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Design, synthesis, and structure–activity relationships of dihydrofuran-2-one and dihydropyrrol-2-one derivatives as novel benzoxazin-3-one-based mineralocorticoid receptor antagonists

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ABSTRACT

Dihydrofuran-2-one and dihydropyrrol-2-one derivatives were identified as novel, potent and selective mineralocorticoid receptor (MR) antagonists by the structure-based drug design approach utilizing the crystal structure of MR/compound complex. Introduction of lipophilic substituents directed toward the unfilled spaces of the MR and identification of a new scaffold, dihydropyrrol-2-one ring, led to potent in vitro activity. Among the synthesized compounds, dihydropyrrol-2-one **11i** showed an excellent in vitro activity (MR binding IC_{50} = 43 nM) and high selectivity over closely related steroid receptors such as the androgen receptor (AR), progesterone receptor (PR) and glucocorticoid receptor (GR) (>200-fold for AR and PR, 100-fold for GR).

1. Introduction

The mineralocorticoid receptor (MR) is a member of the steroid receptor sub-family within the nuclear hormone receptor superfamily, and it plays an important role in blood pressure control through the regulation of body fluid and electrolyte balance.[1] Abnormal activation of the MR by excessive levels of aldosterone, a primary natural ligand for the MR, is known to cause cardiovascular diseases such as hypertension and congestive heart failure.[2] Accordingly, MR blockade is an attractive therapeutic option for such diseases. Indeed, available steroidal MR antagonists, spironolactone[3] and eplerenone,[4] have proven to be clinically useful for those diseases.[5] However, spironolactone treatment is limited by the sex hormone-related side effect such as impotence, gynecomastia, and menstrual irregularity caused by the lack of selectivity over androgen receptor (AR) and progesterone receptor (PR).[6] Meanwhile, the more selective antagonist, eplerenone, shows few side effects but its potency is lower than spironolactone.[7] Accordingly, a new, potent and selective MR antagonist would be a clinically useful drug. Recently, several nonsteroidal MR antagonists were disclosed,[8] but their clinical utilities have not been proven.

We have already reported benzoxazin-3-one derivatives as novel nonsteroidal MR antagonists.[9] Among them, 3-trifluoromethyl pyrazole **1** showed highly potent in vitro activity (MR binding IC_{50} = 41 nM) and good selectivity over the AR, PR, and glucocorticoid receptor (GR) (Fig. 1). While compound **1** showed little affinity for the AR, it still had moderate binding activity for the PR (AR binding IC_{50} >10,000 nM, PR binding IC_{50} = 1900 nM), suggesting that we should seek to reduce PR binding to minimize the potential for sex hormone-related side effects. Thus, we aimed to identify compounds with potent MR binding affinities with little affinities for the AR and PR.

Initially, we explored a new lead compound (Fig. 2). Our previous study of benzoxazin-3-one derivatives suggested that a key pharmacophore for a potent MR binding affinity consisted of three parts: a benzoxazin-3-one ring, a 4-fluorobenzene ring, and a central cycle linking these two aromatic rings at the adjacent position.[9] Among these parts, the central ring moiety tended to allow the easiest introduction of structural diversity. In addition to monocyclic aromatic rings, fused-heterocycles such as triazolothiadiazines and benzothiazines also exhibited potent MR antagonistic activity as central rings. Importantly, in the course of our previous research, we found the modification of central ring moiety affected not only MR binding affinity but also steroid receptor selectivity. Therefore, our early efforts were focused on the identification of new lead scaffolds other than 3-trifluoromethylpyrazole.

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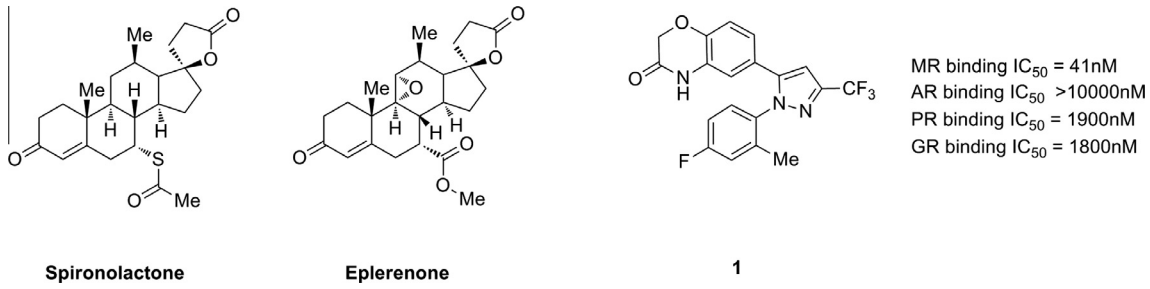


Figure 1. Structure of steroidal and nonsteroidal MR antagonists

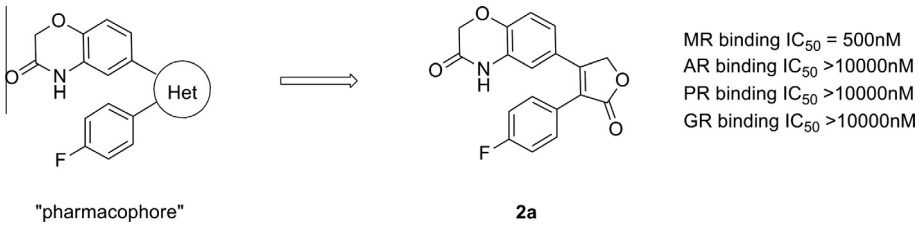


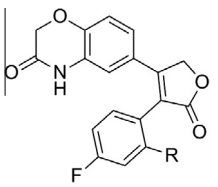
Figure 2. Identification of new lead compound **2a**.

Since non-aromatic heteromonocycles had still been poorly-explored, a variety of five- or six-membered non-aromatic heterocycles were investigated. As a result, we selected the dihydrofuran-2-one derivative **2a** as a lead compound, because it showed moderate MR binding activity (IC_{50} = 500 nM) with low activity for AR, PR and GR (IC_{50} >10,000 nM, for each receptor), and had a 'leadlike' profile, such as low molecular weight and low lipophilicity. In this paper, we describe the lead optimization of **2a** by a structure-based drug design approach utilizing the X-ray co-crystal structure of the MR/compound complex obtained in-house.

To establish the initial SAR, the 4-fluorobenzene ring of **2a** was modified (Table 1). In compound **1**, introduction of a methyl group at the 2-position on the 4-fluorobenzene ring significantly enhanced the binding affinity, probably due to the stabilization effect of the preferred twisted conformation between the central ring and the 4-fluorobenzene ring.[9] Therefore, a fluorine atom or methyl group was introduced into the corresponding position of the lead compound **2a**. However, these compounds (**2b**, **c**) showed no advantage in terms of affinity and selectivity. From this result, we speculated that the carbonyl group of dihydrofuran-2-one ring has already stabilized the twisted conformation and thereby the synergistic effect with the fluorine atom or the methyl group at the 2-position was not observed. Other substituted benzene rings were also investigated; however the 4-fluorobenzene was best among them (data not shown). Accordingly, the 4-fluorobenzene ring was fixed during the following lead optimization process.

The X-ray co-crystal structure of the MR/compound **2b** complex was utilized for compound design (Fig. 3a). As expected, compound **2b** bound to the ligand binding domain of MR in a similar manner to compound **1**. Characteristically, the carbonyl group in the dihydrofuran-2-one scaffold formed a hydrogen bond with Arg817 and Gln776 via two water molecules, and the carbonyl group of the benzoxazin-3-one moiety newly established an additional hydrogen bond interaction with Thr945. These interactions were considered to be important for the binding affinity of dihydrofuran-2-one derivatives. Moreover, Figure 3a revealed that compound **2b** did not fully occupy the ligand binding site of the MR, suggesting that space for lead optimization still remained. On the basis of this finding, we hypothesized that the MR binding affinity could be increased by the introduction of a lipophilic group to fill spaces 'A', 'B' and/or 'C' shown in Figure 3a, or by the introduction of a polar

Table 1
Effect of introduction of substituents into the 4-fluorobenzene ring[a]



Compound No.	R	MR binding IC_{50} (nM)	Selectivity		
			AR binding IC_{50} (nM)	PR binding IC_{50} (nM)	GR binding IC_{50} (nM)
2a	H	500	>10,000	>10,000	>10,000
2b	F	660	>10,000	4600	>10,000
2c	Me	750	>10,000	1800	>10,000

[a] IC_{50} values were shown as the means of duplicate experiments. IC_{50} values are calculated from the concentration–response curves generated by GraphPad Prism.

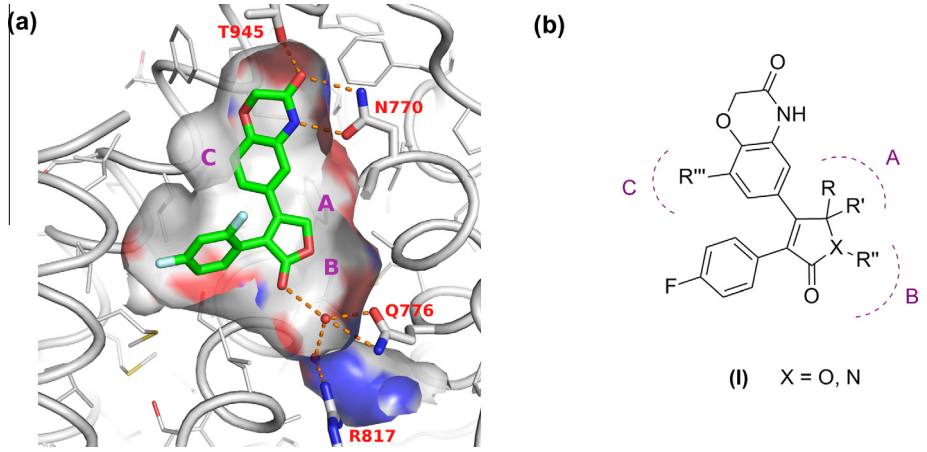


Figure 3. (a) X-ray co-crystal structure of the MR/compound **2b** complex. Orange dot means hydrogen bond. 'A', 'B' and 'C' means the unfilled spaces available for lead optimization. (b) Compound design for potent MR binding affinity.

group at space 'B' to form a hydrogen bond with Arg817 or Gln776, directly or via water molecule. Thus, the substituted dihydrofuran-2-one and dihydropyrrol-2-one derivatives, shown as compound (**I**), were designed (Fig. 3b).

2. Chemistry

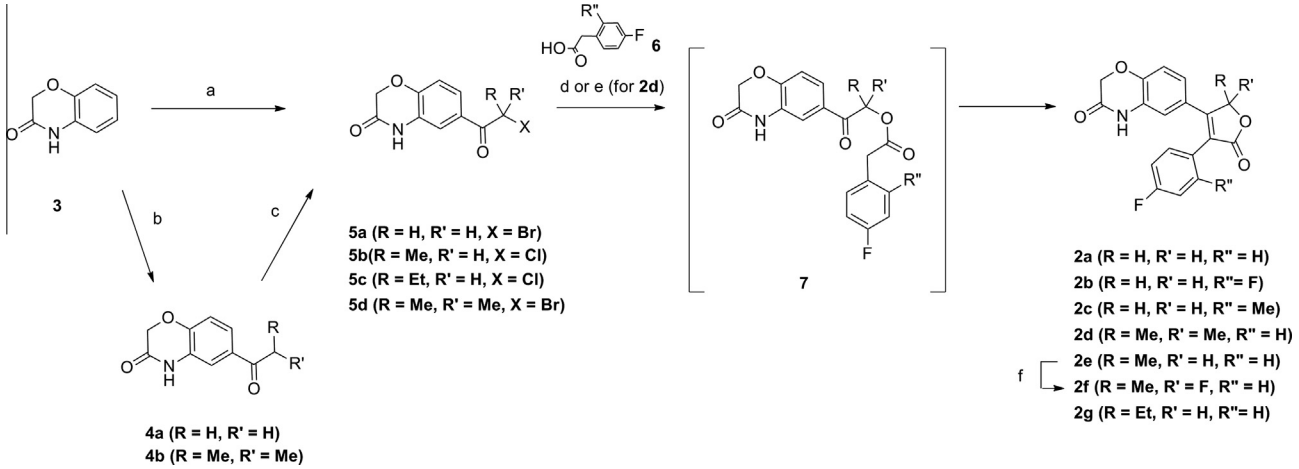
Dihydrofuran-2-ones **2a**–**g** were prepared according to Scheme 1. Condensation of the α-haloketone **5** with the carboxylic acid **6** followed by base-mediated cyclization–dehydration afforded the dihydrofuran-2-ones **2a**–**e**, **g**. Electrophilic fluorination of **2e** with *N*-fluorobenzenesulfonimide gave the 5-fluoro compound **2f**.

The dihydropyrrol-2-ones **11a**–**i** were synthesized as shown in Scheme 2. Condensation of the α-haloketone **5** or **8** with primary amines followed by acylation with 4-fluorophenylacetyl chloride gave the amides **10a**–**f**. The subsequent base-mediated cyclization-dehydration afforded the dihydropyrrol-2-ones **11a**–**d**, **f**, **h**. Acid-mediated deprotection of the tetrahydropyranyl group or of the 4-methoxybenzyl group gave the hydroxyl ethyl compound **11e** or the NH compounds **11g**, **i**, respectively.

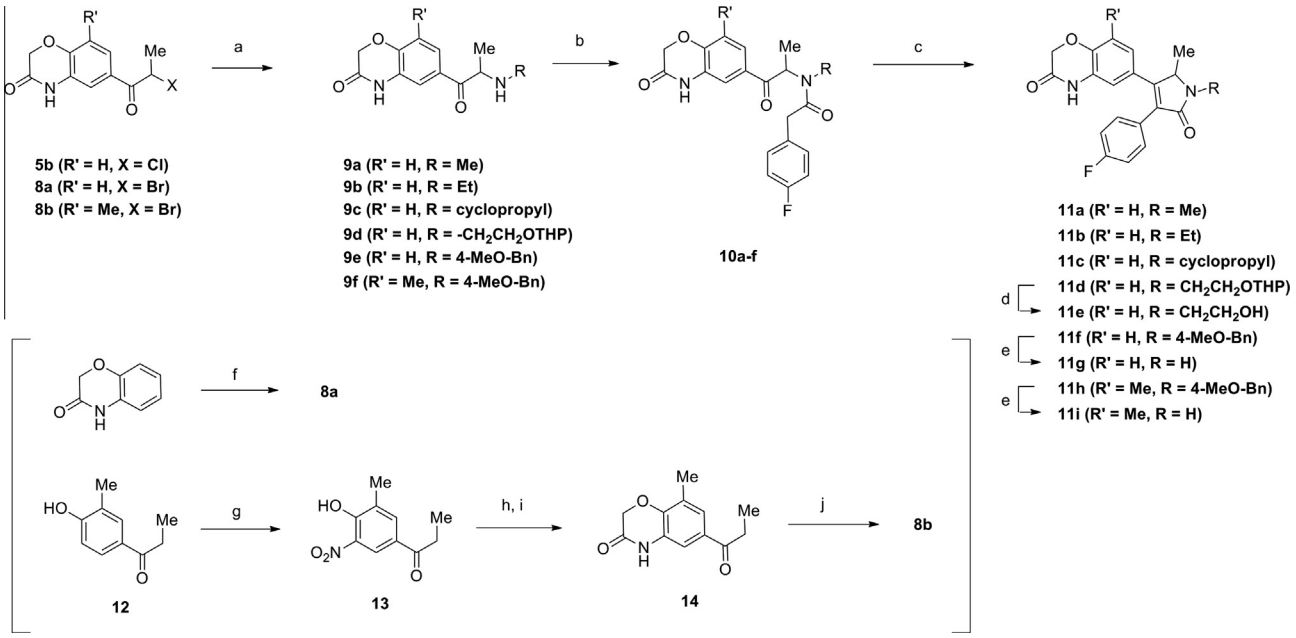
Synthesis of compounds **21a**, **b** is shown in Scheme 3. Compound **17** was prepared by a method similar to that described in Scheme 2, and then converted to triflate **18** as a key precursor for a coupling reaction. Suzuki coupling of **18** with the boronic acid ester **19** gave compound **20** in good yields, and the subsequent deprotection of the 4-methoxybenzyl group afforded the *N*-unsubstituted dihydropyrrol-2-ones **21a**, **b**.

3. Results and discussion

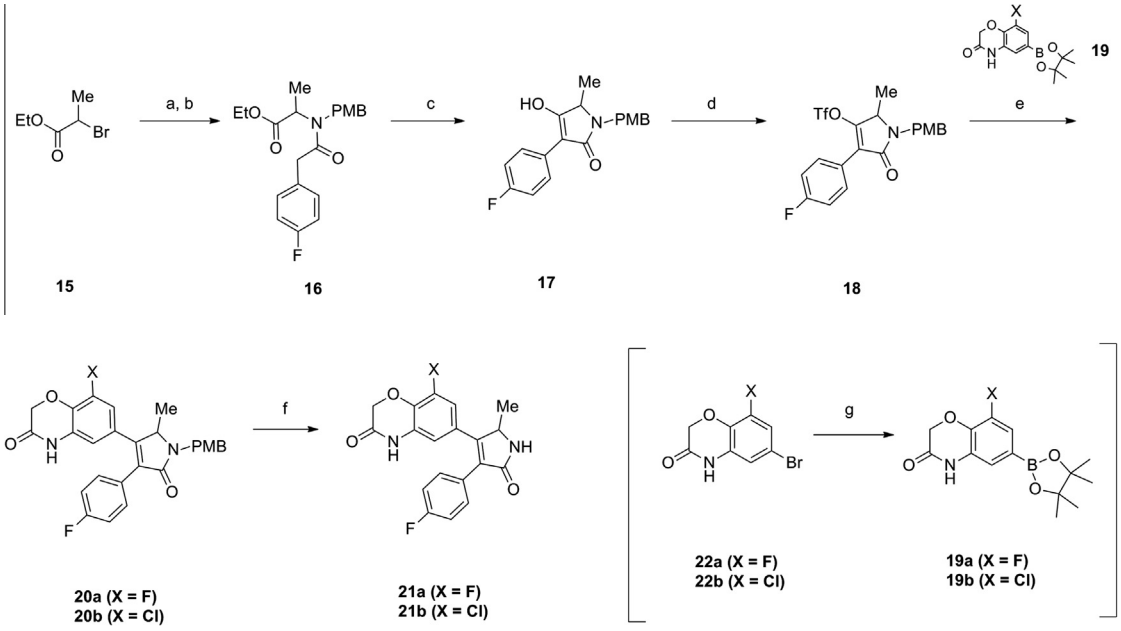
In our first synthetic studies, several alkyl substituents were introduced at the 5-position on the dihydrofuran-2-one ring to fill space 'A' of the ligand binding site (Table 2). The 5,5′-dimethyl analog **2d** showed decreased activity, most likely because the dimethyl group might be too bulky for this position. In contrast, the monomethyl derivative showed a fourfold increase in binding inhibition, as expected (**2e**). Introduction of a fluorine atom onto compound **2e** and a replacement of the methyl group with an ethyl group resulted in decreased binding activity (**2f**, **2g**). The X-ray co-crystal structure obtained with the MR and compound **2e** (racemate) showed that the (5*S*)-methyl group occupied space 'A' with little conformational change of the ligand as designed (Fig. 4), and also explained the SAR described above. Moreover, the (5*R*)-methyl group appeared to clash with the wall of the MR and/or to cause



Scheme 1. Synthesis of dihydrofuran-2-one derivatives **2a**–**g**. Reagents and conditions: (a) $AlCl_3$, $ClCOCRR'Cl$, $ClCH_2CH_2Cl$, 37–99%; (b) $AlCl_3$, $ClCOCRR'H$, $ClCH_2CH_2Cl$, 16%; (c) HBr_3·Py, HBr, AcOH, 69–92%; (d) (1) carboxylic acid **6**, TEA, DMF; (2) DBU, under nitrogen atmosphere, 15–65% in 2 steps; (e) **6**, K_2CO_3, $BnNEt_3Cl$, DMA, 11%; (f) *N*-fluorobenzenesulfonimide, DBU, THF, 24%.



Scheme 2. Synthesis of dihydropyrrol-2-ones **11a–d**, **11g**, **11i**. Reagents and conditions: (a) RNH_2, THF/H_2O, 20–90%; (b) 4-fluorophenylacetyl chloride, K_2CO_3, THF/H_2O, 25–86%; (c) KO^{t}Bu, ^{t}BuOH, under nitrogen atmosphere, 28–86%; (d) PTSA, MeOH, 61%; (e) TFA, anisole, 64–68%; (f) $AlCl_3$, $ClCOCH(CH_3)Br$, CH_2Cl_2, 89%; (g) $NaNO_2$, H_2SO_4,AcOH, 51%; (h) Zn, AcOH; (i) (1) $ClCOCH_2Cl$, K_2CO_3; (2) reflux, 62% (2 steps); (j) HBr_3·Py, HBr, AcOH, 84%

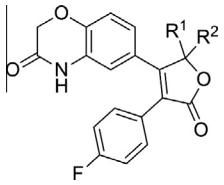


Scheme 3. Synthesis of dihydropyrrol-2-one **21a**,**b**. Reagents and conditions: (a) $PMBNH_2$, THF/H_2O, 90%; (b) 4-F-BnCOCl, K_2CO_3, THF/H_2O, 81%; (c) NaH, DMF, under nitrogen atmosphere, 93%; (d) Tf_2O, TEA, CH_2Cl_2, 71%; (e) boronic acid ester **19**, 20 mol % $PdCl_2(dppf)\cdot CH_2Cl_2$, Cs_2CO_3, THF/H_2O, 42–64%; (f) TFA, anisole, 57–78%; (g) bis(pinacolato)diboron, $PdCl_2(dppf)\cdot CH_2Cl_2$, KOAc, dioxane, 99%.

significant change in the conformation of the ligand. These observations suggested that the (*S*)-enantiomer should be more potent than the (*R*)-enantiomer, and that chiral separation of the racemate would increase the binding affinity. This hypothesis was further supported by the finding that dimethyl compound **2d** was less potent than the monomethyl compound **2e**. As for selectivity towards the other steroidal receptors, the 5-methyl group also increased the binding activity for PR and GR. On the basis of the increase in MR binding, 5-methyl dihydrofuran-2-one ring was selected as the template for further optimization.

In our second synthetic studies, we switched the central scaffold to a dihydropyrrol-2-one ring and introduced various substituents directed toward space 'B', as listed in Table 3. First, a methyl, ethyl or cyclopropyl group was introduced at the 1-position to fill space 'B' (**11a–c**). Whereas the methyl derivative **11a** was slightly less potent than **2e**, the bulkier ethyl and cyclopropyl derivatives **11b**, **c** showed increased binding activity as expected. Next, a hydroxyethyl group was introduced with the aim of forming a hydrogen bond interaction with Arg817, Gln776, and/or a water molecule (**11e**). However, **11e** showed significantly decreased MR

Table 2
SAR summary for space 'A'[a]



Compound No.	R^1	R^2	MR binding IC_{50} (nM)	Selectivity		
				AR binding IC_{50} (nM)	PR binding IC_{50} (nM)	GR binding IC_{50} (nM)
2d	Me	Me	1200	>10,000	1000	4700
2e	Me	H	120	>10,000	740	3800
2f	Me	F	550	>10,000	5000	>10,000
2g	Et	H	1200	>10,000	2200	2000

[a] IC_{50} values were shown as the means of duplicate experiments. IC_{50} values are calculated from the concentration–response curves generated by GraphPad Prism.

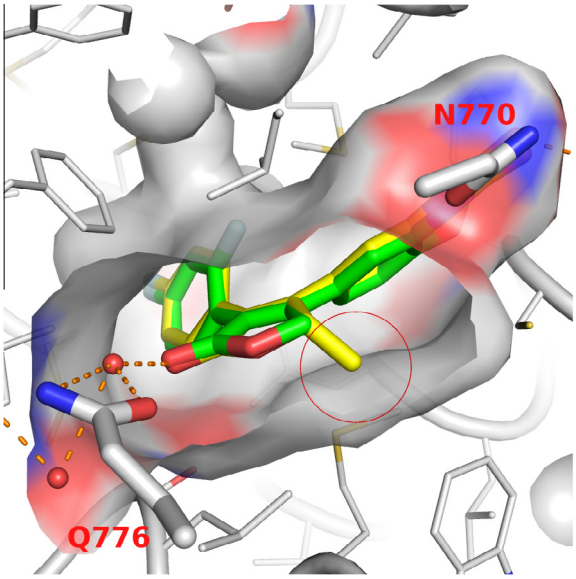


Figure 4. Overlay of a compound **2b** (green) and compound **2e** (yellow) in MR. Cocrystallization of MR and compound **2e** provided only the crystal of MR/(*S*)-enantiomer complex.

binding activity, suggesting that the hydroxyethyl group failed to establish the desired interaction. Unexpectedly, the unsubstituted NH compound **11g** showed a highly potent binding activity (IC_{50} = 94 nM); indeed, it was more potent than the corresponding dihydrofuran-2-one compound **2e**. This result might be explained by reinforcement of the hydrogen-bond with Arg817 and/or Gln776 via a water molecule due to the increased electron density of the carbonyl group. These studies provided important SAR data in relation to the PR binding affinity. While the derivatives substituted with small alkyl groups (**11a**–**c**) showed moderate to potent PR binding (IC_{50} = 61–770 nM), the NH compound **11g** exhibited a weak activity (IC_{50} = 5600 nM). These results suggested that the PR tends to prefer a lipophilic group at space 'B', not to a polar one. Interestingly, we observed a 'selectivity switch' between compounds **11a** and **11g** (**11a**: MR binding IC_{50} = 170 nM, PR binding IC_{50} = 61 nM vs **11g**: MR binding IC_{50} = 94 nM, PR binding IC_{50} = 5600 nM), suggesting that the *N*-methyl derivative **11a** might serve as a new lead compound for a selective PR modulator program.[10] Overall, compound **11g** showed the best balance of MR binding activity and selectivity over AR, PR, and GR among compounds **11a**–**g**, and was therefore selected for further optimization.

In our third round of synthetic studies, we sought to further increase the binding affinity by filling space 'C' with small substituents (F, Cl and Me) introduced at the 5-position of the benzoxazin-3-one ring (Table 4). While introduction of a fluorine atom had little impact on potency (**21a**), incorporation of a chlorine atom (**21b**) or a methyl group (**11i**) increased the binding activity as expected. Notably, their activities were equipotent to that of the spironolactone. Whereas the chlorine atom also increased PR binding activity (**21b**: PR binding IC_{50} = 1600 nM), the methyl group decreased PR binding activity (**11i**: PR binding IC_{50} >10,000 nM). These results indicated that space 'C' in the PR might be slightly narrower than that in the MR. Consequently, compound **11i** showed highly potent MR binding activity (MR binding IC_{50} = 43 nM) and high selectivity over AR, PR, and GR (> 200-fold for AR and PR, 100-fold for GR), suggesting that the potential for sex hormone-related side effects with **11i** may be significantly reduced compared with that of compound **1**.

Functional activity was evaluated in a reporter gene assay in COS-1 cells, and compound **11i** exhibited potent MR antagonistic activity (IC_{50} = 83 nM) comparable to spironolactone (IC_{50} = 60 nM).

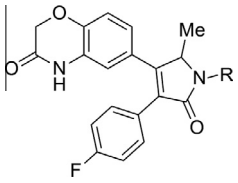
4. Conclusion

In summary, we have discovered dihydrofuran-2-one and dihydropyrrol-2-one derivatives as novel, potent and selective MR antagonists. Exploration of heterocycles in the central ring moiety resulted in the identification of a new lead scaffold, dihydrofuran-2-one ring. X-ray co-crystal structure of the MR/compound complex revealed the binding mode of the dihydrofuran-2-one derivatives and also guided lead optimization. Introduction of lipophilic substituents directed toward the unfilled space of the MR into the central core and into the benzoxazin-3-one ring moiety, as well as scaffold replacement with a dihydropyrrol-2-one ring increased the MR binding activity. The *N*-unsubstituted dihydropyrrol-2-one was identified as a key scaffold for high selectivity against steroid receptors. Thus, we discovered compound **11i**, which showed excellent in vitro activity (MR binding IC_{50} = 43 nM) and high selectivity over AR, PR, and GR (>200-fold for AR and PR, 100-fold for GR). Further optimization of the benzoxazin-3-one derivatives will be reported in due course.

5. Experimental procedure

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Bruker Ultra Shield-300 (300 MHz) or Varian INOVA-

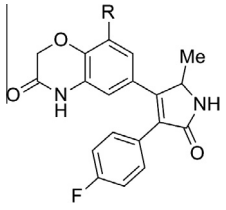
Table 3
SAR summary for space 'B'[a]



Compound No.	R	MR binding IC_{50} (nM)	Selectivity		
			AR binding IC_{50} (nM)	PR binding IC_{50} (nM)	GR binding IC_{50} (nM)
11a	Me	170	>10,000	61	2100
11b	Et	33	>10,000	770	1600
11c	cyclopropyl	68	>10,000	400	2200
11e	CH_2CH_2OH	1600	>10,000	>10,000	>10,000
11g	H	94	>10,000	5600	9100

[a] IC_{50} values were shown as the means of duplicate experiments. IC_{50} values are calculated from the concentration–response curves generated by GraphPad Prism.

Table 4
SAR summary for space 'C'[a]



Compound No.	R	MR binding IC_{50} (nM)	Selectivity		
			AR binding IC_{50} (nM)	PR binding IC_{50} (nM)	GR binding IC_{50} (nM)
11g	H	94	>10,000	5600	9100
21a	F	92	>10,000	3200	5000
21b	Cl	23	>10,000	1600	1100
11i	Me	43	>10,000	>10,000	4900
1	—	41	>10,000	1900	1800
Spironolactone	—	49	120	650	1400
Eplerenone	—	2600	>10,000	>10,000	>10,000

[a] IC_{50} values were shown as the means of duplicate experiments. IC_{50} values are calculated from the concentration–response curves generated by GraphPad Prism.

400 (400 MHz) instruments. Chemical shifts are given in parts per million (ppm) with tetramethylsilane as an internal standard. Peak multi-plicities are expressed as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublets of doublet, br s = broad singlet. Coupling constants (*J* values) are given in hertz (Hz). Elemental analyses were performed by Takeda Analytical Laboratories Ltd. Chemical intermediates were characterized by ^{1}H NMR. The purities of all compounds tested in biological systems were assessed as being >95% using analytical high-performance liquid chromatography (HPLC). The HPLC analyses were performed using a Shimadzu UFLC instrument, equipped with a L-column 2 ODS (3.0 × 50 mm, 2 μm) column, eluting with a gradient of 5–90% solvent B in solvent A (solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in acetonitrile), at a flow rate of 1.2 mL/min, with UV detection at 220 nm. Reaction progress was determined by thin layer chromatography (TLC) analysis on Merck Kieselgel 60 F254 plates or Fuji Silysia NH plates. Chromatographic purification was performed on silica gel columns [(Merck Kieselgel 60, 70–230 mesh size or 230–400 mesh size, Merck) or (Chromatorex NH-DM 1020, 100–200 mesh size)] or on Purif-Pack (SI or NH, particle size: 60 μm, Fuji Silysia Chemical, Ltd). Preparative HPLC purification was performed by using a Waters Corporation UV purification system equipped with a Develosil ODS-UG-10 (4.6 × 150 mm, 5 μm) column, and eluted with a gradient of 5–90% solvent B in solvent A (solvent A was 0.1% TFA in water, and solvent B was 0.1% TFA in acetonitrile), at a flow rate of 150 mL/min, with UV detection at 220 nm. Reagents and solvents were obtained from commercial sources and used without further purification. Abbreviations of solvents are used as follows: $CDCl_3$, deuterated chloroform; DMSO-d_6, dimethyl sulfoxide-d_6; EtOAc, ethyl acetate; DMF, *N*,*N*-dimethylformamide; MeOH, methanol; THF, tetrahydrofuran; EtOH, ethanol; DMSO, dimethyl sulfoxide; DMA, *N*,*N*-dimethylactamide.

5.1. 6-Isobutyryl-2*H*-1,4-benzoxazin-3(4*H*)-one (4b)

To a stirred suspension of 2*H*-1,4-benzoxazin-3(4*H*)-one (15 g, 100 mmol) in 1,2-dichloroethane (180 mL) was added powdered $AlCl_3$ (30 g, 225 mmol) and isobutyryl chloride (12.6 mL, 120.3 mmol) at 0 °C, successively. The mixture was stirred at 80 °C for 3 h, diluted with water and extracted with dichloromethane. The organic layer was dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was crystallized from EtOAc/hexane to give **4b** (3.6 g, 16%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21 (6H, d, J = 7.2 Hz), 3.49 (1H. sep, J = 7.2 Hz), 4.70 (2H, s), 7.03 (1H, d, J = 9.0 Hz), 7.50 (1H, s), 7.61 (1H, d, J = 9.0 Hz), 8.16 (1H, br s).

5.2. 6-(Bromoacetyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (5a)

To a stirred mixture of **4a** (20 g, 104.6 mmol), acetic acid (180 mL) and 25% hydrogen bromide in acetic acid (45 mL) was added portionwise pyridinium hydrobromide perbromide (35 g, 109.8 mmol) at room temperature. The mixture was stirred at room temperature for 3 h, and then the solvent was removed in vacuo. The residue was suspended in EtOAc. The solid was collected by filtration, and washed with water and EtOAc to give **5a** (26.1 g, 92%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.71 (2H, s), 4.81 (2H, s), 7.07 (1H, d, J = 8.3 Hz), 7.49 (1H, d, J = 2.3 Hz), 7.66 (1H, dd, J = 8.3, 2.3 Hz), 10.91 (1H, s).

5.3. 6-(2-Chloropropanoyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (5b)

To a suspension of 2*H*-1,4-benzoxazin-3(4*H*)-one (5 g, 33.5 mmol) in 1,2-dichloroethane (50 mL) was added powdered $AlCl_3$ (9.8 g, 73.7 mmol) and 2-chloropropionyl chloride (3.9 mL, 40.2 mmol) at 0 °C, successively. The mixture was stirred at room temperature for 12 h, poured into ice-cooled water. The precipitate was collected by filtration and washed with 1 N HCl, water and IPE to give **5b** (8.0 g, 99%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.60 (3H, d, J = 6.6 Hz), 4.71 (2H, s), 5.65 (1H, q, J = 6.6 Hz), 7.08 (1H, d, J = 8.7 Hz), 7.54 (1H, d, J = 2.3 Hz), 7.70 (1H, dd, J = 8.7, 2.3 Hz), 10.91 (1H, s).

5.4. 6-(2-Chlorobutanoyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (5c)

The compound **5c** was prepared in a manner similar to that described for **5b**. Yield (37%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.98 (3H, t, J = 7.2 Hz), 1.78–2.13 (2H, m), 4.71 (2H, s), 5.50 (1H, dd, J = 7.8, 5.5 Hz), 7.08 (1H, d, J = 8.3 Hz), 7.53 (1H, d, J = 2.3 Hz), 7.71 (1H, dd, J = 8.3, 2.3 Hz), 10.91 (1H, s).

5.5. 6-(2-Bromo-2-methylpropanoyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (5d)

The compound **5d** was prepared in a manner similar to that described for **5a**. Yield (69%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.03 (6H, d, J = 7.2 Hz), 4.70 (2H, s), 7.00 (1H, d, J = 8.7 Hz), 7.64 (1H, d, J = 1.8 Hz), 7.96 (1H, dd, J = 1.8, 8.7 Hz), 8.10 (1H, br s).

5.6. 6-[4-(2,4-Difluorophenyl)-5-oxo-2,5-dihydrofuran-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (2b)

To a stirred solution of **5a** (300 mg, 1.11 mmol) and triethylamine (281 μL, 2.02 mmol) in DMF (5 mL) was added 2.4-difuluorophenylacetic acid (173 mg, 1.01 mmol) at room temperature. The mixture was stirred at room temperature for 3 h. To the mixture was added DBU (304 μL, 2.02 mmol). The mixture was stirred at room temperature for 12 h under nitrogen atmosphere, diluted with water, and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with hexane/EtOAc (1:1) as an elutant, and crystallized from hexane/EtOAc to give **2b** (74 mg, 19%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.62 (2H, s), 5.43 (2H, s), 6.83 (1H, s), 7.01 (2H, s), 7.12–7.29 (1H, m), 7.30–7.60 (2H, m), 10.82 (1H, br s). Anal. Calcd for $C_{18}H_{11}F_2NO_4$: C, 62.98; H, 3.23; N, 4.08. Found: C, 62.81; H, 3.29; N, 3.98.

The compound **2a**, **2c**–**e**, **2g** were prepared in a manner similar to that described for **2b**.

5.7. 6-[4-(4-Fluorophenyl)-5-oxo-2,5-dihydrofuran-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (2a)

Yield (56%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.62 (2H, s), 5.43 (2H, s), 6.83 (1H, s), 6.98–7.02 (2H, m), 7.12–7.29 (1H, m), 7.30–7.60 (2H, m), 10.82 (1H, s). Anal. Calcd for $C_{18}H_{12}FNO_4$: C, 66.46; H, 3.72; N, 4.31. Found: C, 66.40; H, 3.75; N, 4.35.

5.8. 6-[4-(4-Fluoro-2-methylphenyl)-5-oxo-2,5-dihydrofuran-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (2c)

Yield (15%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.13 (3H, s), 4.54 (2H, s), 5.29 (2H, d, J = 2.1 Hz), 6.77–6.86 (3H, m), 6.95–7.18 (3H, m), 10.75 (1H, s). Anal. Calcd for $C_{19}H_{14}FNO_4$: C, 67.25; H, 4.16; N, 4.13. Found: C, 66.97; H, 4.22; N, 4.08.

5.9. 6-[4-(4-Fluorophenyl)-2-methyl-5-oxo-2,5-dihydrofuran-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (2e)

Yield (42%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.34 (3H, t, J = 6.8 Hz), 4.63 (2H, s), 5.76 (1H, q, J = 6.8 Hz), 6.80 (1H, d, J = 1.9 Hz), 6.90–7.07 (2H, m), 7.18–7.20 (2H, m), 7.33–7.42 (2H, m), 10.75 (1H, s). Anal. Calcd for $C_{19}H_{14}FNO_4$: C, 67.25; H, 4.16; N, 4.13. Found: C, 67.10; H, 4.29; N, 4.06.

5.10. 6-[2-Ethyl-4-(4-fluorophenyl)-5-oxo-2,5-dihydrofuran-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (2g)

Yield (65%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.80 (3H, t, J = 7.3 Hz), 1.41–1.56 (1H, m), 1.80–1.96 (1H, m), 4.63 (2H, s), 5.72 (1H, dd, J = 7.3, 3.6 Hz), 6.79–6.80 (1H, m), 6.89–7.11 (2H, m), 7.17–7.31 (2H, m), 7.31–7.55 (2H, m), 10.74 (1H, s). Anal. Calcd for $C_{20}H_{16}FNO_4 \cdot 0.2H_2O$: C, 67.30; H, 4.63; N, 3.92. Found: C, 67.15; H, 4.75; N, 3.60.

5.11. 6-[4-(4-Fluorophenyl)-2,2-dimethyl-5-oxo-2,5-dihydrofuran-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (2d)

A mixture of **5d** (300 mg, 1.01 mmol), 4-fluorophenylacetic acid (155 mg, 1.01 mmol), potassium carbonate (277 mg, 2.01 mmol) and benzyltriethylammonium chloride (11.4 mg, 0.05 mmol) in DMA (5 mL) was stirred at 50 °C for 2 days and at 100 °C for 3 days, diluted with 1 N HCl and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with hexane/EtOAc (5:2) as an elutant to give **2d** (38 mg, 11%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.54 (6H, s), 4.63 (2H, s), 6.76–6.89 (2H, m), 7.00 (1H, d, J = 8.7 Hz), 7.11–7.22 (2H, m), 7.29–7.48 (2H, m), 10.72 (1H, br s). Anal. Calcd for $C_{20}H_{16}FNO_4$: C, 67.98; H, 4.56; N, 3.96. Found: C, 67.64; H, 4.64; N, 4.04.

5.12. 6-[2-Fluoro-4-(4-fluorophenyl)-2-methyl-5-oxo-2,5-dihydrofuran-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (2f)

Under argon atmosphere, to a stirred solution of **2e** (500 mg, 1.47 mmol) in THF (10 mL) were added DBU (666 μL, 4.33 mmol) and a solution of N-fluorobenzenesulfonimide (700 mg, 2.21 mmol) in THF (4 mL) at −78 °C, successively. The mixture was stirred for 1 h, and then DBU (666 μL, 4.33 mmol) and a solution of *N*-fluorobenzenesulfonimide (700 mg, 2.21 mmol) in THF (4 mL) were added. The mixture was stirred at −78 °C for 30 min, quenched with ammonium chloride solution and 1 N HCl and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with hexane/EtOAc as an elutant and by preparative HPLC to give **2f** (129 mg, 24%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.82 (3H, d, J = 18.9 Hz), 4.65 (2H, s), 6.84–7.06 (2H, m), 7.21–7.36 (2H, m), 7.37–7.47 (2H, m), 10.80 (1H, br s). Anal. Calcd for $C_{19}H_{13}F_2NO_4$: C, 63.87; H, 3.67; N, 3.92. Found: C, 63.70; H, 3.62; N, 4.08.

5.13. 6-(2-Bromopropanoyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (8a)

The compound **8a** was prepared in a manner similar to that described for **5b**. Yield (89%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.76 (3H, d, J = 6.8 Hz), 4.71 (2H, s), 5.69 (1H, q, J = 6.8 Hz), 7.07 (1H, d, J = 8.3 Hz), 7.54 (1H, d, J = 2.3 Hz), 7.70 (1H, dd, J = 8.3, 2.3 Hz), 10.90 (1H, s).

5.14. 6-(2-Bromopropanoyl)-8-methyl-2*H*-1,4-benzoxazin-3(4*H*)-one (8b)

The compound **8b** was prepared in a manner similar to that described for **5a**. Yield (84%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.75 (3H, d, J = 6.4 Hz), 2.22 (3H, s), 4.72 (2H, s), 5.68 (1H, q, J = 6.4 Hz), 7.40 (1H, d, J = 1.9 Hz), 7.62 (1H, d, J = 1.9 Hz), 10.85 (1H, s).

5.15. 6-[2-(Methylamino)propanoyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (9a)

A mixture of **5b** (1 g, 4.17 mmol) and 40% methylamine (15 mL) in THF (15 mL) was stirred at 40 °C for 12 h, diluted with water and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on NH-silica gel with EtOAc as an elutant to give **9a** (200 mg, 20%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.14 (3H, d, J = 6.8 Hz), 2.20 (3H, s), 4.12 (1H, q, J = 6.8 Hz), 4.68 (2H, s), 7.04 (1H, d, J = 8.3 Hz), 7.53 (1H, d, J = 1.9 Hz), 7.67 (1H, dd, J = 8.3, 1.9 Hz), 10.85 (1H, br s).

The compound **9b**, **c** was prepared in a manner similar to that described for **9a**.

5.16. 6-[2-(Ethylamino)propanoyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (9b)

Yield (51%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.98 (3H, t, J = 7.0 Hz), 1.14 (3H, d, J = 6.8 Hz), 2.35–2.49 (2H, m), 4.22 (2H, q, J = 6.8 Hz), 4.69 (2H, s), 7.05 (1H, d, J = 8.5 Hz), 7.53 (1H, d, J = 1.9 Hz), 7.68 (1H, dd, J = 8.5, 1.9 Hz), 10.85 (1H, br s).

5.17. 6-[2-(Cyclopropylamino)propanoyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (9c)

The crude product was used for the next reaction.

5.18. 2-(4-Fluorophenyl)-*N*-[1-oxo-1-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)propan-2-yl]-*N*-[2-(tetrahydro-2*H*-pyran-2-yloxy)ethyl]acetamide (9d)

The crude product was used for the next reaction.

5.19. 6-{2-[(4-Methoxybenzyl)amino]propanoyl}-2*H*-1,4-benzoxazin-3(4*H*)-one (9e)

A mixture of **8a** (2 g, 7.04 mmol), 4-methoxybenzylamine (2.9 g, 21.1 mmol) and triethylamine (1.9 mL, 14.08 mmol) in THF/water (20 mL/5 mL) was stirred at 50 °C for 2 h, diluted with water and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was suspended in IPE and the solid was collected by filtration to give **9d** (2.15 g, 90%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.18 (3H, d, J = 7.0 Hz), 3.43–3.68 (2H, m), 3.72 (3H, s), 4.22 (1H, d, J = 7.0 Hz), 4.69 (2H, s), 6.80–6.91 (2H, m), 7.03 (1H, d, J = 8.3 Hz), 7.18–7.21 (2H, m), 7.51 (1H, d, J = 1.9 Hz), 7.57–7.66 (1H, m). 10.34 (1H, br s).

5.20. 6-{2-[(4-Mthoxybenzyl)amino]propanoyl}-8-methyl-2*H*-1,4-benzoxazin-3(4*H*)-one (9f)

A mixture of **8b** (1 g, 3.35 mmol), 4-methoxybenzylamine (1.38 g, 10.1 mmol) and triethylamine (0.89 mL, 6.71 mmol) in THF/water (10 mL/5 mL) was stirred at 50 °C for 2 h, diluted with water and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was crystallized form EtOAc/hexane to give **9e** (0.86 g, 72%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.18 (3H, d, J = 6.8 Hz), 2.13–2.46 (4H, m), 3.47–3.67 (2H, m), 3.72 (3H, s), 4.21 (1H, q, J = 6.8 Hz), 4.70 (2H, s), 6.84–6.85 (2H, m), 7.19–7.23 (2H, m), 7.36 (1H, d, J = 1.9 Hz), 7.47–7.48 (1H, m), 10.56 (1H. br s).

5.21. 2-(4-Fluorophenyl)-*N*-methyl-*N*-[1-oxo-1-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)propan-2-yl]acetamide (10a)

To a stirred solution of **9a** (200 mg, 0.85 mmol) in THF (2 mL) were added a solution of potassium carbonate (354 mg, 2.56 mmol) in water (2 mL) and 4-fluorophenylacetyl chloride (162 mg, 0.94 mmol) at 0 °C, successively. The mixture was stirred at room temperature for 30 min, diluted with water, and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was suspended in diisopropyl ether and the solid was collected by filtration to give **10a** (240 mg, 76%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.24 (3H, d, J = 6.8 Hz), 2.86 (3H, s), 3.61–3.67 (2H, m), 4.67 (2H, s), 5.51 (1H, q, J = 6.8 Hz), 6.93–7.15 (5H, m), 7.32–7.44 (2H, m), 10.88 (1H, s).

The compound **10b**–**f** was prepared in a manner similar to that described for **10a**.

5.22. *N*-Ethyl-2-(4-fluorophenyl)-*N*-[1-oxo-1-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)propan-2-yl]acetamide (10b)

Yield (53%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.13 (3H, t, J = 7.0 Hz), 1.41 (2H, d, J = 6.8 Hz), 3.17–3.49 (2H, m), 3.69 (2H, s), 4.67 (2H, s), 5.97 (1H, q, J = 6.8 Hz), 6.84–6.99 (3H, m), 7.04–7.20 (2H, m), 7.49 (1H, d, J = 2.3 Hz), 7.54–7.67 (1H, m), 9.39 (1H, s).

5.23. *N*-Cyclopropyl-2-(4-fluorophenyl)-*N*-[1-oxo-1-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)propan-2-yl]acetamide (10c)

Yield (25% in 2 steps). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.80–1.00 (4H, m), 1.32 (3H, d, J = 6.8 Hz), 2.67–2.77 (1H, m), 3.57–3.93 (2H, m), 4.65 (2H, s), 4.99 (1H, q, J = 6.8 Hz), 6.87–7.03 (5H, m), 7.16–7.32 (2H, m), 10.84 (1H, s).

5.24. 6-(2-{[2-(Tetrahydro-2*H*-pyran-2-yloxy)ethyl]amino}propanoyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (10d)

Yield (71% in 2 steps). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.40–1.80 (9H, m), 3.36–3.83 (8H, m), 4.26–4.54 (1H, m), 4.63 (2H, s), 5.49–5.80 (1H, m), 6.85–7.12 (5H, m), 7.26–7.60 (2H, m), 9.80 (1H, br s).

5.25. 2-(4-Fluorophenyl)-*N*-(4-methoxybenzyl)-*N*-[1-oxo-1-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)propan-2-yl]acetamide (10e)

Yield (70%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.08–1.30 (3H, m), 3.52–3.78 (5H, m), 4.63 (2H, s), 4.67 (2H, s), 5.29 (1H, q, J = 6.8 Hz), 6.84–7.04 (6H, m), 7.09–7.39 (5H, m), 10.84 (1H, s).

5.26. 2-(4-Fluorophenyl)-*N*-(4-methoxybenzyl)-*N*-[1-(8-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)-1-oxopropan-2-yl]acetamide (10f)

Yield (86%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.05–1.32 (3H, m), 2.09–2.17 (3H, m), 3.48–3.70 (2H, m), 3.70–3.83 (3H, m), 4.54–4.76 (4H, m), 5.26 (1H, q, J = 6.8 Hz), 6.69–6.95 (2H, m), 6.98–7.03 (4H, m), 7.09–7.34 (4H, m), 10.78 (1H, br s).

5.27. 6-[4-(4-Fluorophenyl)-1,2-dimethyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (11a)

Under argon atmosphere, to a stirred solution of **10a** (240 mg, 0.65 mmol) in THF (5 mL) was added dropwise a solution of potassium *tert*-buthoxide (181 mg, 1.62 mmol) in *tert*-buthanol (4 mL) at 0 °C. The mixture was stirred at room temperature for 30 min, diluted with water, and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with EtOAc/hexane as an elutant and crystallized form EtOAc/hexane to give **11a** (123 mg, 54%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.13 (3H, d, J = 7.0 Hz), 2.99 (3H, s), 4.58–4.68 (3H, m), 6.73 (1H, d, J = 1.9 Hz), 6.78–6.88 (1H, m), 6.92–7.00 (1H, m), 7.13–7.24 (2H, m), 7.26–7.38 (2H, m), 10.70 (1H, s). Anal. Calcd for $C_{20}H_{17}FN_2O_3$: C, 68.17; H, 4.86; N, 7.95. Found: C, 68.07; H, 4.85; N, 8.07.

The compound **11b**–**e**, **11g** was prepared in a manner similar to that described for **11a**.

5.28. 6-[1-Ethyl-4-(4-fluorophenyl)-2-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (11b)

Yield (28%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.05–1.21 (6H, m), 3.16–3.29 (1H, m), 3.63–3.81 (1H, m), 4.60 (2H, s), 4.68–4.77 (1H, m), 6.73 (1H, d, J = 1.9 Hz), 6.83–6.91 (1H, m), 6.93–6.99 (1H, m), 7.09–7.22 (2H, m), 7.26–7.37 (2H, m), 10.68 (1H, s). Anal. Calcd for $C_{21}H_{19}FN_2O_3$: C, 68.84; H, 5.23; N, 7.65. Found: C, 68.79; H, 5.25; N, 7.63.

5.29. 6-[1-Cyclopropyl-4-(4-fluorophenyl)-2-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (11c)

Yield (78%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.67–0.80 (2H, m), 0.81–0.93 (2H, m), 1.20 (3H, d, J = 6.8 Hz), 2.55–2.65 (1H, m), 4.53–4.68 (3H, m), 6.72 (1H, d, J = 1.9 Hz), 6.81–6.89 (1H, m), 6.92–7.02 (1H, m), 7.08–7.24 (2H, m), 7.24–7.36 (2H, m), 10.67 (1H, br s).

5.30. 6-{4-(4-fluorophenyl)-2-methyl-5-oxo-1-[2-(tetrahydro-2*H*-pyran-2-yloxy)ethyl]-2,5-dihydro-1*H*-pyrrol-3-yl}-2*H*-1,4-benzoxazin-3(4*H*)-one (11d)

Yield (62%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.24 (3H, d, J = 6.9Hz), 1.40–1.90 (6H, m), 3.35–3.58 (2H, m), 3.62–3.72 (1H, m), 3.75–4.02 (2H, m), 4.04–4.22 (1H, m), 4.57–4.78 (2H, m), 4.63 (2H, s), 6.57 (1H, d, J = 1.8 Hz), 6.80 (1H, dd, J = 1.8, 8.1 Hz), 6.91–7.03 (3H, m), 7.32–7.41 (2H, m), 8.13 (1H, s).

5.31. 6-[4-(4-Fluorophenyl)-1-(2-hydroxyethyl)-2-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (11e)

A mixture of **11d** (0.51 g, 1.1 mmol) and *p*-toluenesulfonic acid (22.5 mg, 0.13 mmol) in methanol (50 mL) was stirred at room temperature for 2 h, diluted with sat. $NaHCO_3$ and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with EtOAc/hexane as an elutant and crystallized form EtOAc to give **11e** (256 mg, 61%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.13 (3H, d, J = 6.6 Hz), 3.19–3.35 (1H, m), 3.53–3.65 (2H, m), 3.75–3.86 (1H, m), 4.59 (2H, s), 4.78–4.95 (2H, m), 6.62–6.85 (2H, m), 6.88–6.97 (1H, m), 7.09–7.22 (2H, m), 7.28–7.37 (2H, m), 10.66 (1H, s). Anal. Calcd for $C_{21}H_{19}FN_2O_4$: C, 65.96; H, 5.01; N, 7.33. Found: C, 65.72; H, 5.07; N, 7.31.

5.32. 6-[4-(4-Fluorophenyl)-1-(4-methoxybenzyl)-2-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (11f)

Yield (86%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.10 (3H, d, J = 6.8 Hz), 3.74 (3H, s), 4.35 (1H, d, J = 15.1 Hz), 4.47 (1H, q, J = 6.8 Hz), 4.59 (2H, s), 4.89 (1H, d, J = 15.1 Hz), 6.67–6.73 (1H, m), 6.76–6.83 (1H, m), 6.87–6.97 (3H, m), 7.14–7.29 (4H, m), 7.31–7.42 (2H, m), 10.60 (1H, s).

5.33. 6-[4-(4-Fluorophenyl)-1-(4-methoxybenzyl)-2-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-8-methyl-2*H*-1,4-benzoxazin-3(4*H*)-one (11h)

Yield (40%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.10 (3H, d, J = 6.8 Hz), 2.08 (3H, s), 3.74 (3H, s), 4.33 (1H, d, J = 15.1 Hz), 4.46 (1H, q, J = 6.8 Hz), 4.60 (2H, s), 4.90 (1H, d, J = 15.1 Hz), 6.53 (1H, d, J = 1.9 Hz), 6.73 (1H, d, J = 1.9 Hz), 6.86–6.97 (2H, m), 7.12–7.30 (4H, m), 7.30–7.45 (2H, m), 10.53 (1H, s).

5.34. 6-[4-(4-Fluorophenyl)-2-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (11g)

A mixture of **11e** (2 g, 4.36 mmol) and anisole (470 mg, 4.36 mmol) in trifluoroacetic acid (15 mL) was stirred at 65 °C for 12 h, and concentrated under reduced pressure. The residue was diluted with water, and extracted with EtOAc. The organic layer was separated, and stirred at room temperature. The precipitate was collected by filtration and purified by recrystallization from ethanol to give **11g** (950 mg, 64%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.09 (3H, d, J = 6.8 Hz), 4.60 (2H, s), 4.67 (1H, q, J = 6.8 Hz), 6.73 (1H, d, J = 1.9 Hz), 6.81–6.90 (2H, m), 6.91–6.99 (1H, m), 7.10–7.29 (2H, m), 7.27–7.37 (2H, m), 8.61 (1H, s), 10.67 (1H, s). Anal. Calcd for $C_{19}H_{15}FN_2O_3$: C, 67.45; H, 4.47; N, 8.28. Found: C, 67.11; H, 4.63; N, 8.26.

5.35. 6-[4-(4-Fluorophenyl)-2-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-8-methyl-2*H*-1,4-benzoxazin-3(4*H*)-one (11i)

The compound **11i** was prepared in a manner similar to that described for **11g**. Yield (68%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.09 (3H, d, J = 6.8 Hz), 2.12 (3H,s), 4.49–4.72 (3H, m), 6.55 (1H, d, J = 1.9 Hz), 6.79 (1H, d, J = 1.9 Hz), 7.09–7.23 (2H, m), 7.21–7.45 (2H, m), 8.59 (1H, s), 10.59 (1H, s). Anal. Calcd for $C_{20}H_{17}FN_2O_3$·0.1 (H_2O): C, 67.83; H, 4.90; N, 7.91. Found: C, 67.46; H, 5.18; N, 7.53.

5.36. 1-(4-Hydroxy-3-methyl-5-nitrophenyl)propan-1-one (13)

To a stirred solution of **12** (18.3 g, 111.4 mmol) were added a solution of sulfuric acid (10 mL) in water (20 mL) and a solution of sodium nitrite (23.1 g, 334.4 mmol) in water (40 mL) at 0 °C, successively. The mixture was stirred at 0 °C for 3 h, and poured into water. The precipitate was collected by filtration and washed with water to give **13** (11.8 g, 51%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.07 (3H, t, J = 7.2 Hz), 2.31 (3H, s), 3.03 (2H, q, J = 7.2 Hz), 8.11 (1H, d, J = 1.9 Hz), 8.35 (1H, d, J = 1.9 Hz), 11.05 (1H, s).

5.37. 8-Methyl-6-propionyl-2*H*-1,4-benzoxazin-3(4*H*)-one (14)

To a stirred suspension of **13** (11.5 g, 54.9 mmol) was added portionwise zinc powder (35.9 g, 549.7 mmol) under water bath. The mixture was stirred for 10 min and the insoluble material was removed by filtration. The filtrate was concentrated under reduced pressure. To the residue were added 4-methy-2-pentanone (100 mL) and a solution of sodium carbonate (17.4 g, 164.9 mmol) in water (100 mL) and chloroacetyl chloride (6.2 g, 54.9 mmol) at 0 °C, successively. The mixture was stirred at 0 °C for 2 h and at 50 °C for 12 h, diluted with water and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was crystallized form EtOAc to give **14** (7.5 g, 62% in 2 steps). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.06 (3H, t, J = 7.2 Hz), 2.21 (3H, s), 2.93 (2H, q, J = 7.2 Hz), 4.68 (2H, s), 7.33 (1H, d, J = 1.9 Hz), 7.50 (1H, d, J = 1.9 Hz), 10.79 (1H, s).

5.38. Ethyl *N*-[(4-fluorophenyl)acetyl]-*N*-(4-methoxybenzyl)alaninate (16)

The compound **16** was prepared in a manner similar to that described for **9e** and **10a**. Yield (81% in 2 steps). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.06–1.16 (3H, m), 1.17–1.26 (3H, m), 3.65–3.81 (5H, m), 3.93–4.06 (2H, m), 4.10–4.20 (1H, m), 4.49–4.99 (2H, m), 6.79–6.96 (2H, m), 7.06–7.35 (6H, m).

5.39. 3-(4-Fluorophenyl)-4-hydroxy-1-(4-methoxybenzyl)-5-methyl-1,5-dihydro-2*H*-pyrrol-2-one (17)

To a stirred suspension of 60% sodium hydride (2.16 g, 53. 91 mmol), in DMF (90 mL) was added dropwise a solution of **16** (18.3 g, 49. 0 mmol) in DMF (90 mL) at 0 °C. The mixture was stirred at room temperature for 12 h under nitrogen atmosphere, diluted with 1 N HCl and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was crystallized from methanol to give **17** (15 g, 93%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.30 (3H, d, J = 6.8 Hz), 3.68–3.83 (4H, m), 4.14 (1H, d, J = 15.3 Hz), 4.82 (1H, d, J = 15.3 Hz), 6.80–6.96 (2H, m), 7.08–7.25 (4H, m), 7.98–8.15 (2H, m), 11.46 (1H, s).

5.40. 4-(4-Fluorophenyl)-1-(4-methoxybenzyl)-2-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl trifluoromethanesulfonate (18)

Under nitrogen atmosphere, to a stirred solution of **17** (15 g, 45.82 mmol) and triethylamine (6.7 mL, 50.40 mmol) in dichloromethane (150 mL) was added dropwise trifluoromethanesulfonic anhydride (14.2 g, 50.40 mmol) at 0 °C. The mixture was stirred for 3 h, diluted with water. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with EtOAc/hexane as an elutant and crystallized form EtOH/water to give **18** (15 g, 71%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.41 (3H, d, J = 6.8 Hz), 3.74 (3H, s), 4.33–4.51 (2H, m), 4.79 (1H, d, J = 15.5 Hz), 6.87–7.00 (2H, m), 7.27 (2H, d, J = 8.7 Hz), 7.31–7.45 (2H, m), 7.70–7.82 (2H, m).

5.41. 8-Fluoro-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (19a)

A mixture of 6-bromo-8-fluoro-2*H*-1,4-benzoxazin-3(4*H*)-one (8.0 g, 32.5 mmol), bis(pinacolato)diboron (9.08 g, 35.8 mmol), [1,1-1,1′-bis(diphenylphosphino)ferrocene-palldium(II)dichloride dichloromethane complex (1.33 g, 1.63 mmol) and potassium acetate (11.2 g, 114 mmol) in 1,4-dioxane (320 mL) was stirred at 90 °C for 13 h under an argon atmosphere, diluted with water and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with EtOAc/hexane as an elutant and crystallized from EtOAc/hexane to give **19a** (9.48 g, 99%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.28 (12H, s), 4.70 (2H, s), 6.99–7.08 (2H, m), 10.90 (1H, s).

5.42. 8-Chloro-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (19b)

The compound **19b** was prepared in a manner similar to that described for **19a**. Yield (99%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.28 (12H, s), 4.74 (2H, s), 7.14 (1H, d, J = 1.5 Hz), 7.23 (1H, d, J = 1.5 Hz), 10.89 (1H, s).

5.43. 8-Fluoro-6-[4-(4-fluorophenyl)-1-(4-methoxybenzyl)-2-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (20a)

Under argon atmosphere, a mixture of **19a** (500 mg, 1.71 mmol), **18** (784 mg, 1.71 mmol), 1,1′-bis(diphenylphosphino)ferrocene-palldium(II)dichloride dichloromethane complex (278 mg, 0.34 mmol) and cesium carbonate (1.67 g, 5.21 mmol) in THF/water (20 mL/5 mL) was stirred at 100 °C for 12 h, diluted with 1 N HCl and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with EtOAc/hexane as an elutant and crystallized form EtOAc/hexane to give **20a** (522 mg, 64%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.11 (3H, d, J = 6.8 Hz), 3.74 (3H, s), 4.35 (1H, dJ = 15.1 Hz), 4.52 (1H, q, J = 6.8 Hz), 4.68 (2H, s), 4.88 (1H, d, J = 15.1 Hz), 6.51 (1H, d, J = 1.5 Hz), 6.82–6.98 (3H, m), 7.16–7.45 (6H, m), 10.80 (1H, s).

5.44. 8-Chloro-6-[4-(4-fluorophenyl)-1-(4-methoxybenzyl)-2-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (20b)

The compound **20b** was prepared in a manner similar to that described for **20a**. Yield (42%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.10 (3H, d, J = 6.8 Hz), 3.32 (3H, s), 4.35 (1H, dJ = 15.1 Hz), 4.55 (1H, q, J = 6.8 Hz), 4.72 (2H, s), 4.88 (1H, d, J = 15.1 Hz), 6.63 (1H, d, J = 1.9 Hz), 6.92–7.03 (3H, m), 7.15–7.44 (6H, m), 10.78 (1H, s).

5.45. 8-Fluoro-6-[4-(4-fluorophenyl)-2-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (21a)

The compound **21a** was prepared in a manner similar to that described for **11g**. Yield (78%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.10 (3H, d, J = 6.4 Hz), 4.62–4.77 (3H, m), 6.52 (1H, s), 6.89–6.94 (1H, m), 7.07–7.24 (2H, m), 7.27–7.41 (2H, m), 8.67 (1H, s), 10.85 (1H, s). Anal. Calcd for $C_{19}H_{14}F_2N_2O_3$: C, 64.04; H, 3.96; N, 7.86. Found: C, 63.66; H, 4.05; N, 7.79.

5.46. 8-Chloro-6-[4-(4-fluorophenyl)-2-methyl-5-oxo-2,5-dihydro-1*H*-pyrrol-3-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (21b)

The compound **21b** was prepared in a manner similar to that described for **11g**. Yield (57%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.09 (3H, d, J = 6.8 Hz), 4.62–4.79 (3H, m), 6..64 (1H, d, J = 1.9 Hz), 7.05 (1H, d, J = 1.9 Hz), 7.14–7.23 (2H, m), 7.27–7.39 (2H, m), 8.67 (1H, s), 10.84 (1H, br s). Anal. Calcd for $C_{19}H_{14}ClFN_2O_3$: C, 61.22; H, 3.79; N, 7.51. Found: C, 60.98; H, 4.03; N, 7.23.

5.47. Radioligand binding assay

Binding displacement assays were carried out in 96-well v-bottom polypropylene plates with a final volume of 50.5 μL of TEGM buffer (10 mM Tris–HCl (pH 7.2), 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM 2-mercaptoethanol, 10 mM sodium molybdate, Protease inhibitor Cocktail (Roche)) containing [^{3}H]-Aldosterone (final concentration, 10 nM), serially diluted test compounds, and 0.75–1.5 mg/mL of cytosolic protein prepared from human MR transiently transfected FreeStyleTM 293 cells (Invitrogen). Each concentration was run in duplicate. The cytosols were incubated for 16 h at 4 °C. Unbound radioactivity was removed by the addition of 35 μL of dextran/gelatin coated charcoal suspension (5% charcoal, 0.5% dextran T-70 (GE Healthcare UK Ltd), 0.1% gelatin (Sigma–Aldrich Co.),and 10 mM Tris–HCl (pH 7.2), 1 mM EDTA). The mixture was incubated for 10 min at 4 °C and centrifuged at 910 × *g* for 10 min at 4 °C. Then 30 μL of the supernatant from each well was transferred to a 96-well white plate with 150 μL of scintillation fluid and the radioactivity was measured by TopCountTM (PerkinElmer Inc.). For the determination of non-specific binding, cold aldosterone instead of drug was added to reaction mixture at 100 μM. Specific binding was determined by subtracting the value of non-specific binding component from the total binding value. The raw data for the specifically bound counts were normalized between 0% and 100% activity and nonlinear fitted to a sigmoidal equation to calculate the IC_{50} values using PRISM 3.0 (GraphPadSoftware Inc.). Other steroid receptor (GR, AR, and PR) binding assays were carried out by a method similar to MR binding assay except for ligands. [^{3}H]-Dexamethasone, [^{3}H]-Testosterone or [^{3}H]-Progesterone was used as a ligand in GR, AR or PR binding assay, respectively. In the case of PR binding assay, ligand concentration was 5 nM.

5.48. MR antagonist assay (luciferase reporter gene assay)

COS-1 cells were inoculated at 5 × 10^6 cells/F150 in D-MEM (low glucose) supplemented with 10% FBS and 50 mg/mL gentamicin and then cultured at 37 °C in 5% CO_2 for 1 day. To prepare DNA:transfection reagent complexes, a solution of 2.5 mL Opti-MEM, 100 μL Plus Reagent (Invitrogen), 9 μg pMCMYneo-hMR, 5 μg pMAM-Luc and 1 μg pRL-TK was mixed with a solution of 2.5 mL Opti-MEM and 125 μL Lipofectamin Reagent (Invitrogen). The mixture was maintained at room temperature for 15 min. After substitution of culture media with Opti-MEM, the mixture was added to the cells. After 3 h incubation, 25 mL D-MEM (low glucose) supplemented with 0.1% BSA and 50 μg/mL gentamicin was added and then the cells were incubated at 37 °C in 5% CO_2 for 1 day. The transfected cells were harvested and re-suspended at 3.3 × 10^5 cells/mL in D-MEM (low glucose) supplemented with 0.1% BSA and 50 μg/mL gentamicin. Then 40 μL of the cell suspension was transferred in 96-well plate (Corning#3688). After incubation, 5 μL/well of test compounds at various concentrations and 5 μL/well of aldosterone (final concentration: 1 nM) were added to the cells. After 1 day incubation at 37 °C in 5% CO_2, the medium was removed. 20 μL/well of twofold diluted pikkagene (NIPPON GENE CO., Ltd) solution with HBSS was added to each well and the luciferase activity was measured. The data were nonlinear fitted to a sigmoidal equation to calculate the IC_{50} values.

5.49. Crystallization and structure determination

The human MR-LBD triple mutant, 712–984 (C808S/S810L/A976V), was prepared as described previously.[9] The ligands were added with a final concentration of 100 μM in all steps of sample preparation. The purified proteins were concentrated to a protein concentration of 7–10 mg/mL in buffer containing 25 mM HEPES pH 7.2, 0.2 M sodium chloride, 10 mM DTT, 10% glycerol, 0.05% β-octyl glucoside, 100 μM ligand. Crystals of the MR-LBD complexed with compound **2b** or compound **2e** were obtained at 20 °C by sitting-drop vapor diffusion method. The reservoir solution contained 0.04 M potassium dihydrogen phosphate, 16% PEG 8000, 20% glycerol for the MR-LBD/**2b** complex, and contained 0.1 M Tris pH 7.8, 23% ethanol for the MR-LBD/**2e** complex. Crystals were immersed in the reservoir solution containing 25% ethylene glycol and flash-frozen with liquid nitrogen. Diffraction data were collected at the Advanced Light Source beamline 5.0.3. The data were processed using HKL2000[11] and evaluated using SCALA[12] of the CCP4 program suite.[13] The structures were solved by molecular replacement with MOLREP[14] of the CCP4 program suite[13] using the MR-LBD structure (PDB code: 3VHV) as a search model. Several cycles of model building with Coot[15] and refinement with Refmac[16] were performed for improving the quality of the model. The dictionary files for the ligands were prepared using AFITT (OpenEye Scientific Software, USA). The final models were validated using Molprobity.[17] All structural figures were generated using PyMOL (Schrödinger, USA). Crystallographic processing and refinement statistics are summarized in Table 5. The coordinates and structure

Table 5
Data collection and refinement statistics

Crystal	MR-LBD/compound **2b**	MR-LBD/compound **2e**
Data collection		
Space group	$P3_121$	$P2_12_12_1$
Unit cell dimensions		
a, *b*, *c* (Å)	51.9, 51.9, 206	58.4, 66.8, 75.3
α, β, γ (°)	90, 90, 120	90, 90, 90
Resolution range (Å)	50–2.05 (2.10–20.5)	50–1.40 (1.43–1.40)
Observed reflections	156,531	269,767
Unique reflections	21,157	56,402
Redundancy	7.4 (6.6)	4.8 (3.4)
Completeness (%)	99.9 (99.4)	96.4 (74.7)
I/σ	23.1 (3.4)	22.9 (2.6)
R_{sym} [a]	0.079 (0.484)	0.058 (0.369)
R_{meas} [b]	0.084 (0.523)	0.066 (0.426)
R_{pim} [b]	0.030 (0.197)	0.029 (0.208)
$CC_{1/2}$ [c]	0.999 (0.868)	0.998 (0.886)
Molecules in ASU	1	1
Refinement		
Resolution (Å)	40–2.05 (2.10–20.5)	40–1.40 (1.44–1.40)
Reflections	20,007	53,493
R_{work} [d]	0.186 (0.232)	0.164 (0.262)
R_{free} [d]	0.216 (0.248)	0.179 (0.265)
Number of atoms		
Protein	2128	2089
Ligand/ion	30	77
Water	94	200
Average *B* value (Å^2) [e]	38.8	15
RMS deviation from ideal geometry		
Bond lengths (Å)	0.009	0.01
Bond angles (°)	1.404	1.452
Ramachandran plot (%) [f]		
Preferred regions	96.8	97.5
Allowed regions	2.4	2.5
Outliers	0.8	0
PDB code	3WFF	3WFG

[a] $R_{sym} = \Sigma_h\Sigma_i|I(h)_i - \langle I(h)\rangle|/\Sigma_h\Sigma_i\langle I(h)\rangle$, where $\langle I(h)\rangle$ is the mean intensity of symmetry-related reflections.

[b] $R_{meas} = \Sigma_h[N/(N-1)]^{1/2}\Sigma_i|I(h)_i - \langle I(h)\rangle|/\Sigma_h\Sigma_i\langle I(h)\rangle$, $R_{pim} = \Sigma_h[1/(N-1)]^{1/2}\Sigma_i|I(h)^i - \langle I(h)\rangle|/\Sigma_h\Sigma_i\langle I(h)\rangle$.

[c] $CC_{1/2}$, Pearson correlation coefficient between independently merged halves of the data set.

[d] $R_{work} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$. R_{free} was calculated for randomly chosen 5% of reflections excluded from refinement.

[e] *B*-Factor includes contributions from TLS parameters.

[f] Calculated with Coot. Values in parentheses are for the highest resolution shell.

factors have been deposited in PDB with accession codes 3WFF and 3WFG for **2b** and **2e**, respectively.

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