ph-CH_2–N),3.75(3H, s, O═C–O–CH_3), 3.67(3H, s, Ph-C═O–OCH_3), 3.06(1H, d, J = 6.0 Hz, HN–C*H*(C═O)CH(CH$_3$)$_2$), 1.96(1H, m, J = 6.8 Hz, HN–CH(C═O)C*H*(CH$_3$)$_2$), 1.84(1H,s, *H*N–CH(C═O)CH(CH$_3$)$_2$), 0.98(6H, dd, J_1 = 17.6 Hz, J_2 = 10.4 Hz, HN–CH(C═O)CH(CH_3)$_2$); ESI-MS(m/z): 374.2 $[M+1]^+$.

4.1.27. (*S*)-Methyl 4-fluoro-4′-((*N*-(1-methoxy-3-methyl-1-oxobutan-2-yl)butyramido)methyl)biphenyl-2-carboxylate (16)

To a solution of **15** (1.935 g, 5.3 mmol), DIPEA (1 mL, 15.7 mmol) and dichloromethane (15 mL) was added dropwise *n*-butyryl chloride(0.81 mL, 7.8 mmol) at 0 °C. The mixture was stirred for 6 h at 28 °C. The resulting mixture was poured into ice water, the organic layer was washed with diluted hydrochloric acid, sodium bicarbonate solution (15 mL × 3) and brine(30 mL × 3), dried by $MgSO_4$, and concentrated in vacuo. The product was purified by chromatography with petroleum ether–ethyl acetate (15/1) as eluant. The product was obtained as colorless oil. Yield: 76.2%. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.40–7.29 (7H, m, Ph-H), 4.97–4.66 (1H, two d, J_1 = 15.2 Hz, J_2 = 17.6 Hz, –NCH_2–), 4.90(1H, two d, J = 10.4 Hz, –N–C*H*–), 4.60–4.25 (2H, two d, J_1 = 15.2 Hz, J_2 = 17.6 Hz, –NCH_2–), 3.60–3.50 (3H, two s, -OCH_3), 3.39–3.34 (3H, two s, –PhCOOCH_3), 2.34–2.19 (3H, m, –CH_2CH$_2$CH$_3$ and –C*H*(CH$_3$)$_2$), 1.79–1.61 (2H, m, –CH$_2$CH_2CH$_3$), 0.99–0.72 (9H, m). ESI-MS (m/z): 459.2 $[M+1]^+$.

4.1.28. (*S*)-4′-((*N*-(1-Carboxy-2-methylpropyl)butyramido)methyl)-4-fluorobiphenyl–2-carboxylic acid (4)

Compound **4** was prepared according to the procedure of **3**. Mp: 215–218 °C. Yield: 99.5%. Anal. Calcd for $C_{23}H_{26}FNO_5$: C, 66.49; H, 6.31; N, 3.37. Found: C, 66.48; H, 6.30; N, 3.39. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.56(1H, dd, J_1 = 8.0 Hz, J_2 = 2.4 Hz, Ph-H), 7.28–7.10

(6H, m, Ph-H), 4.94–4.74 (2H, two d, J_1 = 16.0 Hz, J_2 = 17.6 Hz, –NCH_2–), 4.54–4.31 (2H, two d, J_1 = 15.2 Hz, J_2 = 17.6 Hz, –NCH_2–), 4.30–4.03 (1H, two d, J = 10.4 Hz, –N–CH –), 2.63–2.32 (3H, m, –CH_2CH$_2$CH$_3$ and–CH(CH$_3$)$_2$), 1.76–1.61(2H, m, –CH$_2$CH_2CH$_3$), 0.98–0.86(3H, t, –CH$_2$CH$_2$CH_3); 0.84–0.80(6H, d, –CH(CH_3)$_2$). ^{13}C NMR (DMSO, 400 MHz) δ: 178.68, 175.82, 172.48, 165.09, 162.62, 142.06, 140.93, 140.88, 139.16, 137.50, 134.91, 134.22, 130.59, 129.57, 121.06, 119.64, 68.56, 53.61, 38.00, 29.84, 22.20, 21.11, 16.11; ESI-MS(m/z): 416.2 $[M+1]^+$.

4.2. Radioligand binding assay

The vascular smooth muscle cells (VSMCs) were obtained from thoracic aorta of SD rats and cultured by the tissue explants methods.[14] One section of aorta was removed and placed in Dulbecco's Modified Eagle's Medium (DMEM). Adherent fat and connective tissue were gently removed with fine sterile forceps. The aorta was minced into small cube-shaped specimens and digested with collagenase for 1 h, at 37 °C. The homogenate was centrifuged at 10000×g for 5 min. They were then incubated with 1 mL of DMEM supplemented with 15% fetal bovine serum (FBS) at 37 °C in 95% air 5% CO_2. Cells at passage 3–7 were used for the experiments.

Each 250 μL incubate contained the following: 0.1 nM $^{[125]}$Angiotensin II (Northern Biotechnology Company, China) and concentration of test compounds. The final concentrations were 1×10^{-6}–1×10^{-12} M. They were incubated in 24-well plates with VSMCs for 60 min at 37 °C. Nonspecific binding was measured in the presence of 1 μM Ang II and represented 5–10% of total binding. After the reaction, removed the liquid immediately, washed five times with PBS, and digested cells with 0.1 M NaOH for 10 min. The radioactivity was counted with a γ-counter (Wallac 1470 Wizard, PerkinElmer, Finland). The IC_{50} value (concentration for 50% displacement of the specifically bound $^{[125]}$Angiotensin II) was determined by regression analysis of displacement curves.[15] The inhibition constant (K_i value) was calculated from the formula $K_i = IC_{50}/(1 + [L]/k_d)$,[16] where [$L$] was the concentration of radioligand present in tubes.

4.3. In vivo study of anti-hypertensive effect

For preparing renal hypertensive rats, the left renal arteries of Sprague-Dawley rats (250–350 g, Second Military Medical University, China) were completely ligated under sodium pentobarbital (40 mg/kg) anesthesia. Thereafter, the rats with SBP higher than 160 mmHg were selected and used as renal hypertensive rats. Spontaneous hypertensive rats (250–300 g) came from Second Military Medical University, China too. Than both spontaneous and renal hypertensive rats were randomly divided into different experimental groups of 10 animals (negative control group, positive control groups, compound low-dose groups and high-dose groups). Each compound was suspended in a 0.5% solution of sodium carboxymethyl cellulose and administered orally at the dose of 5 and 10 mg/kg separately. Losartan (10 mg/kg) and Valsartan (10 mg/kg) (SanXin Zhujiang Chemical Engineering Company) were taken as positive control groups. The negative control group was administered the same volume of sodium carboxymethyl cellulose solution. To measure the blood pressure, the animals were anesthetized with sodium pentobarbital (40 mg/kg, ip). The right carotid artery and jugular vein were cannulated for arterial pressure measurement and drug administration, respectively. The arterial catheter was connected to a pressure transducer and displayed on a computer and analyzed with a biological signal analysis system (MPA-2000, Alcott Biotech, China). The parameters were measured continuously for more than 10 h, and again at 24 h.[17]

4.4. In vitro study of anticancer activity

The MTT assays were performed as previously described.[18] Briefly, cells (1×10^5 cells) were seeded in 96-well culture plates and cultured overnight at 37 °C, 5% CO_2 atmosphere. LNCaP cells were incubated with Ang II (1×10^{-7} M), Ang II (1×10^{-7} M) + Losartan (10 μM), Ang II (1×10^{-7} M) + compound **4** (10 μM), Losartan (10 μM), compound **4** (10 μM), respectively. The control group received drug-free medium with 0.05% v/v DMSO. Subsequently, MTT (5 mg/mL) was added to each well after 24, 48, 72 and 96 h separately. After incubation at 37 °C for 4 h, the medium was discarded and 150 μL DMSO was added into each well. The optical density was measured by a microplate reader at a test wavelength of 570 nm.

4.5. In vivo study of antitumor activity

The antitumor activity of compound **4** was determined in athymic nude mice bearing LNCap tumors. LNCap cells (10^7) were injected into the flank region of athymic nude mice (4–6 weeks old), and treatment was started on day 8 when the tumor measured 5 mm in diameter.[12] Each mouse was received one of two different doses of compound **4** (5.0 or 10.0 mg/kg/day) and Losartan (5.0 or 10.0 mg/kg/day) was taken as positive group. The control group was received water containing sodium hypochlorite (10 ppm). Each group consisted of 10 animals. Tumors were measured with a caliper every 7 days. The volume of the tumor was calculated using the formula: tumor volume (mm^3) = length × (width) ^{2} × 0.5.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.09.065.

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