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Identification of Benzoxazin-3-one Derivatives as Novel, Potent, and Selective Nonsteroidal Mineralocorticoid Receptor Antagonists†

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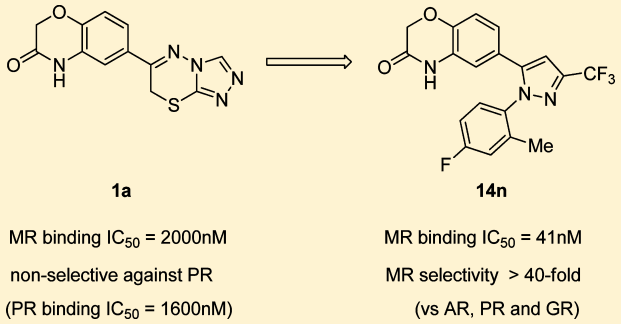
Supporting Information

ABSTRACT: Mineralocorticoid receptor (MR) blockade has come into focus as a promising approach for the treatment of cardiovascular diseases such as hypertension and congestive heart failure. In order to identify a novel class of nonsteroidal MR antagonists that exhibit significant potency and good selectivity over other steroidal hormone receptors, we designed a novel series of benzoxazin-3-one derivatives and synthesized them from 6-(7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazin-6-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (**1a**), high-throughput screening (HTS) hit compound. Our design was based on a crystal structure of an MR/compound complex and a docking model. In the course of lead generation from **1a**, a 1,2-diaryl framework was characterized as a key structure with high binding affinity. On the basis of scaffold hopping and optimization studies, benzoxazin-3-one derivatives possessing 1-phenyl-3-trifluoromethylpyrazol-5-yl moiety at the 6-position were identified as a novel series of potent and selective MR antagonists. Among these compounds, 6-[1-(4-fluoro-2-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (**14n**) showed highly potent activity and good selectivity and also exhibited a significant antihypertensive effect in deoxycorticosterone acetate−salt hypertensive rats. On the basis of these results, compound **14n** was progressed for further pharmacological evaluation.



INTRODUCTION

Mineralocorticoid receptor (MR) is a member of the superfamily of nuclear hormone receptors (NHRs), which have structures that share high homology with those of other steroid hormone receptors, exemplified by androgen receptor (AR), progesterone receptor (PR), and glucocorticoid receptor (GR). Aldosterone, a primary natural ligand for MR, regulates body fluid and electrolyte balance through binding to MR in kidney and plays an important role in blood pressure control.[1] Recently, it has been reported that aldosterone is also synthesized in heart and blood vessels, and an elevated level of aldosterone is closely associated with the development of congestive heart failure and renal dysfunction in addition to hypertension.[2,3] Thus, aldosterone blocking has attracted great attention as a promising therapeutic option for such diseases.

Indeed, MR antagonists, such as spironolactone[4] and eplerenone,[5] have shown antihypertensive effects in patients with essential hypertension.[6] It was noticeable that the addition of MR antagonists to standard antihypertensive therapy effectively reduced blood pressure in refractory hypertension patients.[7] In addition, the large scale clinical trials (RALES and EPHESUS) have proved the utility of MR antagonists in heart failure therapy.[8,9] Furthermore, a recent study revealed that MR antagonists have a therapeutic potential for diabetic nephropathy.[10]

However, spironolactone treatment has limitations like sexual adverse effects, such as impotence, gynecomastia, and menstrual irregularity, caused by nonselective binding to AR and/or PR.[11] Meanwhile, eplerenone was a more selective MR antagonist and had few sexual adverse effects but its MR antagonistic activity and antihypertensive effect were less potent than those of spironolactone.[12] It is expected that an MR antagonist with sufficient potency and selectivity to prevent sexual adverse effects would be a clinically convenient and valuable agent. Although several nonsteroidal MR antagonists have been reported,[13] their clinical utilities have not been confirmed. Thus, the goal of our project was to discover a novel series of potent and selective nonsteroidal MR antagonists.

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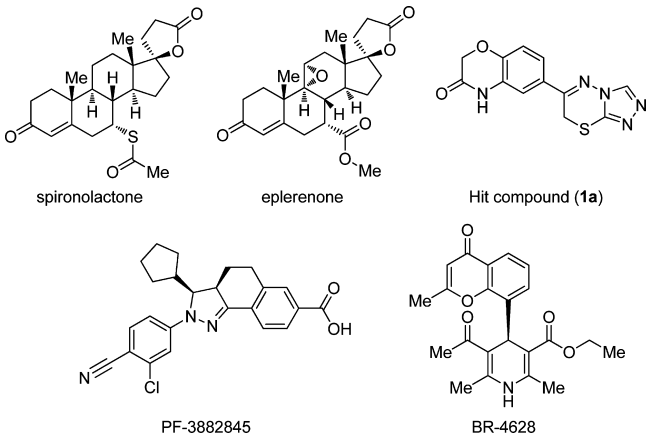
We performed competitive radiometric binding high-throughput screening (HTS) assay and identified benzoxazin-3-one **1a** as an MR antagonist (Chart 1, IC_{50} = 2000 nM in binding assay)

Chart 1



with suitable properties for a hit compound such as low molecular weight and low lipophilicity. In this paper, we report the lead generation from **1a** and the subsequent optimization by utilizing an X-ray crystal structure, which led to the identification of 6-[1-(4-fluoro-2-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (**14n**) as a novel nonsteroidal MR antagonist. The compound **14n** showed highly potent in vitro activity, good selectivity over the other steroid hormone receptors, and significant antihypertensive effect in deoxycorticosterone acetate (DOCA)−salt hypertensive rats and was therefore selected for further pharmacological evaluation.

CHEMISTRY

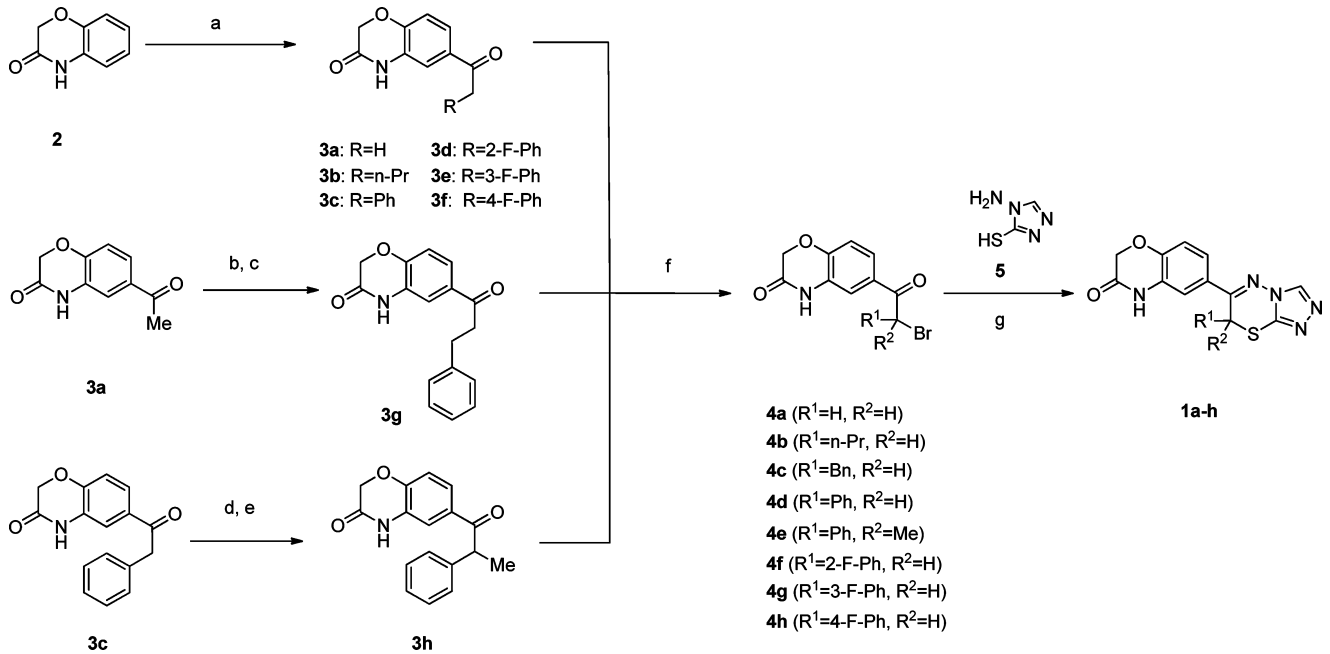
Triazolothiadiazines **1a−h** were prepared according to Scheme 1. Acetophenone **3a** was commercially available, and the ketones **3b−f** were obtained by Friedel−Crafts acylation of 2*H*-1,4-benzoxazin-3(4*H*)-one **2**. Condensation of acetophenone **3a** with benzaldehyde and subsequent hydrogenation afforded phenethyl ketone **3g**. Benzyl ketone **3c** was converted to the 2-methyl derivative **3h** in two steps by a manner similar to that described for **3g**. Ketones **3a−h** were brominated and subsequently condensed with 4-amino-3-mercapto-4*H*-1,2,4-triazole **5** to give triazolothiadiazines **1a−h** in good yield.

Compounds **7**, **8**, **9**, and **10** were prepared by condensation of bromoketone **4c** with 1-amino-2-mercaptoimidazole **6**, 2-aminothiophenol, thioacetamide, and acetamidine, respectively (Scheme 2). Imidazole **6** was prepared in two steps from commercially available thiadiazole **11**.

The synthesis of 3-substituted pyrazoles **14a−o** and **16** is described in Scheme 3. The acetophenone **3a** was converted to the diketones **13a−f** in good yields. The acid-mediated condensation of diketones **13a−f** with arylhydrazines afforded the 3-substituted pyrazoles **14a−o**. The 3-cyanopyrazole **16** was obtained in three steps from ester **14o** via dehydration of the carboxamide group of compound **15**. The ethyl ester of compound **14o** was reduced by lithium aluminum hydride to afford 3-hydroxymethylpyrazole **17**. Hydrazine **18** was prepared by the reduction of the diazonium salt of 4-fluoro-2-methylaniline.

The 4-trifluoromethylpyrazole **21** was prepared as shown in Scheme 4. The acetophenone **3a** was reacted with *N*,*N*-dimethylformamide dimethyl acetal (DMFDMA) and subsequently condensed with 4-fluorophenylhydrazine to give 3,4-unsubstituted pyrazole **19**. Iodination at the 4-position by *N*-iodosuccinimide and subsequent copper-catalyzed trifluoromethylation afforded the 4-trifluoromethylpyrazole **21**.

Scheme 1. Synthesis of Triazolothiadiazines 1a−h[a]



[a]Reagents and conditions: (a) RCH_2COCl, $AlCl_3$, $ClCH_2CH_2Cl$, 25−88%; (b) PhCHO, NaOMe, MeOH, 60 °C, 87%; (c) H_2, Pd/C, EtOH, 25%; (d) $Me_2NCH_2NMe_2$, Ac_2O, CH_2Cl_2, 56%; (e) H_2, Pd/C, THF, 66%; (f) HBr_3·Py, HBr, AcOH, 85−99%; (g) 4-amino-3-mercapto-4*H*-1,2,4-triazole **5**, EtOH/toluene, reflux, 24−97%.

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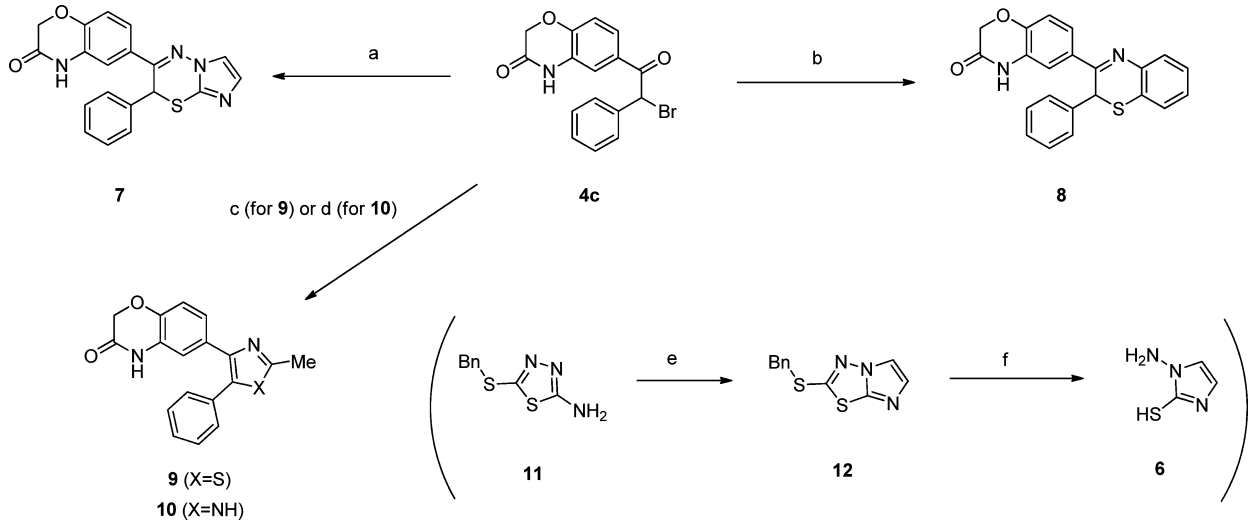
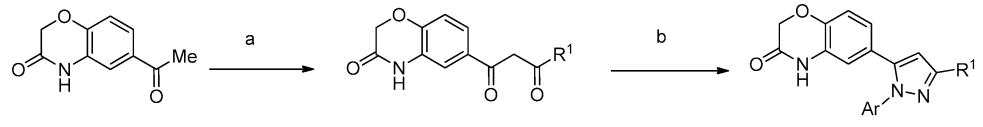
Scheme 2. Synthesis of Imidazothiadiazine 7, Benzothiazine 8, Thiazole 9, and Imidazole 10[a]



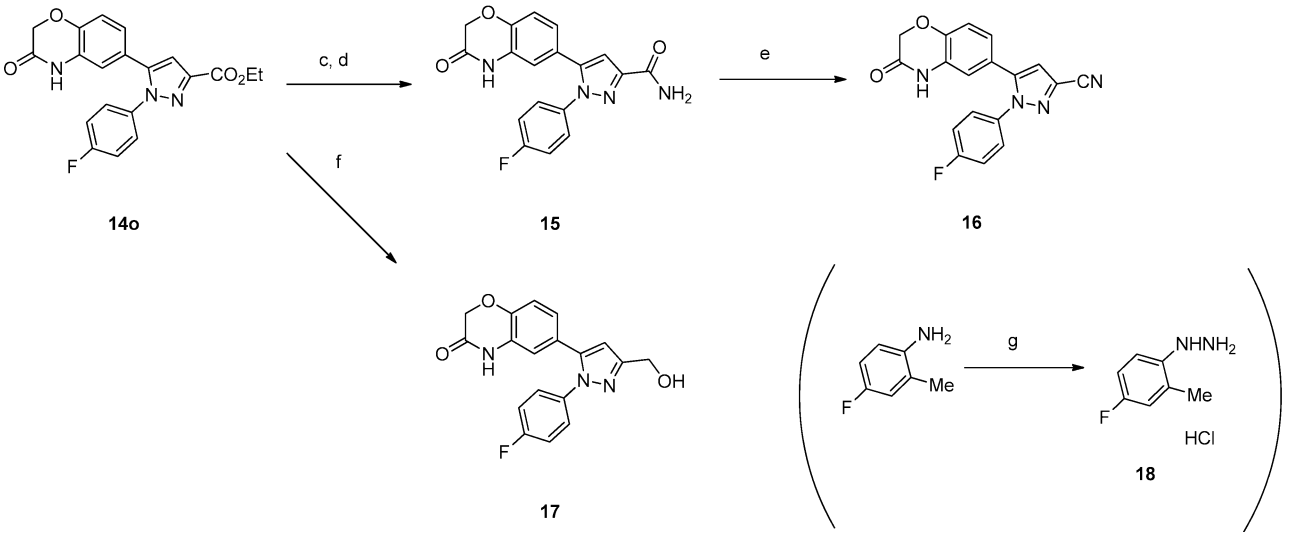
[a]Reagents and reaction conditions: (a) **6**, EtOH, reflux, 17%; (b) 2-aminothiophenol, EtOH, reflux, 19%; (c) thioacetamide, NaOEt, EtOH, reflux, 23%; (d) acetamidine, K_2CO_3, EtOH, reflux, 14%; (e) 45% chloroacetaldehyde, EtOH/toluene, reflux, 23%; (f) hydrazine monohydrate, EtOH, reflux, 32%.

Scheme 3. Synthesis of 3-Substituted Pyrazoles 14a–o, 16, and 17[a]



13a (R^1=Me) **13d** (R^1=CF_3)
13b (R^1=Et) **13e** (R^1=CF_2CF_3)
13c (R^1=^{i}Pr) **13f** (R^1=CO_2Et)

14a (R^1=Me, Ar = Ph)
14b (R^1=Me, Ar = 4-F-Ph)
14c (R^1=Et, Ar = 4-F-Ph)
14d (R^1=CF_3, Ar = 4-F-Ph)
14e (R^1=CF_2CF_3, Ar = 4-F-Ph)
14f (R^1=^{i}Pr, Ar = 4-F-Ph)
14g (R^1=CF_3, Ar = 2-F-Ph)
14h (R^1=CF_3, Ar = 3-F-Ph)
14i (R_1=CF_3, Ar = 2-Cl-Ph)
14j (R^1=CF_3, Ar = 4-Cl-Ph)
14k (R^1=CF_3, Ar = 2-Me-Ph)
14l (R^1=CF_3, Ar = 4-Me-Ph)
14m (R^1=CF_3, Ar = 2-Et-Ph)
14n (R^1=CF_3, Ar = 2-Me-4-F-Ph)
14o (R^1=CO_2Et, Ar = 4-F-Ph)



[a]Reagents and conditions: (a) 60% NaH, 2,4-dibenzo-18-crown-6, R^1CO_2Et, THF, reflux, 43–95%; (b) $ArNH_2NH_2$, 2-propanol, TFA, reflux, 12–86%; (c) 1 N NaOH, MeOH, 75%; (d) 2 M NH_3 in MeOH, EDCI, HOBt, DMF, 76%; (e) TFAA, pyridine, dioxane, 38%; (f) $LiAlH_4$, THF, 34% (g) (1) $NaNO_2$, HCl; (2) $SnCl_2$, 48% in two steps.

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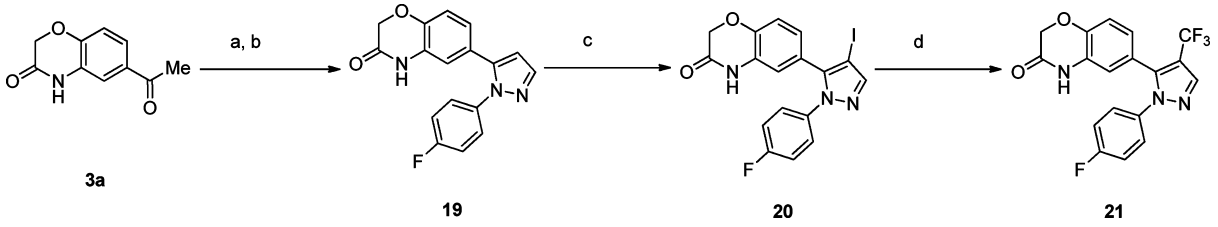
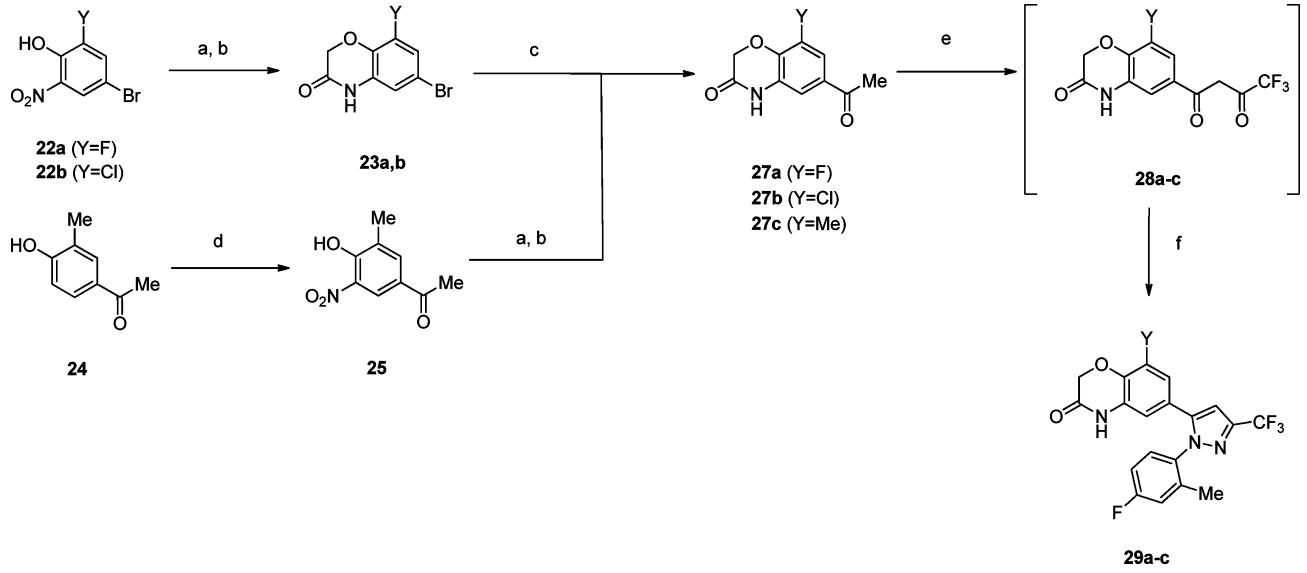
Scheme 4. Synthesis of 4-Trifluoromethylpyrazole 21[a]



[a]Reagents and conditions: (a) DMFDMA, 80 °C, 73%; (b) 4-fluorophenylhydrazine, EtOH, 40 °C, 44%; (c) NIS, DMF, 55 °C, 50%; (d) $FSO_2CF_2CO_2Et$, CuI, DMF, 100 °C, 5%.

Scheme 5. Synthesis of 8-Substituted Benzoxazin-3-one Derivatives 29a–c[a]



[a]Reagents and conditions: (a) $BrCH_2CO_2Et$, K_2CO_3, DMF, 65 °C; (b) Zn, AcOH, 100 °C, 34–89% in two steps; (c) 1) 4-(vinyloxy)butan-1-ol, $PdCl_2P(o\text{-tolyl})_3$, DMF/$H_2O$, 80 °C; (2) 2 N HCl, reflux, 72% in two steps; (d) HNO_3, AcOH, room temperature, 59%; (e) NaH, 2,4-dibenzo-18-crown-6, CF_3CO_2Et, THF, 60 °C; (f) 1-(4-fluoro-2-methylphenyl)hydrazine hydrochloride **18**, TFA, 2-propanol, reflux, 16–30% in two steps.

The synthesis of 8-substituted benzoxazin-3-one derivatives **29a–c** is described in Scheme 5. Alkylation of nitrophenol **22** and subsequent Zn-mediated reductive cyclization afforded the benzoxazin-3-one **23**. Heck reaction of compound **23** and subsequent acid hydrolysis gave 8-halobenzoxazin-3-ones **27a,b**. The acetophenone **24** was converted to the nitro derivative **25** by nitric acid. 8-Methylbenzoxazin-3-one **27c** was prepared from **25** by a manner similar to that described for benzoxazin-3-one **23**. The acetophenones **27a–c** were converted to diketones **28a–c** and subsequent acid-mediated pyrazole-ring formation afforded 8-substituted benzoxazin-3-ones **29a–c** in good yield.

RESULTS AND DISCUSSION

The synthesized compounds were evaluated for their inhibitory activity against the binding of [^{3}H]aldosterone to MR. Further, the selectivity of these compounds over other steroid hormone receptors was determined by radioligand binding assays (AR, [^{3}H]testosterone; PR, [^{3}H]progesterone; GR, [^{3}H]-dexamethasone). The results are presented in Tables 1–4 as IC_{50} values.

Lead Generation. The issues of HTS hit compound **1a** that were initially addressed were potency (IC_{50} = 2000 nM) and selectivity over PR (IC_{50} = 1600 nM in PR binding assay, Table 1). A docking model of **1a** with MR, constructed based on the X-ray cocrystal structure of MR with spironolactone obtained in-house, is illustrated in Figure 1a. This model suggested that compound **1a** binds to the steroid binding site of MR similarly to spilonolactone and interacts via hydrogen bonds with Asn770 and Gln776. Notably, compound **1a** did not fill a hydrophobic pocket that was occupied by the thioester group of spironolactone. This finding led us to hypothesize that filling this pocket with a lipophilic substituent would increase the binding affinity by hydrophobic interaction. Therefore, several lipophilic substituents were introduced at the 2-position of the triazolothiadiazine ring (Table 1). Introduction of a propyl (**1b**) or a benzyl group (**1c**) had little influence on potency. In contrast, the phenyl compound **1d**, containing 1,2-diaryl framework, showed a 4-fold increase in potency (IC_{50} = 510 nM). Moreover, compound **1d** showed improved selectivity against PR. Introduction of an additional methyl group at the 2-position of compound **1d** led to a large decrease in activity (**1e**).

Figure 1b shows an X-ray crystal structure of **1d** with MR. To our knowledge, this is the first example of an X-ray structure of a nonsteroidal compound bound to MR. This crystal structure revealed the binding mode of compound **1d**, in which interactions between benzoxazin-3-one moiety and Asn770 and among nitrogen atoms of the triazole, Gln776, and Arg817 were observed, and the phenyl group at the 2-position of the

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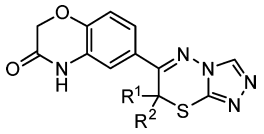
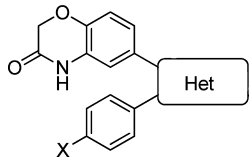
Table 1. Effects of Introduction of Substituents at the 2-Position of Triazolothiadiazine Ring[a]



compd	R^1	R^2	MR binding IC_{50} (nM)	selectivity, IC_{50} (nM) AR binding	PR binding	GR binding
1a	H	H	2000 (1500−2800)	>10000	1600 (1100−2300)	>10000
1b	*n*-Pr	H	4100 (3300−5100)	nt[b]	nt[b]	nt[b]
1c	Bn	H	1300 (990−1800)	nt[b]	nt[b]	nt[b]
1d	Ph	H	510 (340−770)	3600 (2800−4700)	>10000	5600 (3100−10000)
1e	Ph	Me	>10000	nt[b]	nt[b]	nt[b]
1f	2-F-Ph	H	600 (500−710)	5900 (3300−10000)	>10000	5500 (3100−9600)
1g	3-F-Ph	H	310 (210−470)	4700 (2700−8400)	>10000	>10000
1h	4-F-Ph	H	270 (190−400)	>10000	>10000	>10000

[a]IC_{50} values are shown as the mean of duplicate experiments. IC_{50} values and 95% confidence limits are calculated from the concentration−response curves generated by GraphPad Prism. [b]Not tested.

Table 2. SAR Summary for Central Ring Moiety[a]



compound No.	Het	X	MR binding IC_{50} (nM)	selectivity AR binding IC_{50} (nM)	PR binding IC_{50} (nM)	GR binding IC_{50} (nM)
7		H	36 (23–57)	95 (74–120)	350 (300–420)	3500 (2600–4500)
8		H	69 (51–94)	1200 (770–2000)	1400 (820–2300)	950 (180–4900)
9		H	>10000	n.t.[b]	n.t.[b]	n.t.[b]
10		H	>10000	n.t.[b]	n.t.[b]	n.t.[b]
14a		H	5300 (2700–10000)	>10000	>10000	>10000
14b		F	1100 (620–1900)	>10000	>10000	6900 (3500–13000)

[a]IC_{50} values are shown as the mean of duplicate experiments. IC_{50} values and 95% confidence limits are calculated from the concentration−response curves generated by GraphPad Prism. [b]Not tested.

triazolothiadiazine ring filled the hydrophobic pocket as designed. The structure also showed a small space around the introduced phenyl ring. Therefore, a fluorine atom was introduced at the ortho-, meta-, or para-position of the benzene ring with the expectation of an increase in binding affinity. Among these compounds, the 3-fluoro and 4-fluorophenyl compounds showed increased binding affinities (**1g**, IC_{50} = 310 nM; **1h**, IC_{50} = 270 nM). Interestingly, the selectivities of all these fluorinated compounds over the other steroid hormone receptors were improved compared to that of compound **1d**. In particular, the 4-fluorophenyl compound **1h** showed significantly improved selectivity (vs AR, PR, and GR > 35-fold). On the basis of this result, we rationalized that the corresponding hydrophobic pockets of AR, PR, and GR are smaller than that of MR.

Although compound **1h** exhibited good potency and selectivity, **1h** and related analogues showed poor PK profiles in rats (data not shown), probably because of the metabolically

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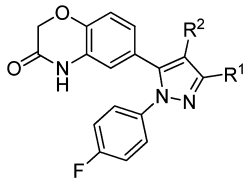
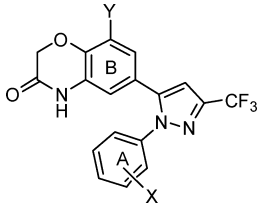
Table 3. SAR Summary for Substituent on Pyrazole Ring[a]



compd	R^1	R^2	MR binding IC_{50} (nM)
14c	Et	H	960 (670–1400)
14d	CF_3	H	260 (220–310)
14e	CF_2CF_3	H	570 (480–670)
14f	*i*-Pr	H	>10000
16	CN	H	920 (750–1100)
17	CH_2OH	H	6800 (4000–12000)
21	H	CF_3	2700 (1900–4000)

[a] IC_{50} values are shown as the means of duplicate experiments. IC_{50} values and 95% confidence limits are calculated from the concentration–response curves generated by GraphPad Prism.

Table 4. SAR Summary for Benzene Ring (A-Ring) at 1-Position of Pyrazole Ring and Benzene Ring (B-Ring) in the Benzoxazin-3-one Moiety[a]



compd	X	Y	MR binding IC_{50} (nM)
14g	2-F	H	440 (350–560)
14h	3-F	H	1100 (880–1300)
14i	2-Cl	H	430 (350–530)
14j	4-Cl	H	320 (240–430)
14k	2-Me	H	80 (65–100)
14l	4-Me	H	590 (400–860)
14m	2-Et	H	700 (500–990)
14n	2-Me, 4-F	H	41 (32–53)
29a	2-Me, 4-F	F	76 (64–90)
29b	2-Me, 4-F	Cl	12 (9.4–15)
29c	2-Me, 4-F	Me	21 (19–24)

[a] IC_{50} values are shown as the mean of duplicate experiments. IC_{50} values and 95% confidence limits are calculated from the concentration–response curves generated by GraphPad Prism.

labile thioether and cyclic imine of triazolothiadiazine ring (**1h**, the metabolic clearance in rat microsomes of 60 μL min^{-1} mg^{-1}). Therefore, we concentrated our efforts to investigate alternative scaffolds without metabolically labile groups. Table 2 shows structure–activity relationships (SAR) of the central ring moiety. Replacement of triazolothiadiazine ring with an imidazothiadiazine (**7**) or a benzothiazine ring (**8**) was investigated for an initial SAR, while these compounds still possessed the metabolic labile groups. Surprisingly, these compounds exhibited significant binding affinities despite the decrease or lack of hydrogen-bonding interactions with Gln776 and Arg817, which we had assumed to be a key interaction for binding to MR (**7**, IC_{50} = 36 nM; **8**, IC_{50} = 69 nM). These results indicated that the interaction with Gln776 and Arg817 did not significantly contribute to binding activity of **1h** and that the hydrogen-bonding interaction with Asn770 and the hydrophobic interaction with the phenyl group were more important for potent binding affinity in this benzoxazin-3-one series. The reason for the increase in binding affinity of these compounds could be explained by the increase in lipophilicity of the central ring. Further, these results suggested that more drastic modification of the central ring moiety could be tolerated and encouraged us to explore a new "leadlike" scaffold such as a five-membered heteroaromatic ring. Replacement of the bicyclic cores by a thiazole or an imidazole ring led to a significant decrease in activity (**9**, **10**). In contrast, a pyrazole derivative **14a** showed moderate binding affinity. In anticipation of increasing the binding affinity of **14a**, the fluorine atom was introduced at the para-position of the benzene ring. As expected, the fluorinated compound **14b** showed a 5-fold improvement in the binding affinity over **14a** and importantly exhibited good selectivity over the other steroid hormone receptors. While compound **14b** was still metabolically unstable (metabolic clearance in rat microsomes of 164 μL min^{-1} mg^{-1}), it was presumed that this metabolic instability could be improved by modifying the methyl group, which was the putative metabolic site of **14b**. On the basis of the advantageous features such as good selectivity, lower molecular weight, elimination of the chiral center, and the potential for improved metabolic clearance, the pyrazole ring was selected as a lead scaffold for further modification. Thus, the compound **14b** was advanced into lead optimization.

Lead Optimization. Initially, our effort was focused on changing the methyl group on the pyrazole ring to increase the binding affinity and improve the metabolic clearance of compound **14b** (Table 3). The docking model of compound **14b** with MR, shown in Figure 2, suggested that the 3-methylpyrazole moiety did not fully occupy the space filled by the triazolothiadiazine ring of compound **1d** in the MR binding site. Accordingly, larger substituents were introduced at the pyrazole 3-position. Compounds **14c–e**, bearing an ethyl, trifluoromethyl, or pentafluoroethyl group, showed increased potency as predicted. In particular, 3-trifluoromethylpyrazole **14d** showed a 4-fold improvement in activity over **14b** (IC_{50} = 260 nM vs 1100 nM). Furthermore, compound **14d** showed improved PK profile in rats (BA = 28%), likely due to improved metabolic clearance (metabolic clearance in rat microsomes: **14b**, 164 μL min^{-1} mg^{-1}; **14d**, 24 μL min^{-1} mg^{-1}). In contrast, isopropyl analogue **14f** showed significantly decreased potency, suggesting that the branched substituents were too large for this position. The MR binding model of 3-methylpyrazole **14b** further showed that the 3-position substituent of the pyrazole ring was oriented toward Gln776 and Arg817. Hydrogen bond accepting or donating groups were introduced with a hope to establish an interaction with these residues. However, replacement of the methyl group with a cyano or a hydroxymethyl group did not result in a significant improvement in activity (**16**, IC_{50} = 920 nM; **17**, IC_{50} = 6800 nM). Transposition of the trifluoromethyl group from the 3- to the 4-position also led to decreased binding activity (**21**, IC_{50} = 2700 nM). Consequently, the 3-trifluoromethylpyrazole was optimal among the substituents explored here.

Optimization of the substituents on the benzene rings (A and B) at the 1-position of the pyrazole ring and in the benzoxazin-3-one moiety was performed within the 3-trifluoromethylpyrazole series, and the results are shown in Table 4. Among the three fluorophenyl derivatives (**14d**, **14g**, and **14h**), the 4-fluoro compound **14d** showed the most potent binding affinity. The

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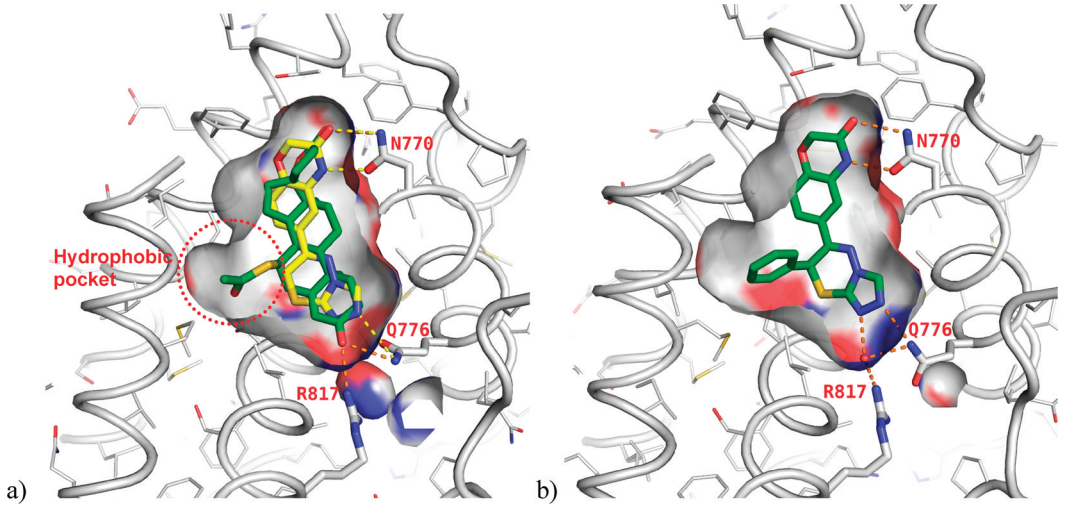

Figure 1. (a) Overlay of a compound **1a** (yellow) and spironolactone (green) in MR. Compound **1a** was docked to MR in the X-ray crystal structure of MR/spironolactone obtained in-house. (b) X-ray crystal structure of MR/compound **1d** complex. The hydrogen bonds are shown as orange or yellow dotted lines.

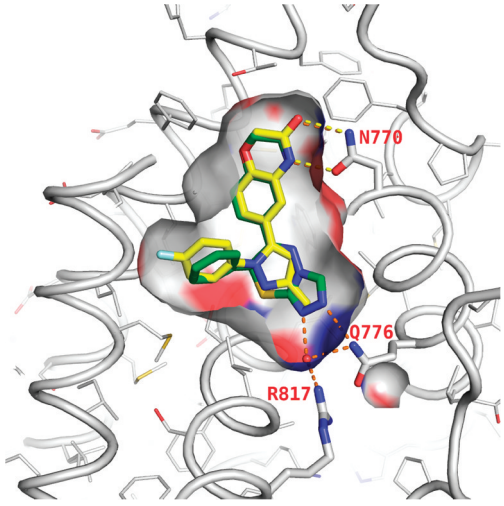

Figure 2. Overlay of the compound **14b** (yellow) and compound **1d** (green) in MR. Compound **14b** was docked to the X-ray crystal structure of MR/compound **1d** complex. The hydrogen bonds are shown as orange or yellow dotted lines.

incorporation of a chlorine atom gave similar results as that of the fluorine atom. The 4-chloro compound **14j** showed more potent activity than the 2-chloro compound **14i**. Surprisingly, installation of a methyl group at the 2-position resulted in a marked increase in binding affinity and its activity was 5-fold more potent (**14k**, IC_{50} = 80 nM) than the 2-fluoro and the 2-chloro compounds (**14g** and **14i**). This effect of high potency unique to the 2-methyl group could be explained by a hydrophobic interaction with Leu769, Leu814, and Met852 around the 2-position of the A-ring and/or a stabilization effect of preferred twisted conformation between the pyrazole ring and A ring. In contrast, installation of a methyl group at the 4-position (**14l**) led to a 3-fold decrease in potency relative to the 4-fluoro and 4-chloro compounds (**14d** and **14j**). Replacement of the 2-methyl group with an ethyl group decreased the activity (**14m**, IC_{50} = 700 nM). A synergistic effect was observed when combining the optimal 2-methyl (**14k**, IC_{50} = 80 nM) and the 4-fluoro (**14d**, IC_{50} = 260 nM) substituents, resulting in 2-methyl-4-fluoro compound **14n** with significantly potent binding activity (IC_{50} = 41 nM). Compound **14n** was docked to MR, and its binding mode was similar to that of **14b**.[14] These docking models showed a small pocket around the 8-position of the B-ring. In the 8-position of **14n**, substitution with a fluorine atom, chlorine atom, or methyl group was performed in anticipation of further enhancement of potency. While the installation of a fluorine atom had little influence on the binding activity (**29a**, IC_{50} = 76 nM), introduction of a chlorine atom or a methyl group led to about 2- or 3-fold more potent activity than **14n** (**29b**, IC_{50} = 12 nM; **29c**, IC_{50} = 21 nM).

The five compounds with potent binding activities (**14k**, **14n**, **29a**, **29b**, and **29c**) were evaluated for their selectivity over the other steroid hormone receptors and MR antagonistic activity in a reporter gene assay in COS-1 cells (Table 5). The 2-methyl compound **14k** showed an approximately 3-fold loss in functional activity relative to spironolactone. In contrast, the 4-fluoro-2-methyl compound **14n** showed equipotent MR antagonistic activity (IC_{50} = 43 nM) to spironolactone, and it also showed good selectivity over the other steroidal hormone receptors (vs AR, PR, and GR > 40-fold). It was noteworthy that the selectivity of **14n** over AR and PR was greatly improved compared to those of spironolactone (**14n**, for AR > 240-fold, for PR = 45-fold; spironolactone, for AR = 3-fold, for PR = 15-fold). An in vivo safety evaluation will be conducted for an estimation of the sexual side effects in clinical use. Compound **29a** also showed good functional activity and selectivity, but it was inferior to **14n**. Although compounds **29b** and **29c** showed potent functional activity, their selectivity over GR was less than that of spironolactone. Compound **14n** showed the best balance of potency and selectivity and in addition showed a superior PK profile in rats (Table 6). On the basis of these results, compound **14n** was evaluated for MR antagonist activity in vivo.

First, the potassium-sparing and natriuretic activity of compound **14n** was evaluated in Wistar rats (Figure 3A). Compound **14n** (10, 30, or 100 mg/kg) and spironolactone (30 mg/kg) were orally administered 30 min before the aldosterone injection (3 μg/kg, sc) and oral saline load (25 mL/kg). Urine (5 h after saline load) was collected, and urinary Na^+ and K^+ concentrations were measured. Compound **14n** increased the urinary Na^+/K^+ ratio in a dose-dependent manner, and the effect at doses of

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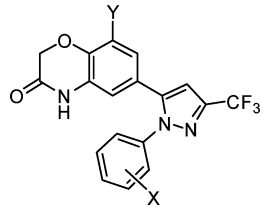
Table 5. Steroid Hormone Receptor Selectivities and MR Antagonistic Activities of Selected Compounds[a]



				selectivity, IC_{50} (nM)			
compd	X	Y	MR binding	AR binding	PR binding	GR binding)	MR antagonistic activity IC_{50} (nM) [b]
14k	2-Me	H	80	>10000	4900	2500	150
			(65−100)		(3000−8000)	(1600−3700)	(80−280)
14n	2-Me, 4-F	H	41	>10000	1900	1800	43
			(32−53)		(1400−2700)	(1300−2200)	(24−79)
29a	2-Me, 4-F	F	76	>10000	1800	1700	85
			(64−90)		(1200−2500)	(1200−2400)	(42−170)
29b	2-Me, 4-F	Cl	12	4500	930	200	70
			(9.5−15)	(3000−8000)	(730−1200)	(150−260)	(45−110)
29c	2-Me, 4-F	Me	21	>10000	2400	470	15
			(19−24)		(1600−3700)	(370−590)	(8.2−26)
spironolactone			49	120	650	1400	60
			(36−66)	(100−140)	(460−920)	(880−2100)	(32−110)
eplerenone			2600	>10000	>10000	>10000	1300
			(1900−3400)				(700−2400)

[a] IC_{50} values are shown as the mean of duplicate or triplicate experiments. IC_{50} values and 95% confidence limits are calculated from the concentration−response curves generated by GraphPad Prism. [b] Inhibitory activity of transcription of aldosterone (reporter gene assay).

Table 6. Pharmacokinetic Profiles of Compound 14n in SD Rats[a]

CL (mL h^{-1} kg^{-1})	Vd_{ss} (mL/kg)	$AUC_{0-24h,po}$ (μg·h/mL)	MRT_{po} (h)	BA (%)
1328 ± 88	4626 ± 315	3.829 ± 1.172	4.39 ± 0.12	50.7 ± 15.9

[a] Rats were administered the drug intravenously at 3 mg/kg and orally at 10 mg/kg (n = 3).

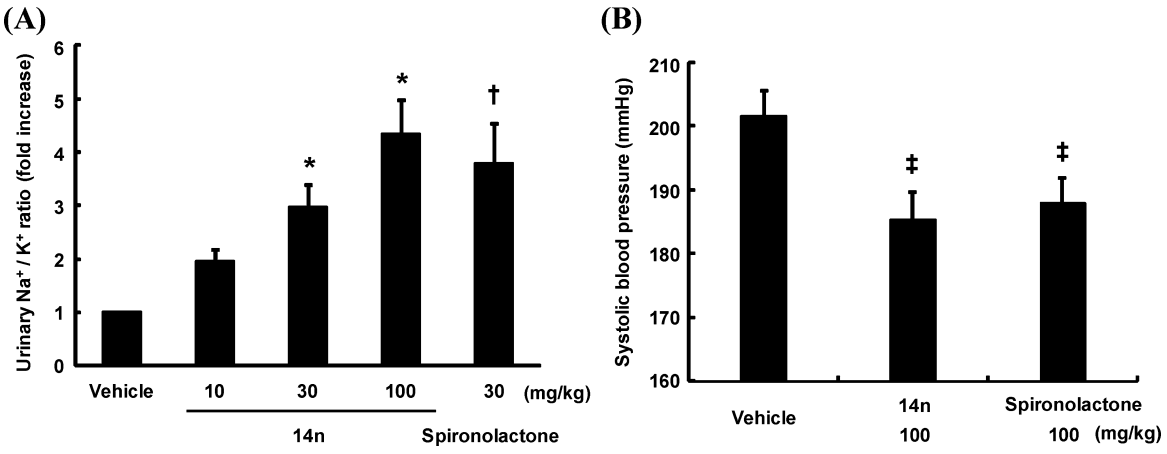


Figure 3. Potassium-sparing and natriuretic (A) and antihypertensive (B) effects of compound **14n** in rats. (A) Wistar rats were administered vehicle, compound **14n** at 10, 30, or 100 mg/kg, or spironolactone at 30 mg/kg 30 min before aldosterone injection (3 μg/kg, sc) and oral saline load (25 mL/kg). After the saline load, 5 h urine was collected and urinary Na^+ and K^+ were measured: mean ± SEM (n = 5); *, $p < 0.025$ vs vehicle by one-tailed Williams' test; †, $p < 0.05$ vs vehicle by Aspin−Welch test. (B) DOCA−salt hypertensive rats were treated with vehicle, compound **14n**, or spironolactone at a dose of 100 mg/kg for 13 days. SBP and HR were measured by tail-cuff method approximately 24 h after the last administration. Pretreatment values of SBP in each group were 193−194 mmHg: mean ± SEM (n = 7−8); ‡, $p < 0.05$ vs vehicle by Student's t test.

30 and 100 mg/kg was significant compared to vehicle treatment. Similarly, spironolactone (30 mg/kg) increased the urinary Na^+/K^+ ratio significantly. The effect of both compounds at 30 mg/kg was comparable. Second, the antihypertensive effect of **14n** was evaluated in established DOCA−salt hypertensive rats (Figure 3B). Compound **14n** (100 mg/kg, q.d.) and spironolactone (100 mg/kg, q.d.) were administered orally for 13 days, and systolic blood pressure (SBP) and heart rate (HR) were measured by tail-cuff method approximately 24 h after the last administration. Both compounds significantly lowered SBP at a dose of 100 mg/kg,

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and their antihypertensive effects were similar. In addition, these compounds did not affect the HR (data not shown). These results indicated that compound **14n** exhibited MR antagonistic activity and blood pressure-lowering effect in vivo, comparable to that of spironolactone.

CONCLUSION

We have discovered benzoxazin-3-one derivatives as a novel class of potent and selective nonsteroidal MR antagonists and described the lead generation and optimization from hit compound **1a**. Installation of a phenyl group at the 2-position in the triazolothiadiazine ring revealed that a 1,2-diaryl framework was a key structure for potent activity in this series. Subsequent scaffold hopping led to a central pyrazole ring in the lead compound **14b** showing selective MR binding activity. Optimization of the substituents of **14b** resulted in the identification of 6-[1-(4-fluoro-2-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (**14n**). Compound **14n** showed excellent in vitro potency and good selectivity, and it also exhibited significant blood-pressure-lowering effect in DOCA−salt hypertensive rats. On the basis of these results, compound **14n** was progressed for further pharmacological evaluation. Additional results of continued investigation of this benzoxazin-3-one series will be reported in due course.

EXPERIMENTAL SECTION

General. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Bruker Ultra Shield-300 (300 MHz) or Varian INOVA-400 (400 MHz) instruments. Chemical shifts are given in parts per million (ppm) with tetramethylsilane as an internal standard. Abbreviations used are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublets of doublet, brs = broad singlet. Coupling constants (*J*) are given in hertz (Hz). The acidic protons of diketones, carboxylic acids, alcohols, or anilines were not frequently observed in ^{1}H NMR spectra. Elemental analyses and high-resolution mass spectrometry (HRMS) 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 mm × 50 mm, 2 μm). Elution was 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. Reagents and solvents were obtained from commercial sources and used without further purification. 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 mm × 150 mm, 5 μm) column, and elution was 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. Abbreviations of the solvents are used as follows: EtOAc, ethyl acetate; THF, tetrahydrofuran; MeOH, methanol; EtOH, ethanol; DMF, *N*,*N*-dimethylformamide; CH_3CN, acetonitrile; IPE, diisopropyl ether.

6-Pentanoyl-2*H*-1,4-benzoxazin-3(4*H*)-one (3b). To a suspension of 2*H*-1,4-benzoxazin-3(4*H*)-one (10 g, 67 mmol) in 1,2-dichloroethane (120 mL) were added powdered $AlCl_3$ (20 g, 150 mmol) and valeryl chloride (9.6 mL, 80.9 mmol) at 0 °C, successively. The mixture was stirred at 80 °C for 3 h, poured into ice-cooled water, and extracted with dichloromethane. The organic layer was washed with saturated $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was crystallized from MeOH to give **3b** (12.0 g, 77%) as white crystals. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.89 (3H, t, J = 7.4 Hz), 1.24−1.40 (2H m), 1.50−1.64 (2H, m), 2.91 (2H, t, J = 7.2 Hz), 4.68 (2H, s), 7.03 (1H, d, J = 8.4 Hz), 7.48 (1H, d, J = 2.0 Hz), 7.61 (1H, dd, J = 8.4, 2.0 Hz), 10.85 (1H, brs).

Compound **3c−f** were prepared in a manner similar to that described for **3b**.

6-(Phenylacetyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (3c). Yield, 46%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.22 (2H, s), 4.69 (2H, s), 7.00 (1H, d, J = 8.4 Hz), 7.22−7.36 (5H, m), 7.48−7.49 (1H, d, J = 2.1 Hz), 7.68 (1H, dd, J = 8.4, 2.1 Hz), 8.10 (1H, brs).

6-[(2-Fluorophenyl)acetyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (3d). Yield, 88%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.26 (2H, s), 4.70 (2H, s), 7.02−7.29 (5H, m), 7.51 (1H, d, J = 1.8 Hz), 7.70 (1H, dd, J = 1.8, 8.4 Hz), 8.15−8.30 (1H, brs).

6-[(3-Fluorophenyl)acetyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (3e). Yield, 25%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.35 (2H, s), 4.69 (2H, s), 7.01−7.15 (4H, m), 7.30−7.40 (1H, m), 7.52 (1H, d, J = 2.0 Hz), 7.73 (1H, dd, J = 2.0, 8.5 Hz), 10.89 (1H, brs).

6-[(4-Fluorophenyl)acetyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (3f). Yield, 36%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.31 (2H, s), 4.69 (2H, s), 7.02−7.20 (3H, m), 7.23−7.33 (2H, m), 7.52 (1H, d, J = 1.9 Hz), 7.73 (1H, dd, J = 1.9, 8.3 Hz), 10.88 (1H, brs).

6-(3-Phenylpropanoyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (3g). To a stirred mixture of **3a** (4 g, 20.9 mmol) and benzaldehyde (2.7 g, 25.5 mmol) in MeOH (40 mL) was added 28% sodium methoxide in MeOH (4.4 g, 22.8 mmol) at room temperature. The mixture was stirred at 50 °C for 24 h and concentrated under reduced pressure. The residue was treated with water and 10% hydrochloric acid. The precipitate was collected by filtration and washed with MeOH (40 mL) to give 6-(3-phenylprop-2-enoyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (5.07 g, 87%) as a white solid. A mixture of 6-(3-phenylprop-2-enoyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (4.0 g, 14.3 mmol), 10% palladium on carbon (2.0 g) in EtOH (80 mL), and THF (80 mL) was stirred at room temperature for 2 h and filtered. The filtrate was concentrated under reduced pressure. The residue was crystallized from MeOH to give **3g** (1.0 g, 25%) as white crystals. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.91 (2H, t, J = 7.6 Hz), 3.27 (2H, t, J = 7.6 Hz), 4.68 (2H, s), 7.03 (1H, d, J = 8.3 Hz), 7.12−7.33 (5H, m), 7.49 (1H, d, J = 2.1 Hz), 7.63 (1H, dd, J = 8.3, 2.1 Hz), 10.84 (1H, brs).

6-(2-Phenylpropanoyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (3h). To a stirred mixture of **3a** (7.0 g, 36.6 mmol) and *N*,*N*,*N*′,*N*′-tetramethyldiaminomethane (10.5 mL, 77.0 mmol) in dichloromethane (14 mL) was added acetic anhydride (10.5 mL, 111 mmol) at 0 °C. The mixture was stirred at room temperature for 72 h and concentrated under reduced pressure. The residue was diluted with water and extracted with EtOAc. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was suspended in MeOH (70 mL), and the resulting solid was collected by filtration to give 6-(2-phenylacryloyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (5.75 g, 56%). A mixture of 6-(2-phenylacryloyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (3.0 g, 10.7 mmol) and 10% palladium on carbon (1.0 g) in THF (60 mL) was stirred under hydrogen atmosphere at room temperature for 1 h and filtered. The filtrate was concentrated under reduced pressure. The residue was purified by column chromatography on basic silica gel with hexane/EtOAc (1:1) as eluant to give **3h** (1.98 g, 66%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.38 (3H, d, J = 6.8 Hz), 4.63 (2H, s), 4.81 (1H, q, J = 6.8 Hz), 6.96 (1H, d, J = 8.6 Hz), 7.13−7.34 (5H, m), 7.50 (1H, d, J = 2.2 Hz), 7.64 (1H, dd, J = 8.6, 2.2 Hz), 10.84 (1H, brs).

6-(2-Bromopentanoyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (4b). To a stirred mixture of **3b** (10 g, 42.9 mmol), acetic acid (80 mL), and 25% hydrogen bromide in acetic acid (20 mL) was added portionwise pyridinium hydrobromide perbromide (14.4 g, 45.0 mmol) at room temperature. The mixture was stirred at room temperature for 2 h and poured into water (300 mL). The precipitate was collected by filtration

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to give **4b** (13.1 g, 98%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.99 (3H, t, J = 7.4 Hz), 1.32−1.70 (2H, m), 2.01−2.26 (2H, m), 4.73 (2H, s), 5.07 (1H, dd, J = 7.6, 6.7 Hz), 7.04 (1H, d, J = 8.5 Hz), 7.56 (1H, d, J = 2.0 Hz), 7.67 (1H, dd, J = 8.5, 2.0 Hz), 8.56 (1H, s).

Compounds **4a**, **4c**, and **4e**−**h** were prepared in a manner similar to that described for **4b**.

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

6-(2-Bromo-3-phenylpropanoyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (4c). Yield, 95%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.23 (1H, dd, J = 14.3, 7.3 Hz), 3.52 (1H, dd, J = 14.3, 7.3 Hz), 4.70 (2H, s), 5.82 (1H, t, J = 7.3 Hz), 7.05 (1H, d, J = 8.4 Hz), 7.16−7.39 (5H, m), 7.52 (1H, d, J = 2.0 Hz), 7.74 (1H, dd, J = 8.4, 2.0 Hz), 10.87 (1H, brs).

6-(2-Bromo-2-phenylpropanoyl)-2*H*-1,4-benzoxazin-3(4*H*)-one (4e). Yield, 97%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.14 (3H, s), 4.63 (2H, s), 6.82 (1H, d, J = 8.4 Hz), 7.15 (1H, dd, J = 8.4, 2.1 Hz), 7.29−7.50 (6H, m), 10.88 (1H, brs).

6-[Bromo(2-fluorophenyl)acetyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (4f). Yield, 85%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.71 (2H, s), 6.66 (1H, s), 6.99−7.36 (4H, m), 7.55−7.65 (3H, m), 8.79 (1H, brs).

6-[Bromo(3-fluorophenyl)acetyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (4g). Yield, 99%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.69 (2H, s), 6.97−7.11 (2H, m), 7.13−7.26 (1H, m), 7.29−7.57 (4H, m), 7.78 (1H, dd, J = 8.5, 2.1 Hz), 10.91 (1H, brs).

6-[Bromo(4-fluorophenyl)acetyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (4h). Yield, 96%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.69 (2H, s), 7.01−7.12 (2H, m), 7.23 (2H, t, J = 8.90 Hz), 7.54 (1H, d, J = 2.0 Hz), 7.56−7.65 (2H, m), 7.78 (1H, dd, J = 2.0, 8.5 Hz), 10.93 (1H, s).

6-[Bromo(phenyl)acetyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (4d). To a stirred mixture of **3c** (25 g, 93.5 mmol), AcOH (280 mL), and 25% hydrogen bromide in acetic acid (70 mL) was added portion-wise pyridinium hydrobromide perbromide (30.4 g, 95.1 mmol) at room temperature. The mixture was stirred at room temperature for 30 min. Aqueous $Na_2S_2O_3$ solution was added, and the supernatant was decanted. The residue was diluted with 10% aqueous citric acid and extracted with EtOAc. The organic layer was washed with saturated $NaHCO_3$ solution, dried over anhydrous $MgSO_4$, passed through silica gel, and concentrated under reduced pressure. The residue was suspended in EtOAc/IPE and the solid was collected by filtration to give **4d** (28.2 g, 87%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.70 (2H, s), 6.30 (1H, s), 6.98 (1H, d, J = 8.7 Hz), 7.29−7.53 (6H, m), 7.62 (1H, dd, J = 8.7, 2.1 Hz), 8.64 (1H, brs).

6-(7*H*-[1,2,4]Triazolo[3,4-*b*][1,3,4]thiadiazin-6-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (1a). A mixture of **4a** (0.2 g, 0.74 mmol), 4-amino-3-mercapto-4*H*-1,2,4-triazole (0.10 g, 0.89 mmol) in EtOH (4 mL), and toluene (2 mL) was stirred at reflux for 24 h and concentrated under reduced pressure. To the residue were added MeOH (30 mL) and 10% aqueous potassium carbonate (10 mL). The mixture was stirred at room temperature for 3 h, and the resulting solid was collected by filtration and washed with water and MeOH to give **1a** (180 mg, 85%) as white crystals. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.38 (2H, s), 4.70 (2H, s), 7.12 (1H, d, J = 9.0 Hz), 7.51−7.59 (2H, m), 9.14 (1H, s), 10.95 (1H, brs). Anal. Calcd for $C_{12}H_9N_5O_2S$: C, 50.17; H, 3.16; N, 24.38. Found: C, 49.80; H, 3.19; N, 24.24.

6-(7-Propyl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazin-6-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (1b). A mixture of **3b** (0.50 g, 1.60 mmol), 4-amino-4*H*-1,2,4-triazole-3-thiol (0.19 g, 1.63 mmol) in EtOH (10 mL), and toluene (5 mL) was stirred at reflux for 12 h and then concentrated under reduced pressure. The residue was diluted with saturated $NaHCO_3$ solution and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was crystallized from MeOH to give **1b** (0.34 g, 65%) as white crystals. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.85 (3H, t, J = 7.0 Hz), 1.20−1.66 (4H, m), 4.70 (2H, s), 4.87 (1H, dd, J = 9.1, 5.0 Hz), 7.13 (1H, d, J = 9.2 Hz), 7.54−7.62 (2H, m), 9.17 (1H, s),10.96 (1H, brs). Anal. Calcd for $C_{15}H_{15}N_5O_2S$: C, 54.70; H, 4.59; N, 21.26. Found: C, 54.67; H, 4.48; N, 21.19.

Compounds **1c**−**d** and **1f**−**g** were prepared in a manner similar to that described for **1b**.

6-(7-Benzyl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazin-6-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (1c). Yield, 97%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.77 (1H, dd, J = 14.1, 9.2 Hz), 3.01 (1H, dd, J = 14.1, 5.7 Hz), 4.69 (2H, s), 5.14 (1H, dd, J = 9.2, 5.7 Hz), 7.06 (1H, d, J = 8.6 Hz), 7.11−7.31 (5H, m), 7.51 (1H, dd, J = 8.6, 2.2 Hz), 7.58 (1H, d, J = 2.2 Hz), 9.12 (1H, s), 10.95 (1H, brs). Anal. Calcd for $C_{19}H_{15}N_5O_2S$: C, 60.46; H, 4.01; N, 18.56. Found: C, 60.43; H, 4.66; N, 16.55.

6-(7-Phenyl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazin-6-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (1d). Yield, 24%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.67 (2H, s), 6.32 (1H, s), 7.03−7.22 (3H, m), 7.26−7.41 (3H, m), 7.47 (1H, dd, J = 8.7, 2.3 Hz), 7.59 (1H, d, J = 2.3 Hz), 9.25 (1H, s), 10.93 (1H, s). Anal. Calcd for $C_{18}H_{13}N_5O_2S$·0.3EtOAc: C, 59.16; H, 3.98; N, 17.97. Found: C, 58.83; H, 4.18; N, 17.61.

6-[7-(2-Fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazin-6-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (1f). Yield, 37%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.66 (2H, s), 6.44 (1H, s), 6.76−6.81 (1H, m), 7.04−7.10 (2H, m), 7.30−7.43 (2H, m), 7.47−7.50 (1H, m), 7.53−7.54 (1H, m), 9.28 (1H, s), 10.92 (1H, brs). ESI-HRMS calcd for $C_{18}H_{12}FN_5O_2S$ m/z 382.0769 (M + H), found 382.0754 (M + H).

6-[7-(3-Fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazin-6-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (1g). Yield, 73%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.68 (2H, s), 6.35 (1H, s), 6.87−6.94 (1H, m), 7.04−7.12 (2H, m), 7.12−7.22 (1H, m), 7.32−7.42 (1H, m), 7.47 (1H, dd, J = 2.5, 8.5 Hz), 7.58 (1H, d, J = 2.5 Hz), 9.28 (1H, s), 10.96 (1H, brs). ESI-HRMS calcd for $C_{18}H_{12}FN_5O_2S$ m/z 382.0769 (M + H), found 382.0741 (M + H).

6-[7-(4-Fluorophenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazin-6-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (1h). Yield, 69%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.68 (2H, s), 6.35 (1H s), 7.09 (1H, d, J = 8.5 Hz), 7.12−7.26 (4H, m), 7.46 (1H, dd, J = 2.3, 8.5 Hz), 7.58 (1H, d, J = 2.3 Hz), 9.27 (1H, s), 10.95 (1H, brs). Anal. Calcd for $C_{18}H_{12}FN_5O_2S$·0.1EtOAc: C, 56.64; H, 3.31; N, 17.95. Found: C, 56.25; H, 3.27; N, 17.79.

6-(7-Methyl-7-phenyl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazin-6-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (1e). A mixture of **4e** (0.3 g, 0.83 mmol), 4-amino-4*H*-1,2,4-triazole-3-thiol (0.29 g, 2.50 mmol), and triethylamine (3 mL) in EtOH (3 mL) was stirred at 80 °C for 6 h and then concentrated under reduced pressure. The residue was diluted with saturated $NaHCO_3$ solution 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/MeOH (20:1) as eluant to give **1e** (0.2 g, 64%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.02 (3H, s), 4.62 (2H, s), 6.86−6.94 (2H, m), 7.05 (1H, s), 7.28−7.49 (5H, m), 9.26 (1H, s), 10.75 (1H, brs). ESI-HRMS calcd for $C_{19}H_{15}N_5O_2S$ m/z 378.1019 (M + H), found 378.0989 (M + H).

2-(Benzylthio)imidazo[2,1-*b*][1,3,4]thiadiazole (12). A mixture of 5-(benzylthio)-1,3,4-thiadiazol-2-amine (5.0 g, 20.2 mmol) and 45% chloroacetaldehyde (3.9 g, 22.3 mmol) in EtOH (20 mL) and toluene (10 mL) was refluxed for 12 h and concentrated under reduced pressure. The residue was diluted with saturated $NaHCO_3$ solution 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 eluant to give **12** (1.27 g, 23%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.44 (2H, s), 7.30−7.40 (6H, m), 7.68 (1H, m).

1-Amino-1*H*-imidazole-2-thiol (6). A mixture of **12** (1.2 g, 5.1 mmol) and hydrazine monohydrate (2.4 g, 47.94 mmol) in EtOH (20 mL) was stirred under reflux for 50 h and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with EtOAc as eluant to give **6** (0.19 g, 32%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.62 (2H, s), 6.80 (1H, d, J = 2.3 Hz), 7.04 (1H, d, J = 2.3 Hz), 12.06 (1H, brs).

6-(2-Phenyl-2*H*-imidazo[2,1-*b*][1,3,4]thiadiazin-3-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (7). Compound 7 was prepared in a manner

similar to that described for **1e**. Yield, 17%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.65 (2H, s), 6.14 (1H, s), 6.99−7.00 (1H, m), 7.04−7.07 (1H, m), 7.13−7.16 (2H, m), 7.27−7.34 (3H, m), 7.41−7.45 (1H, m), 7.57−7.58 (1H, m), 7.77−7.79 (1H, m), 10.92 (1H, brs). ESI-HRMS calcd for $C_{19}H_{14}N_4O_2S$ m/z 363.0910 (M + H), found 363.0877 (M + H).

6-(2-Phenyl-2*H*-1,4-benzothiazin-3-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (8). A suspension of **4c** (0.50 g, 1.44 mmol) and 2-amionothiophenol (0.18 g, 1.44 mmol) in EtOH (10 mL) was refluxed for 6 h and concentrated under reduced pressure. The residue was dissolved in $CHCl_3$, and the solution was washed with water, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with hexane/EtOAc (4:1) as eluant and crystallized from EtOAc/IPE to give **8** (0.10 g, 19%) as white crystals. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.67 (2H, s), 5.17 (1H, s), 6.98 (1H, d, J = 8.4 Hz), 7.03−7.32 (8H, m), 7.44−7.57 (2H, m), 7.63 (1H, s), 7.98 (1H, brs). Anal. Calcd for $C_{22}H_{16}N_2O_2S$·0.1EtOAc: C,70.95; H, 4.33; N, 7.52. Found: C, 70.14; H, 4.55; N, 7.24.

6-(2-Methyl-5-phenyl-1,3-thiazol-4-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (9). Compound **9** was prepared in a manner similar to that described for **8**. Yield, 23%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.74 (3H, s), 4.61 (2H, s), 6.83 (1H, d, J = 8.4 Hz), 7.00−7.07 (2H, m), 7.33 (5H, s), 7.63 (1H, brs). ESI-HRMS calcd for $C_{18}H_{14}N_2O_2S$ m/z 323.0849 (M + H), found 323.0833 (M + H).

6-(2-Methyl-5-phenyl-1*H*-imidazol-4-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (10). A mixture of **4c** (200 mg, 0.58 mmol), acetamidine hydrochloride (273 mg, 2.89 mmol), and K_2CO_3 (399 mg, 2.89 mmol) in 2-propanol (5 mL) was stirred at reflux for 12 h and concentrated under reduced pressure. The residue was diluted with water and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on basic silica gel with EtOAc/MeOH (10:1) as eluant and crystallized from EtOAc/hexane to give **10** (24 mg, 14%) as white crystals. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.32 (3H, s), 4.57 (2H, brs), 6.76−7.54 (8H, m), 10.58−10.77 (1H, m), 11.99 (1H, brs). ESI-HRMS calcd for $C_{18}H_{15}N_3O_2$ m/z 306.1237 (M + H), found 306.1212 (M + H).

4,4,4-Trifluoro-1-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)butane-1,3-dione (13d). To a stirred mixture of 60% NaH (2.51 g, 105 mmol) in THF (100 mL) were added ethyl 2,2,2-trifluoroacetate (12.5 mL, 105 mmol), **3a** (5.00 g, 26.2 mmol), EtOH (2.5 mL), and a solution of 2,4-dibenzo-18-crown-6 (150 mg, 0.418 mmol) in THF (50.0 mL), successively. The mixture was stirred at reflux for 16 h, treated with 10% H_2SO_4 (200 mL), and extracted with EtOAc (200 mL). The organic layer was washed with water (200 mL) and saturated $NaHCO_3$ solution (200 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was crystallized from diethyl ether to give **13c** (6.67 g, 80%) as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.74 (2H, s), 6.50 (1H, s), 7.08 (1H, d, J = 8.7 Hz), 7.44 (1H, d, J = 1.9 Hz), 7.60 (1H, dd, J = 8.7, 1.9 Hz), 8.08 (1H, brs).

Compound **13a−c**, **13e**, and **13f** were prepared in a manner similar to that described for **13d**.

1-(3-Oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)butane-1,3-dione (13a). Yield, 51%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.19 (3H, s), 4.70 (2H, s), 6.10 (1H, s), 7.01 (1H, d, J = 8.2 Hz), 7.51 (1H, dd, J = 8.2, 2.0 Hz), 7.40 (1H, d, J = 2.0 Hz), 8.26 (1H, brs).

1-(3-Oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)pentane-1,3-dione (13b). Yield, 80%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (4H, t, J = 7.6 Hz), 2.46 (3H, d, J = 7.6 Hz), 4.70 (2H, s), 6.10 (1H, s), 7.02 (1H, d, J = 8.7 Hz), 7.40 (1H, d, J = 1.9 Hz), 7.52 (1H, dd, J = 8.7 Hz, 1.9 Hz), 8.23 (1H, brs).

4-Methyl-1-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)-pentane-1,3-dione (13c). Yield, 43%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.22 (6H, d, J = 7.2 Hz), 2.61 (1H, sep, J = 7.2 Hz), 4.70 (2H, s), 6.10 (1H, s), 7.02 (1H, d, J = 8.5 Hz), 7.42 (1H, d, J = 1.9 Hz), 7.53 (1H, dd, J = 8.5, 1.9 Hz), 8.64 (1H, brs).

4,4,5,5,5-Pentafluoro-1-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)pentane-1,3-dione (13e). Yield, 87%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.59 (2H, s), 5.80 (1H, s), 6.91 (1H, d, J = 8.2 Hz), 7.34 (1H, dd, J = 8.2, 2.0 Hz), 7.38 (1H, d, J = 2.0 Hz), 10.71 (1H, s).

Ethyl 2,4-Dioxo-4-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)butanoate (13f). Yield, 95%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.18−1.25 (3H, m), 4.02−4.13 (2H, m), 4.58 (2H, s), 5.18 (1H, s), 6.80−6.82 (1H, m), 7.20−7.40 (2H, m), 10.68 (1H, brs).

6-(3-Methyl-1-phenyl-1*H*-pyrazol-5-yl)-2*H*-1,4-benzoxazin-3(4*H*)-one (14a). Compound **14a** was prepared in a manner similar to that described for **14b**. Yield, 61%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.38 (3H, s), 4.70 (2H, s), 6.24 (1H, s), 6.63 (1H, s), 6.80−6.92 (2H, m), 7.25−7.40 (5H, m), 7.83 (1H, brs). Anal. Calcd for $C_{18}H_{15}N_3O_2$: C, 70.81; H, 4.95; N, 13.76. Found: C, 70.72; H, 5.09; N, 13.60.

6-[1-(4-Fluorophenyl)-3-methyl-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14b). To a stirred mixture of (4-fluorophenyl)hydrazine hydrochloride (105 mg, 0.64 mmol) and triethylamine (90 μL, 0.64 mmol) in 2-propanol (5 mL) were added 2,2,2-trifluoroacetic acid (99 μL, 1.29 mmol) and **13a** (150 mg, 0.64 mmol) at room temperature, successively. The reaction mixture was stirred at 80 °C for 12 h and concentrated under reduced pressure. The residue was diluted with saturated $NaHCO_3$ solution and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with EtOAc/hexane (1:1) as eluant and crystallized from EtOAc/hexane to give **14b** (70 mg, 34%) as white crystals. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.35 (3H, s), 4.63 (2H, s), 6.25 (1H, s), 6.64 (1H, d, J = 1.9 Hz), 6.78 (1H, dd, J = 8.2, 1.9 Hz), 6.89 (1H, d, J = 8.2 Hz), 7.00−7.08 (2H, m), 7.20−7.28 (2H, m), 8.35 (1H, brs). Anal. Calcd for $C_{18}H_{14}N_3O_2F$: C, 66.87; H, 4.36; N, 13.00. Found: C, 66.75; H, 4.35; N, 12.90.

6-[3-Ethyl-1-(4-fluorophenyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14c). To a stirred mixture of (4-fluorophenyl)-hydrazine hydrochloride (99 mg, 0.61 mmol) and triethylamine (85 μL, 0.61 mmol) in 2-propanol (5 mL) were added 2,2,2-trifluoroacetic acid (93 μL, 1.21 mmol) and **13b** (150 mg, 0.61 mmol), successively. The reaction mixture was stirred at 80 °C for 12 h and concentrated under reduced pressure. The residue was diluted with saturated $NaHCO_3$ solution and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by preparative HPLC and crystallized from EtOAc/hexane to give **14c** (51 mg, 25%) as white crystals. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.32 (3H, t, J = 7.6 Hz), 2.74 (2H, q, J = 7.6 Hz), 4.63 (2H, s), 6.29 (1H, s), 6.67 (1H, d, J = 2.0 Hz), 6.79 (1H, dd, J = 8.4, 2.0 Hz), 6.98 (1H, d, J = 8.4 Hz), 7.00−7.08 (2H, m), 7.22−7.30 (2H, m), 8.72 (1H, brs). Anal. Calcd for $C_{19}H_{16}N_3O_2F$: C, 67.65; H, 4.78; N, 12.46. Found: C, 67.74; H, 4.84; N, 12.44.

6-[1-(4-Fluorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14d). To a stirred mixture of (4-fluorophenyl)hydrazine hydrochloride (133 mg, 0.82 mmol) and triethylamine (113 μL, 0.82 mmol) in 2-propanol (4.6 mL) were added 2,2,2-trifluoroacetic acid (129 μL, 1.68 mmol) and **13c** (225 mg, 0.74 mmol), successively. The reaction mixture was stirred at 60 °C for 12 h and concentrated under reduced pressure. The residue was treated with water, and 1 N NaOH was added to the solution to adjust the pH to 5−6. The precipitate was collected by filtration and suspended in diethyl ether. The resulting crystals were collected by filtration to give **14d** (198 mg, 67%) as a pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.65 (2H, s), 6.65 (1H, d, J = 2.0 Hz), 6.71 (1H, s), 6.80 (1H, dd, J = 8.4, 2.0 Hz), 6.93 (1H, d, J = 8.4 Hz), 7.05−7.16 (2H, m), 7.25−7.38 (2H, m), 8.28 (1H, brs). Anal. Calcd for $C_{18}H_{11}N_3O_2F_4$: C, 57.30; H, 2.94; N, 11.14. Found: C, 57.31; H, 2.90; N,11.10.

6-[1-(4-Fluorophenyl)-3-(perfluoroethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14e). Compound **14e** was prepared in a manner similar to that described for **14d**. Yield, 65%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.66 (2H, s), 6.66 (1H, d, J = 2.0 Hz), 6.72 (1H, s), 6.81 (1H, dd, J = 8.2, 2.0 Hz), 6.93 (1H, d, J = 8.2 Hz), 7.09−7.15 (2H, m), 7.31−7.38 (2H, m), 8.26 (1H, brs). Anal. Calcd

for $C_{19}H_{11}N_3O_2F_6$: C, 53.41; H, 2.59; N, 9.83. Found: C, 53.48; H, 2.74; N, 9.52.

6-[1-(4-Fluorophenyl)-3-(1-methylethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14f). Compound **14f** was prepared in a manner similar to that described for **14c**. Yield, 12%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.16 (6H, d, J = 6.8 Hz), 2.90−3.05 (1H, sep, J = 6.8 Hz), 4.59 (2H, s), 6.66 (1H, s), 6.97 (1H, d, J = 8.3 Hz), 7.28−7.46 (4H, m), 7.51−7.64 (2H, m), 10.69 (1H, brs). ESI-HRMS calcd for $C_{20}H_{18}N_3O_2F$ m/z 352.1456 (M + H), found 352.1434 (M + H).

Compounds **14g−m** were prepared in a manner similar to that described for **14d**.

6-[1-(2-Fluorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14g). Yield, 85%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.63 (2H, s), 6.65 (1H, d, J = 2.0 Hz), 6.73 (1H, s), 6.80 (1H, dd, J = 8.4, 2.0 Hz), 6.90 (1H, d, J = 8.4 Hz), 7.08−7.18 (1H, m), 7.23−7.30 (1H, m), 7.45−7.58 (2H, m), 7.62 (1H, brs). Anal. Calcd for $C_{18}H_{11}N_3O_2F_4$: C, 57.30; H, 2.94; N, 11.14. Found: C, 57.24; H, 2.99; N, 11.08.

6-[1-(3-Fluorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14h). Yield, 86%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.67 (2H, s), 6.66 (1H, d, J = 1.9 Hz), 6.71 (1H, s), 6.84 (1H, dd, J = 8.4, 1.9 Hz), 6.96 (1H, d, J = 8.4 Hz), 7.08−7.18 (3H, m), 7.35−7.40 (1H, m), 7.81 (1H, brs). Anal. Calcd for $C_{18}H_{11}N_3O_2F_4$: C, 57.30; H, 2.94; N, 11.14. Found: C, 57.33; H, 3.02; N, 11.12.

6-[1-(2-Chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14i). Yield, 76%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.62 (2H, s), 6.63 (1H, d, J = 2.0 Hz), 6.79 (1H, dd, J = 8.4, 2.0 Hz), 6.88 (1H, d, J = 8.4 Hz), 6.97 (1H, d, J = 2.0 Hz), 7.40−7.47 (4H, m), 7.66 (1H, brs). ESI-HRMS calcd for $C_{18}H_{11}N_3O_2F_3Cl$ m/z 392.0419 (M + H), found 392.0415 (M + H).

6-[1-(4-Chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14j). Yield, 66%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.66 (2H, s), 6.65 (1H, d, J = 2.0 Hz), 6.70 (1H, s), 6.82 (1H, dd, J = 8.4, 2.0 Hz), 6.95 (1H, d, J = 8.4 Hz), 7.27−7.30 (2H, m), 7.37−7.40 (2H, m), 7.86 (1H, brs). Anal. Calcd for $C_{18}H_{11}N_3O_2ClF_3$: C, 54.91; H, 2.82; N, 10.67. Found: C, 54.70; H, 2.87; N, 10.56.

6-[1-(2-Methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14k). Yield, 73%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.97 (3H, s), 4.61 (2H, s), 6.53 (1H, d, J = 2.0 Hz), 6.75−6.93 (3H, m), 7.26−7.34 (3H, m), 7.34−7.40 (1H, m), 7.69 (1H, brs). Anal. Calcd for $C_{19}H_{14}N_3O_2F_3$: C, 61.13; H, 3.78; N, 11.26. Found: C, 61.00; H, 3.82; N, 11.18.

6-[1-(4-Methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14l). Yield, 83%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.39 (3H, s), 4.64 (2H, s), 6.61 (1H, d, J = 2.0 Hz), 6.69 (1H, s), 6.84 (1H, dd, J = 8.4, 2.0 Hz), 6.93 (1H, d, J = 8.4 Hz), 7.18−7.20 (1H, 2 m), 7.23−7.27 (3H, m), 7.73 (1H, brs). Anal. Calcd for $C_{19}H_{14}N_3O_2F_3$: C, 61.13; H, 3.78; N, 11.26. Found: C, 61.22; H, 3.84; N, 11.23.

6-[1-(2-Ethylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14m). Yield, 47%. ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.94 (3H, t, J = 7.6 Hz), 2.22 (2H, q, J = 7.6 Hz), 4.57 (2H, s), 6.66−6.81 (2H, m), 6.79−6.94 (1H, m), 7.11 (1H, s), 7.26−7.38 (2H, m), 7.34−7.59 (2H, m), 10.77 (1H, brs). Anal. Calcd for $C_{20}H_{16}N_3O_2F_3$: C, 62.01; H, 4.16; N, 10.85.Found: C, 62.28; H, 4.31; N,10.65.

6-[1-(4-Fluoro-2-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (14n). To a stirred mixture of **18** (29.0 g, 164 mmol) and triethylamine (22.9 mL, 164 mmol) in 2-propanol (350 mL) were added trifluoroacetic acid (12.6 mL, 164 mmol) and **13d** (47.1 g, 164 mmol), successively. The resulting mixture was stirred at 80 °C for 3 h and then poured into water. The precipitate was collected by filtration and purified by column chromatography on silica gel with hexane/EtOAc (1:1) as eluant to give **14n** (31.4 g, 54%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.90 (3H, s), 4.58 (2H, s), 6.70 (1H, d, J = 2.1 Hz), 6.82 (1H, dd, J = 8.4, 2.1 Hz), 6.92 (1H, d, J = 8.4 Hz), 7.13 (1H, s), 7.11−7.23 (1H, m), 7.28−7.32 (1H, m), 7.41−7.45 (1H, m), 10.78 (1H, brs). Anal. Calcd for $C_{19}H_{13}N_3O_2F_4$: C, 58.32; H, 3.35; N, 10.74. Found: C, 58.25; H, 3.23; N, 10.80.

Ethyl 1-(4-Fluorophenyl)-5-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)-1*H*-pyrazole-3-carboxylate (14o). Compound **14o** was prepared in a manner similar to that described for **14d**. Yield, 80%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.32 (3H, t, J = 7.2 Hz), 4.33 (2H, q, J = 7.2 Hz), 4.60 (2H, s), 6.76 (1H, d, J = 2.0 Hz), 6.80 (1H, dd, J = 8.2, 2.0 Hz), 6.93 (1H, d, J = 8.2 Hz), 7.02 (1H, s), 7.30−7.37 (2H, m), 7.37−7.41 (2H, m), 10.76 (1H, s).

1-(4-Fluorophenyl)-5-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)-1*H*-pyrazole-3-carboxamide (15). To a stirred solution of **14o** (1.26 g, 3.30 mmol) in THF (25 mL) was added 1 N NaOH (8.26 mL). The mixture was stirred at reflux for 12 h and acidified with 1 N HCl. The precipitate was collected by filtration and recrystallized from EtOH to give 1-(4-fluorophenyl)-5-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)-1*H*-pyrazole-3-carboxylic acid (877 mg, 75%) as white crystals. To a stirred mixture of 1-(4-fluorophenyl)-5-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)-1*H*-pyrazole-3-carboxylic acid (170 mg, 0.48 mmol), 1-hydroxybenzotriazole monohydrate (74.4 mg, 0.48 mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (100 mg, 0.52 mmol) in DMF (5 mL) was added 2 M ammonia in MeOH (253 μL, 0.51 mmol) at room temperature. The mixture was stirred for 12 h, diluted with water, and extracted with EtOAc. The organic layer was washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was suspended in EtOAc, and the solid was collected by filtration to give **15** (130 mg, 76%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.60 (2H, s), 6.77−6.80 (2H, m), 6.89 (1H, s), 6.93 (1H, d, J = 8.9 Hz), 7.31−7.42 (5H, m), 7.69 (1H, s), 10.76 (1H, brs).

1-(4-Fluorophenyl)-5-(3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)-1*H*-pyrazole-3-carbonitrile (16). To a stirred solution of **15** (25 mg, 0.07 mmol) in dioxane (1 mL) and pyridine (0.1 mL) was added trifluoroacetic anhydryde (25 μL, 0.18 mmol) at 0 °C. The mixture was stirred for 30 min, quenched 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 (2:1) as eluant to give **16** (9 mg, 38%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.60 (2H, s), 6.75 (1H, d, J = 2.0 Hz), 6.80 (1H, dd, J = 8.6, 2.0 Hz), 6.95 (1H, d, J = 8.6 Hz), 7.08−7.15 (3H, m), 7.23−7.44 (2H, m), 7.60 (1H, brs). ESI-HRMS calcd for $C_{18}H_{11}N_4O_2F$ m/z 392.0419 (M + H), found 392.0415 (M + H).

6-[1-(4-Fluorophenyl)-3-(hydroxymethyl)-l*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (17). To a stirred solution of **14o** (100 mg, 0.26 mmol) in THF (1 mL) was added 1 M lithium aluminum hydride in THF (0.26 mL, 0.26 mmol) at 0 °C. The mixture was stirred at room temperature for 1 h. To the mixture was added 1 M lithium aluminum hydride in THF (0.10 mL, 0.10 mmol) at room temperature. The mixture was stirred at room temperature for 12 h, diluted with THF, and quenched with sodium sulfate decahydrate. The resulting mixture was stirred at room temperature for 12 h and filtered. The filtrate was treated with 1 N NaOH and extracted with EtOAc. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with dichloromethane/MeOH (20:1) as eluant to give **17** (30 mg, 34%) as a white solid.^{1}H NMR (300 MHz, DMSO-d_6) δ 4.49 (2H, d, J = 6.0 Hz), 4.59 (2H, s), 5.17 (1H, t, J = 6.0 Hz), 6.49 (1H, s), 6.65−6.81 (2H, m), 6.92 (1H, d, J = 8.3 Hz), 7.16−7.33 (4H, m), 10.73 (1H, brs). ESI-HRMS calcd for $C_{18}H_{14}N_3O_3F$ m/z 340.1092 (M + H), found 340.1066 (M + H).

1-(4-Fluoro-2-methylphenyl)hydrazine Hydrochloride (18). To a solution of 4-fluoro-2-methylaniline **17** (125 g, 1.00 mol) in concentrated HCl (1000 mL) was added sodium nitrite (137 g, 2.00 mol) at 0 °C. The mixture was stirred at 0 °C for 2 h. To the mixture was added $SnCl_2$ (474 g, 2.50 mol) at 0 °C. The mixture was stirred at room temperature for 12 h and diluted with diethyl ether (250 mL). The aqueous layer was separated, basified with NaOH, and extracted with EtOAc. The organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was dissolved in diethyl ether. To the solution was added 4 N HCl in dioxane. The

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precipitate was collected by filtration to give **18** (85.0 g, 48%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.20 (3H, s), 6.89−7.12 (3H, m), 7.75 (1H, brs), 10.16 (3H, brs).

6-[1-(4-Fluorophenyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (19). A mixture of 6-acetyl-2*H*-1,4-benzoxazin-3(4*H*)-one (4.0 g, 20.9 mmol) and *N*,*N*-dimethylformamide dimethyl acetal (4.46 mL, 33.5 mmol) in EtOH (50 mL) was stirred at 80 °C for 16 h and then cooled to room temperature. The precipitate was collected by filtration and washed with EtOH to give 6-(3-(dimethylamino)-acryloyl)-2*H*-1,4-benzoxazin-3(4*H*)-one as a yellow solid (3.8 g, 73%). To a stirred suspension of 1-(4-fluorophenyl)hydrazine hydrochloride (0.58 g, 3.54 mmol) in MeOH (14 mL) were added 6 N HCl (3.16 mL, 19.0 mmol) and 6-[3-(dimethylamino)acryloyl]-2*H*-1,4-benzoxazin-3(4*H*)-one (0.78 g, 3.16 mmol), successively. The mixture was stirred at 40 °C for 16 h and concentrated under reduced pressure. The residue was washed with diethyl ether and recrystallized from EtOH to give **19** (0.43 g, 44%) as white crystals. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.64 (2H, s), 6.46 (1H, d, J = 2.0 Hz), 6.67 (1H, d, J = 2.0 Hz), 6.80 (1H, J = dd, 8.6. 2.0 Hz), 6.91 (1H, d, J = 8.6 Hz), 7.05−7.18 (2H, m), 7.28−7.38 (2H, m), 7.69 (1H, d, J = 2.0 Hz), 8.54 (1H, brs).

6-[1-(4-Fluorophenyl)-4-iodo-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (20). To a stirred solution of **19** (1.0 g, 3.2 mmol) in DMF (10 mL) was added NIS (0.73 g, 3.2 mmol) at 0 °C. The mixture was stirred at 55 °C for 60 h. The resulting mixture was diluted with water and allowed to cool to 0 °C. The precipitate was collected by filtration and washed with dichloromethane to give **20** (0.70 g, 50%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.67 (2H, s), 6.69 (1H, d, J = 2.0 Hz), 6.83 (1H, dd, J = 8.2, 2.0 Hz), 6.97 (1H, d, J = 8.2 Hz), 6.98−7.10 (2H, m), 7.19−7.22 (2H, m), 7.76 (1H, s), 7.80 (1H, brs).

6-[1-(4-Fluorophenyl)-4-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (21). Under nitrogen atmosphere, a mixture of **20** (200 mg, 0.46 mmol), methyl 2,2-difluoro-2-(fluorosulfonyl)acetate (0.39 mL, 3.03 mmol)m and CuI (96.3 mg, 0.51 mmol) in DMF(3 mL) was stirred at 100 °C for 16 h. The reaction mixture was diluted with water and extracted with EtOAc. The organic layer was washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel with dichloromethane/EtOAc (10:1) as eluant to give **21** (8.2 mg, 5%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.67 (2H, s,), 6.66 (1H, d, J = 2.0 Hz), 6.80−6.83 (1H, m), 6.93−7.10 (3H, m), 7.18−7.30 (2H, m), 7.83 (1H, brs), 7.95 (1H, s). ESI-HRMS calcd for $C_{18}H_{11}N_3O_2F_4$ m/z 376.0715 (M + H), found 376.0715 (M + H).

6-Bromo-8-fluoro-2*H*-1,4-benzoxazin-3(4*H*)-one (23a). A mixture of 4-bromo-2-fluoro-6-nitrophenol (216 g, 917 mmol), methyl 2-bromoacetate (104 mL, 1.10 mol), and K_2CO_3 (633 g, 4.58 mol) in DMF (500 mL) was stirred at 65 °C for 12 h and poured into water. The precipitate was collected by filtration to give methyl (4-bromo-2-nitrophenoxy)acetate (282 g) as a yellow solid. To a solution of methyl (4-bromo-2-nitrophenoxy)acetate (282 g) in acetic acid (1.5 L) was added Zn dust (209 g, 320 mmol) slowly. The mixture was stirred at 100 °C for 12 h and filtered. The filter cake was suspended in DMF and filtered. The filtrates were combined and poured into water. The precipitate was collected by filtration to give **23a** (130 g, 57%) as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.68 (2 H, s), 6.87 (1 H, t, J = 2.0 Hz), 7.21 (1 H, dd, J = 10.0, 2.0 Hz), 10.99 (1 H, brs).

6-Bromo-8-chloro-2*H*-1,4-benzoxazin-3(4*H*)-one (23b). Compound **23b** was prepared in a manner similar to that described for **23a**. Yield, 41% in two steps. ^{1}H NMR (300 MHz, DMSO-d_6) δ 4.72 (2H, s), 6.99 (1H, d, J = 2.5 Hz), 7.30 (1H, d, J = 2.5 Hz), 10.98 (1H, brs).

4-Hydroxy-3-methyl-5-nitroacetophenone (25). To a solution of 4-hydroxy-3-methylacetophenone (100 g, 666 mmol) in acetic acid (444 mL) was added 70% nitric acid (31.0 mL, 732 mmol) at room temperature. The mixture was stirred at room temperature for 24 h and poured into water. The precipitate was collected by filtration to give **25** (77.0 g, 59%) as a yellow solid. ^{1}H NMR (400 MHz, acetone-d_6) δ 2.38 (3H, s), 2.62 (3H, s), 8.18 (1H, s), 8.57 (1H, s), 11.05 (1H, brs).

6-Acetyl-8-fluoro-2*H*-1,4-benzoxazin-3(4*H*)-one (27a). Under nitrogen atmosphere, a mixture of **23a** (93.7 g, 381 mmol), 4-(vinyloxy)butan-1-ol (156 mL, 1.26 mol), dichlorobis(tri-*o*-tolylphosphine)palladium(II) (8.98 g, 11.4 mmol), and K_2CO_3 (105 g, 762 mmol) in DMF (635 mL) and H_2O (38.1 mL) was stirred at 80 °C for 12 h and then poured into 2 N HCl. The resulting mixture was stirred for 1 h and extracted with dichloromethane. 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 (2:1) as eluant to give **27a** (58.0 g, 72%) as a tan solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.53 (3H, s), 4.78 (2H, s), 7.33 (1H, s), 7.64 (1H, d, J = 10.8 Hz), 11.02 (1H, brs).

6-Acetyl-8-chloro-2*H*-1,4-benzoxazin-3(4*H*)-one (27b). Compound **27b** was prepared in a manner similar to that described for **27a**. Yield, 34%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.52 (3H, s), 4.80 (2H, s), 7.40 (1H, s), 7.71 (1H, s), 11.02 (1H, brs).

6-Acetyl-8-methyl-2*H*-1,4-benzoxazin-3(4*H*)-one (27c). Compound **27c** was prepared in a manner similar to that described for **23a**. Yield, 89%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.23 (3H, s), 2.52 (3H, s), 4.68 (2H, s), 7.34 (1H, s), 7.52 (1H, s), 10.78 (1H, brs).

8-Fluoro-6-[1-(4-fluoro-2-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (29a). To a slurry of 60% NaH (44.4 g, 1.10 mol) in THF (4.0 L) was added ethyl 2,2,2-trifluoroacetate (146 mL, 1.10 mol), **27a** (58 g, 0.28 mol), EtOH (1.5 mL), and 2,4-dibenzo-18-crown-6 (1.60 g, 4.43 mmol), successively. The resulting mixture was stirred at 60 °C for 12 h, diluted with 1 N HCl, and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was suspended in diethyl ether. The solid was collected by filtration to give 4,4,4-trifluoro-1-(8-fluoro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-6-yl)-butane-1,3-dione (**28a**) (33.0 g, 39%) as a tan solid. To a stirred mixture of **18** (19.1 g, 108 mmol) and triethylamine (15.1 mL, 108 mmol) in 2-propanol (500 mL) were added trifluoroacetic acid (8.33 mL, 108 mmol) and **28a** (33.0 g, 108 mmol), successively. The resulting mixture was stirred at 80 °C for 3 h and poured into water. The precipitate was collected by filtration and purified by column chromatography on silica gel with EtOAc/hexane (1:3) as eluant to give **29a** (35.2 g, 79%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.91 (3H, s), 4.67 (2H, s), 6.47 (1H, s), 6.91 (1H, dd, J = 11.3, 2.0 Hz), 7.15−7.28 (2H, m), 7.30−7.34 (1H, m), 7.46−7.50 (1H, m), 10.98 (1H, brs). Anal. Calcd for $C_{19}H_{12}N_3O_2F_5$: C, 55.75; H, 2.96; N, 10.27. Found: C, 55.85; H, 3.03; N, 10.21.

8-Chloro-6-[1-(4-fluoro-2-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-2*H*-1,4-benzoxazin-3(4*H*)-one (29b). Compound **29b** was prepared in a manner similar to that described for **29a**. Yield, 28% in two steps. ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.91 (3H, s), 4.71 (2H, s), 6.58 (1H, d, J = 1.9 Hz), 7.07 (1H, d, J = 1.9 Hz), 7.13−7.36 (3H, m), 7.44−7.49 (1H, m), 10.95 (1H, brs). Anal. Calcd for $C_{19}H_{12}N_3O_2ClF_4$: C, 53.60; H, 2.84; N, 9.87. Found: C, 53.58; H, 2.89; N, 9.76.

6-[1-(4-Fluoro-2-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-5-yl]-8-methyl-2*H*-1,4-benzoxazin-3(4*H*)-one (29c). Compound **29c** was prepared in a manner similar to that described for **29a**. Yield, 16% in two steps. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.96 (3H, s), 2.15 (3H, s), 4.64 (2H, s), 6.39 (1H, d, J = 2.0 Hz), 6.70 (1H, m), 6.74 (1H, s), 6.97−7.00 (2H, m), 7.25−7.30 (1H, m), 9.00 (1H, brs). ESI-HRMS calcd for $C_{20}H_{15}N_3O_2F_4$ m/z 406.1173 (M + H), found 406.1146 (M + H).

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 cytosolic protein prepared from human MR transiently transfected FreeStyle 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 TopCount (PerkinElmer Inc.). For the determination of nonspecific binding, cold aldosterone instead of drug was added to the reaction mixture at 100 μM. Specific binding was determined by subtracting the value of the nonspecific 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} and its 95% confidence interval (CI) using PRISM 3.0 (GraphPad Software 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, the ligand concentration was 5 nM.

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 of PLUS reagent (Invitrogen), 9 μg of pMCMYneo-hMR, 5 μg of pMAM-Luc, and 1 μg of pRL-TK was mixed with a solution of 2.5 mL of Opti-MEM and 125 μL of Lipofectamin reagent (Invitrogen). The mixture was maintained at room temperature for 15 min. After substitution of culture medium with Opti-MEM, the mixture was added to the cells. After 3 h of incubation, 25 mL of 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 resuspended 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 no. 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 of incubation at 37 °C in 5% CO_2, the medium was removed. Then 20 μL/well of 2-fold 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} and its 95% confidence interval (CI).

Pharmacological Evaluation in Rats. Male 5-week-old Wistar rats were obtained from CREA Japan, Inc. (Tokyo, Japan) and acclimated for 1 week before experiments. The care and use of the animals and the experimental protocols used in the studies were reviewed and approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company (Osaka, Japan).

Potassium-Sparing and Natriuretic Effects in Wistar Rats. Potassium-sparing and natriuretic effect was examined using the method of Kagawa[15] with some modifications. In brief, rats aged 6 weeks were used. On the day before the experiment, rats were deprived of food and water overnight for approximately 16 h. Compound **14n** and spironolactone were suspended in 0.5% methylcellulose solution and orally administered 30 min before aldosterone (3 μg/kg, sc) and saline (25 mL/kg, po) administration. Urine was collected over the next 5 h. Urinary Na^+ and K^+ concentrations were measured using an electrolyte analyzer EX-2000 (Jokoh Company Ltd.). Urinary Na^+/K^+ ratio was calculated and normalized by the value for the vehicle group as 1.0. Data are shown as the mean ± SEM.

Antihypertensive Effect in DOCA–Salt Hypertensive Rats. Briefly, 6-week-old Wistar rats were anesthetized by injection of pentobarbiturate (50 mg/kg body weight, ip) for subcutaneous implantation of a 25 mg pellet of DOCA. After recovery from anesthesia, rats were housed in standard cages and maintained on standard chow and with ad libitum access to both tap water and a 1% NaCl drinking solution. After 3 weeks, SBP and HR were measured by a tail-cuff method (Softron BP-98A, Softron Co.) and development of hypertension was confirmed. Hypertensive rats were divided into groups with equalization of body weight, SBP, and HR. The groups of rats were treated with vehicle (0.5 w/v% methylcellulose, $n = 8$), compound **14n** ($n = 8$), and spironolactone ($n = 8$) at a dose of 100 mg/kg. Drugs were administrated orally once a day. After 13 days of treatment, SBP and HR were measured approximately 24 h after the last dosing. One rat in vehicle group was excluded from analysis because unexpected large fluctuation in SBP values was observed. Data are shown as the mean ± SEM.

Pharmacokinetic Analysis in Rats. Compound **14d** or **14n** was administered to rats (fasted, 8-week-old) intravenously at 0.1 or 3 mg/kg and orally at 1.0 or 10 mg/kg. After intravenous and oral administration, blood samples were collected at designated time points. The blood samples were centrifuged to obtain the plasma fraction. The plasma samples were deproteinized with acetonitrile containing an internal standard. After centrifugation, the supernatant was diluted with 0.01 mol/L ammonium acetate and centrifuged again. The compound concentrations in the supernatant were measured by a liquid chromatograph equipped with tandem mass spectrometer.

Metabolic Clearance Assay. In vitro oxidative metabolic studies of the tested compounds were carried out using hepatic microsomes obtained from rats. The reaction mixture with a final volume of 0.1 mL consists of 0.2 mg/mL hepatic microsome in 50 mmol/L KH_2PO_4–K_2HPO_4 phosphate buffer (pH 7.4) and 1 μmol/L test compound. After 5 min of preincubation at 37 °C, the reaction was initiated by addition of an NADPH-generating system containing 50 mmol/L $MgCl_2$, 50 mmol/L gulucose 6-phosphate, 5 mmol/L β-$NADP^+$, and 15 unit/mL glucose 6-phosphate dehydrogenase at 10% volume of reaction mixture. After the addition of the NADPH-generating system, the mixture was incubated at 37 °C for 0 and 60 min. The reaction was terminated by addition of an equivalent volume of acetonitrile. After the samples were mixed and centrifuged, the supernatant fractions were subjected to high performance liquid chromatography with UV detection. Test compound in the reaction mixture was measured by an HPLC system equipped with a UV detector. For metabolic clearance determinations, chromatograms were analyzed for parent compound disappearance rate from the reaction mixtures. All incubations were made in duplicate.

Docking Study. The atomic coordinates of the protein structures analyzed in-house were used for docking. To both protein structures were added hydrogens, and then only hydrogens were energetically optimized in MOE (version 2005.06, Chemical Computing Group Inc.) using MMFF94s force field. Each compound was docked into the MR-LBD using the GOLD program (version 3.0, the Cambridge Crystallographic Data Centre) with the default parameter set. Obtained docking poses showing high score (Gold score) were subjected to energy minimization using MMFF94s force field in MOE. During the energy minimization procedure, amino acid residues within 5.0 Å from the each ligand were relaxed and the dielectric constant was set to 2*r*, where *r* is the distance between two interacting atoms.

Crystallography. Several mutants of human MR-LBD were prepared as described previously.[16] The proteins (residues 712–984 or residues 727–984) were expressed in a BL21(DE3) cells (Invitrogen) with the 6×His tag at the C terminus, which was removed by a TEV protease cleavage. Ligands were added with a final concentration of 10–100 μM prior to the IPTG induction of expression. The proteins were then purified using Ni-affinity and size-exclusion chromatography. All buffers were supplemented with 10–100 μM ligand. 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, and 100 μM ligand.

The crystals of the MR-LBD complexed with spironolactone or compound **1d** were grown at 20 °C using the vapor diffusion method in sitting drops by mixing 50 nL of protein solution with 50 nL of reservoir solution. The reservoir solution for the MR C808S/S810L–spironolactone complex contained 0.1 M MES, pH 7.0, 1.26 M lithium

sulfate, and 6% PEG 2000 monomethyl ether, and that for the MR C808S/S810L/A976V–**1d** complex contained 0.1 M HEPES, pH 7.4, 0.88 M potassium/sodium tartrate, and 5% ethylene glycol. Prior to data collection, crystals were immersed in mother liquor solution containing 25% ethylene glycol and flash frozen in liquid nitrogen. Diffraction data were collected from a single crystal at Advance Light Source on beamline 5.0.3 using a Quantum210 CCD detector (ADSC) under a 100 K nitrogen cryostream. The data were reduced and scaled using HKL2000.[17] The structures were solved by molecular replacement with MOLREP[18] from the CCP4 suites[19] using the coordinates of the MRS810L-LBD associated with spironolactone (PDB code 2AB2) as a search model. The structures were refined through an iterative procedure utilizing REFMAC[20] followed by model building in COOT.[21] The dictionary files for the ligands were prepared using AFITT (OpenEye Scientific Software). The final models were validated using Molprobity.[22] Crystallographic processing and refinement statistics are summarized in Supporting Information (Table S1). All structural figures were generated using PyMOL (Schrödinger, LLC).

■ ASSOCIATED CONTENT

Supporting Information

Crystallographic data and details of refinement for compound **1d** and spironolactone; docking model of compound **14n**. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

†Atomic coordinates and structure factors have been deposited in the Protein Data Bank with codes 3VHU and 3VHV for spironolactone and compound **1d**, respectively.

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■ ABBREVIATIONS USED

MR, mineralocorticoid receptor; HTS, high-throughput screening; AR, androgen receptor; PR, progesterone receptor; GR, glucocorticoid receptor; DOCA, deoxycorticosterone acetate; SBP, systolic blood pressure; HR, heart rate; EtOAc, ethyl acetate; THF, tetrahydrofuran; MeOH, methanol; EtOH, ethanol; DMF, *N*,*N*-dimethylformamide; CH_3CN, acetonitrile; IPE, diisopropyl ether

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