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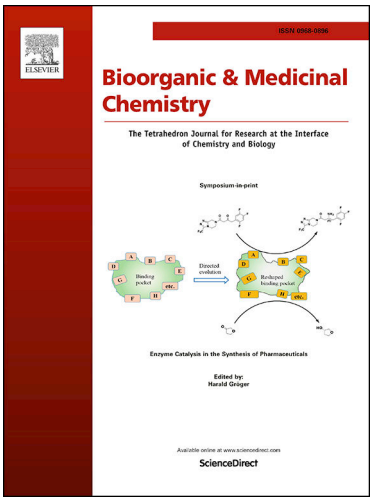

Design, synthesis and biological evaluation of 8-(2-amino-1-hydroxyethyl)-6-hydroxy-1,4-benzoxazine-3(*4H*)-one derivatives as potent β_2-adrenoceptor agonists

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Design, synthesis and biological evaluation of 8-(2-amino-1-hydroxyethyl)-6-hydroxy-1,4-benzoxazine-3(*4H*)-one derivatives as potent β_2-adrenoceptor agonists

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Abstract

A series of β_2-adrenoceptor agonists with an 8-(2-amino-1-hydroxyethyl)-6-hydroxy-1,4-benzoxazine-3(4*H*)-one moiety is presented. The stimulatory effects of the compounds on human β_2-adrenoceptor and β_1-adrenoceptor were characterized by a cell-based assay. Their smooth muscle relaxant activities were tested on isolated guinea pig trachea. Most of the compounds were found to be potent and selective agonists of the β_2-adrenoceptor. One of the compounds, **(R)-18c**, possessed a strong β_2-

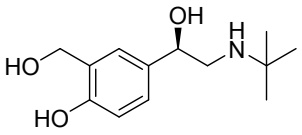
adrenoceptor agonistic effect with an EC_{50} value of 24 pM. It produced a full and potent airway smooth muscle relaxant effect same as olodaterol. Its onset of action was 3.5 min and its duration of action was more than 12 h in an *in vitro* guinea pig trachea model of bronchodilation. These results suggest that **(R)-18c** is a potential candidate for long-acting β_2-AR agonists.

Keyword: Asthma, COPD, β_2-adrenoceptor agonist, isoprenaline, salmeterol, olodaterol.

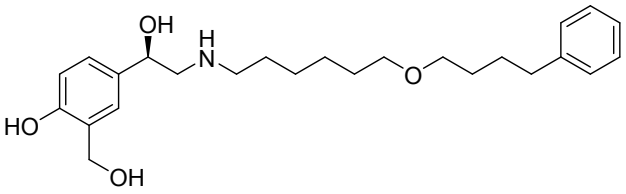
1. Introduction

Chronic obstructive pulmonary disease (COPD), characterized by persistent expiratory limitation and progressive airway inflammation, is a major cause of morbidity and mortality in the world.[1-3] It is the most common noncommunicable disease of the respiratory system secondary to asthma. The death rate of COPD ranks fifth globally nowadays and is expected to rise to the third place in 2030.[4-6] Asthma is a chronic inflammatory disorder of the airway characterized by hyperresponsiveness and structural change of the airway. In susceptible individuals, the inflammation causes recurrent episodes of widespread but variable airflow obstruction.[7] The airflow limitation in asthma is reversible either spontaneously or with treatment. β_2-Adrenoceptor (β_2-AR) agonists are the most effective bronchodilator drugs to treat asthma.[8] The β_2-AR, a member of the G protein-coupled receptor superfamily, is highly expressed in smooth muscles of the respiratory tract. Stimulation of the β_2-AR causes airway smooth muscle relaxation. Thus, β_2-AR agonists such as those presented in **Fig.**

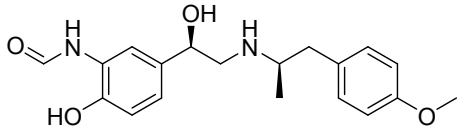
1 are widely used to relieve symptoms of airway obstruction. As highly effective bronchodilators, inhaled long-acting β_2-AR agonists (LABAs) have become standard of care for the control of airway obstruction.[9-11] β_2-AR agonists are mainly categorized into three generations. First-generation drugs, such as salbutamol (**1**),[12] are short acting β_2-AR agonists (SABA) which are characterized by fast action onset and short duration of action (3-6 h). Salmeterol (**2**)[13] and formoterol (**3**)[13] are second-generation β_2-AR agonists with duration of action of about 12 h. Third-generation agents (also called ultra-LABAs) including indacaterol (**4**)[14,15] and olodaterol (**5**)[16,17] with duration of action of about 24 h are used with a once-daily dosing regimen.



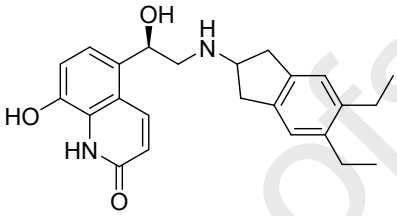
Salbutamol 1



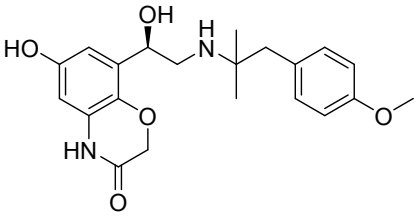
Salmeterol 2



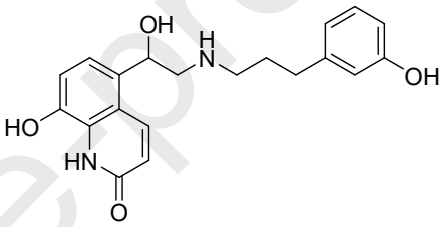
Formoterol 3



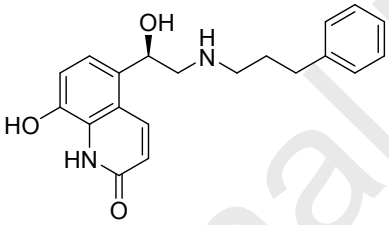
Indacaterol 4



Olodaterol 5



9g

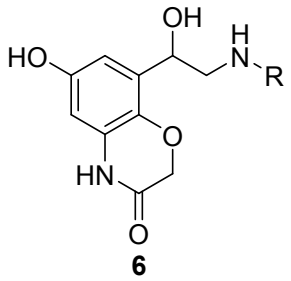


(R)-18c

Fig. 1 Structures of β_2-adrenoceptor agonists: salbutamol (**1**), salmeterol (**2**), formoterol (**3**), indacaterol (**4**), olodaterol (**5**), **9g** (Ref. 18) and **(R)-18c** (Ref. 18).

Using various medicinal chemistry strategies, we aim to discover new LABAs, but it is becoming more difficult to construct truly innovative compounds. Our group has previously synthesized some new β_2-AR agonists. Compounds **9g** (Ref. 18) and **(R)-18c** (Ref. 18) (**Fig. 1**) with a common 5-(2-amino-1-hydroxyethyl)-8-hydroxyquinolin-2(1*H*)-one moiety have shown high β_2-AR agonistic activities and β_2/β_1-selectivities.[18] The difference between the two compounds lies on their tail groups. In the present study,

different tail groups were combined with 8-(2-amino-1-hydroxyethyl)-6-hydroxy-1,4-benzoxazine-3(4*H*)-one **6** (**Fig. 2**), the head group of olodaterol, to produce a series of compounds. Olodaterol is an ultra-LABA developed by Boehringer-Ingelheim.



6

Fig. 2 The structure of 8-(2-amino-1-hydroxyethyl)-6-hydroxy-1,4-benzoxazine-3(4*H*)-one analogues (**6**)

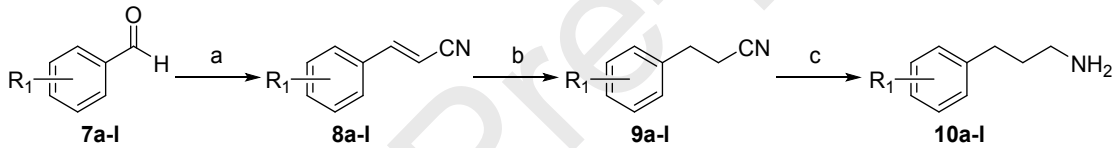
2. Results and discussion

2.1 Chemistry

Firstly, we synthesized a series of tail groups: **10a-l** and **14a-h**. Tail groups **10a-l** were synthesized in three steps from the respective aldehydes **7a-l** which went through the Wittig reaction with serial reduction of the double bond and the cyano group (**Scheme 1**). In addition, different ketones **11a-l** went through the Grignard reaction, the Ritter reaction and deacetylation to yield the respective tail groups **14a-h** (**Scheme 2**).

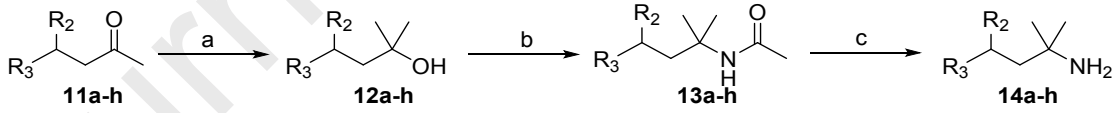
The head group was synthesized and linked to the tail groups. Briefly, acetophenone **15** was prepared as previously described.[19] The key steps involved oxidation of **15** to yield compound **16** and addition of different primary amines (**10a-l** and **14a-h**) to **16** followed by removal of the benzyl protecting group to deliver the target compounds **17a-l** and **18a-h**. Compound **16** reacted with purchased commercially amines (phenethylamine, 4-fluorophenethylamine, 4-methoxyphenethylamine, 4-phenylbutan-

1-amine) to yield **17-1 – 17-4**. To synthesize the chiral target compounds **(R)-18a-h**, acetophenone **15** was first brominated to give compound **19**. Then, (-)-diisopinocampheyl chloroborane ((-)-DIP-Cl) was added in the presence of 2 mol/L NaOH to induce chiral reduction and epoxidation of compound **19** to give the important chiral intermediate **20**. Compounds **14a-h** were reacted with compound **20** and balanced with ethyl acetate-HCl to yield the respective HCl salts, followed by hydrogenation to yield the target compounds **(R)-18a-h** (**Scheme 3**). Biological tests were performed on the free base of salmeterol and compound **18a**, and the HCl salts of olodaterol, isoprenaline and all the new compounds except **18a**.



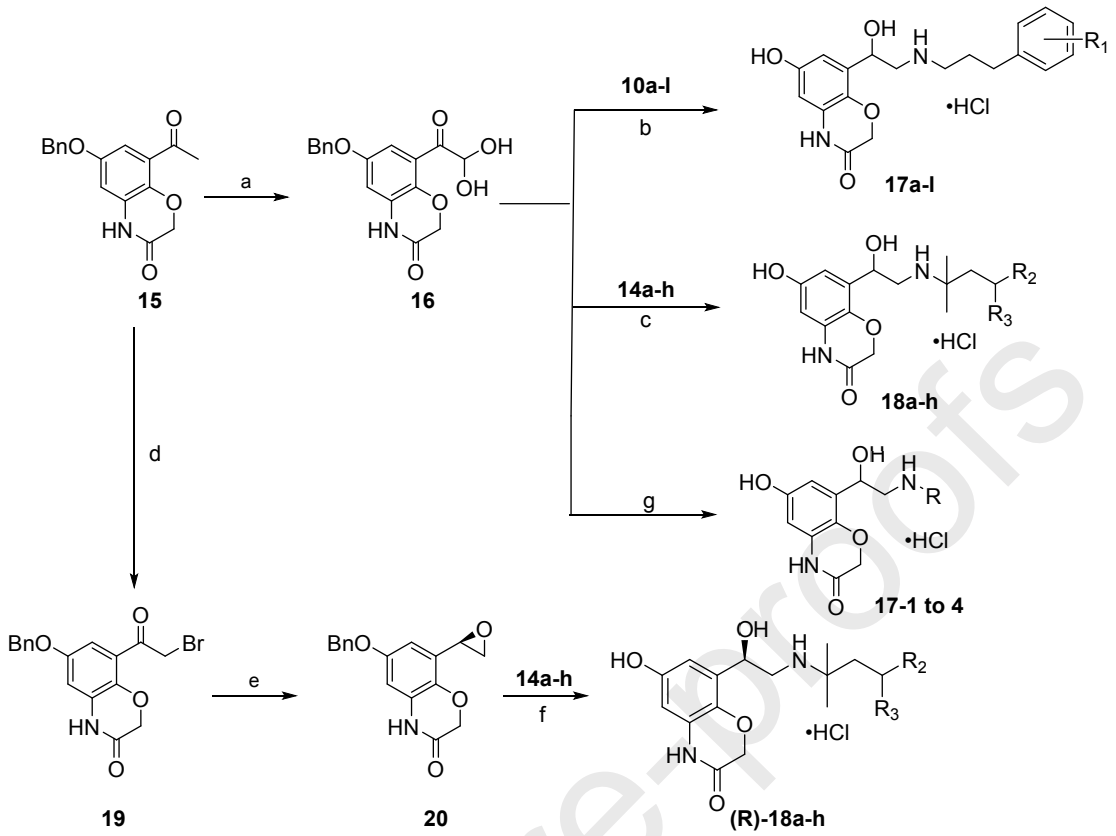
10a:R_1= H **10b:R_1= *p*-F** **10c:R_1= *p*-OH** **10d:R_1= *o*-OH**
10e:R_1= *m*-OH **10f:R_1= *p*-OCH_3** **10g:R_1= *o*-OCH_3** **10h:R_1= *m*-OCH_3**
10i:R_1= *p*-CH_3 **10j:R_1= *m*-CH_3** **10k:R_1= *m*-CF_3** **10l:R_1= 3,5-F,F**

Scheme 1. Reagents and conditions: (a) 2-diethoxyphosphorylacetonitrile, t-BuOK, anhydrous THF, ice-bath 30 min, 90-95%; (b) 5% Pd/C, H_2, ethyl acetate, 1 h, 95-98%; (c) $BH_3 \cdot THF$, anhydrous THF, 60 °C, 2 h, 90-95%.



14a:R_2= H , R_3= -phenyl **14b:R_2= H , R_3= *p*-methoxyphenyl**
14c:R_2= H , R_3= *o*-methoxyphenyl **14d:R_2= H , R_3= *m*-methoxyphenyl**
14e:R_2= H , R_3= *p*-hydroxyphenyl **14f:R_2= H , R_3= *m*-hydroxyphenyl**
14g:R_2= -phenyl , R_3= -phenyl **14h:R_2= *p*-fluorophenyl , R_3= *p*-fluorophenyl**

Scheme 2. Reagents and conditions: (a) MeMgBr, anhydrous THF, ice-bath, 40 min, 85-90%; (b) acetonitrile, concentrated sulfuric acid, ice-bath, 3 h, 70-80%; (c) KOH, ethane-1,2-diol, 200 °C, 15 h, 85-90%;



Scheme 3. Reagents and conditions: (a) 48% HBr, DMSO, 80°C, 1.5 h, 90-98%; (b) (i) $NaBH_4$, DMSO, MeOH, 1 h, 45-60%; (ii) 5% Pd/C, H_2, MeOH, 1 h, 90-98%; (iii) ethyl acetate-HCl, 2 h; (c) (i) $NaBH_4$, DMSO, MeOH, 15 h, 45-60%; (ii) 5% Pd/C, H_2, MeOH, 1 h, 90-98%; (iii) ethyl acetate-HCl, 2 h; (d) $TBABr_3$, 1,4-dioxane, MeOH, 2 h, 85-90% (e) (i) (-)-DIP-Cl, anhydrous THF, ice-bath, 1 h, 80-84%; (ii) 2 mol/L NaOH (aq), ice-bath, 2 h, 95-98%; (f) (i) isopropanol, reflux, 15 h, 65-70%; (ii) 5% Pd/C, H_2, MeOH , 1 h, 90-98%; (iii) ethyl acetate-HCl, 2 h; (g) (i) purchased amines (phenethylamine, 4-fluorophenethylamine, 4-methoxyphenethylamine, 4-phenylbutan-1-amine), isopropanol, reflux, 15 h, 65-70%; (ii) 5% Pd/C, H_2, MeOH , 1 h, 90-98%; (iii) ethyl acetate-HCl, 2 h.

2.2. Biological tests

2.2.1. Activities and selectivities in HEK-293 cells

Respiratory tissues including the lung and the airway express both the β_1-subtype and the β_2-subtype of the β-ARs, in which the β_2-AR is the dominant subtype. The reverse is true for the heart with the β_1-AR being the dominant subtype. Thus, β_2-AR agonists that cross-activate the β_1-AR are more prone to side effects such as tachycardia. In the present study, isoprenaline was used as a reference for non-selective β_1,β_2-AR agonist.[20] In response to stimulation of the β_2-AR, intracellular cAMP levels of airway smooth muscle cells increase and a cascade of intracellular events occur ultimately

leading to airway smooth muscle relaxation.[21] To evaluate the biological activities of the target compounds, cell-based assays were conducted using human embryonic kidney 293 (HEK-293) cells overexpressing β_1-AR or β_2-AR. cAMP accumulation was measured after exposure of the cells to the test compounds for 1 h. The potencies of the compounds were expressed in terms of the pEC_{50} values. The pEC_{50} value at the β_1-AR was subtracted from the pEC_{50} value at the β_2-AR to obtain the β_2/β_1-selectivity (**Table 1**).

We began the study by comparing compounds having different lengths of the carbon chain in the tail group. Compounds **17-1**, **17a** and **17-4** with 2, 3 and 4 carbon atoms between the secondary amine group and the terminal benzene group were found to have pEC_{50} values of 8.62, 9.50 and 9.60, respectively. The potency of **17-1** was one order of magnitude lower than that of **17-4**, and this held true even when the *para*-position of the phenyl group in **17-1** was substituted with a fluoride group (**17-2**) or a methoxy group (**17-3**). Note that both **17-3** and olodaterol have the same backbone with olodaterol having an extra dimethyl group on the α-carbon of the *N*-ethylbenzene group. The pEC_{50} values of **17-4** and **17a** were similar. Based on our previous study with the discovery of a highly potent β_2-adrenoceptor agonist **(R)-18c** (Ref. 18) from the indacaterol derivatives, we deduced that compound **17a** with an extra carbon atom in the carbon chain as compared to olodaterol would be a more promising lead compound as compared to **17-4** for further modification and testing. We explored whether further substitution in the phenyl ring of **17a** might improve activity. A series of compounds (**17b**-**17l**) were synthesized. As exemplified in compounds **17c-e** and **17f-h**, substitution in the *ortho*- or *meta*-position of the phenyl group resulted in higher activity than substitution in the *para*-position. For compounds **17c-e**, substitution with a hydroxyl group in the *meta*-position achieved the best result. But for compounds **17f-h**

(substituted with a methoxy), the *ortho*-position was the best. Comparing compounds **17b** (fluoride) and **17f** (methoxy), compound **17b** was minimally more active than **17f**, suggesting that the ability of the *para*-substituent to supply or absorb electrons makes little difference. The same structure-activity relationship applies to **17i** (methyl) and **17k** (trifluoromethyl) with similar activities as the methyl group and the trifluoromethyl group occupy similar space but have opposite electophilicity. Activities of compounds **17d** and **17e** were at the same level as that of the parent compound **17a**. Interestingly, the compound **17l** bearing two fluorine atoms showed the highest activity in this series with a pEC_{50} value of 9.89. In summary, most compounds in this series **17a-17l** were more active than **17-1, 17-2, 17-3** (compound **17i** was less active than **17-3**), albeit less potent than isoprenaline, salmeterol and olodaterol by about 10 folds.

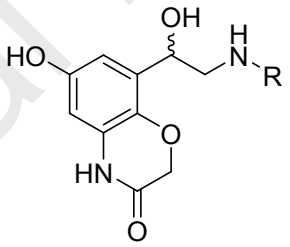
Comparison of the activities of compound **17-3** and olodaterol revealed that introduction of a dimethyl group on the α-carbon of the secondary amine group can greatly increase activity. Therefore, compounds **18a-18h** were synthesized with a dimethyl substitution on the α-carbon of the secondary amine group. The pEC_{50} value of **18a** was 10.49, a potency similar to those of isoprenaline and salmeterol. However, **18a** was less selective to the β_2-AR than salmeterol and olodaterol as suggested by its low β_2-β_1 value. Therefore, we intended to improve the potency of **18a** by introducing a range of substituent groups on the phenyl ring (**18g-18h**). When **18a** was substituted with a methoxy group (**18b-d**), *ortho*-substitution (**18c**) was found to be superior, albeit no observable improvements in activity and selectivity were noted. Among the compounds with a hydroxyl substituent **18e-f**, compound **18f** (*meta*-hydroxyl) with a pEC_{50} value of 10.51 was better than compound **18e** (*para*-hydroxyl). Comparing selectivity, **18f** was similar to **18c** but inferior to **18a** while the selectivity of **18e** was slightly improved. To find out whether steric hindrance had a deleterious influence on

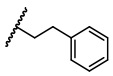
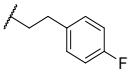
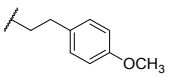
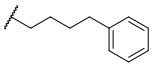
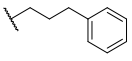
potency, compounds **18g-h** were synthesized and studied. They were found to be less potent than **18a**.

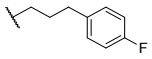
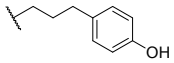
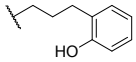
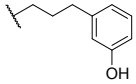
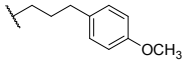
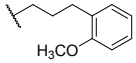
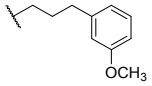
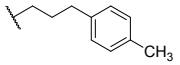
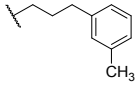
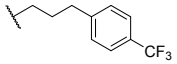
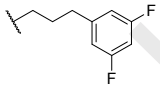
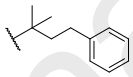
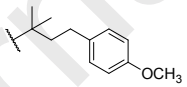
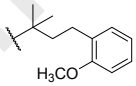
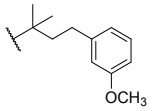
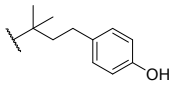
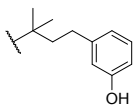
Next, we focused on the stereoselective synthesis of **(R)-18a-h**, and the biological activities of this set were compared with those of **18a-h**. We found that activities of all (R)-enantiomers were enhanced while the effects of the substituent groups on pEC_{50} values largely remained. Except for **(R)-18g-h**, all the pEC_{50} values were above 10.45. The highest one was 11.04 (**(R)-18a**) which was basically the same as that of olodaterol. However, the selectivity of **(R)-18a** was not as good. Compound **(R)-18f** with a slightly lower activity (pEC_{50} = 10.79) exhibited a selectivity approaching those of olodaterol and salmeterol.

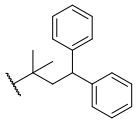
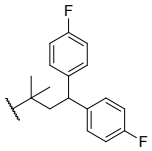
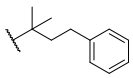
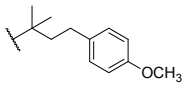
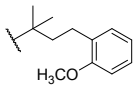
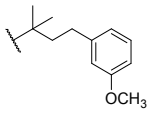
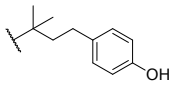
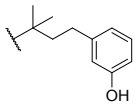
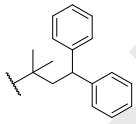
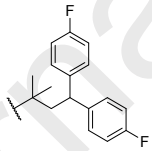
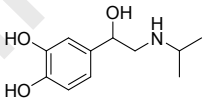
Table 1

Activities of the test compounds in inducing cAMP accumulation in HEK293 cells expressing β_2- or β_1-adrenoceptors. Isoprenaline, salmeterol and olodaterol were used as references.



Compound	R	β_2pEC_{50}[a]	β_1pEC_{50}	β_2-β_1[b]
17-1		8.62		
17-2		8.31		
17-3		8.79		
17-4		9.60		
17a		9.50		

17b		9.13		
17c		9.05		
17d		9.67		
17e		9.76		
17f		9.03		
17g		9.42		
17h		9.37		
17i		8.65		
17j		9.16		
17k		8.87		
17l		9.89		
18a		10.49±0.08	7.83±0.05	2.66
18b		10.31±0.16		
18c		10.54±0.10	8.41±0.62	2.13
18d		10.31±0.16	7.91±0.80	2.40
18e		10.40±0.05	7.54±0.04	2.86
18f		10.51±0.31	8.38±0.58	2.13

18g		9.21		
18h		9.10		
(R)-18a		11.04±0.08	8.91±0.73	2.13
(R)-18b		10.45±0.03	8.09±0.51	2.36
(R)-18c		10.62±0.04	8.85±0.45	1.77
(R)-18d		10.56±0.24	8.25±0.81	2.31
(R)-18e		10.59±0.30	8.27±0.61	2.32
(R)-18f		10.79±0.07	7.44±0.22	3.35
(R)-18g		9.85		
(R)-18h		9.76		
isoprenaline		10.26	8.81±0.19	1.45
salmeterol		10.35	6.50±0.07	3.85
olodaterol		11.07	7.56±0.64	3.51

[a.] Data represent mean ± SEM obtained from at least four independent experiments. HCl omitted from structures.

[b] Selectivity is defined as pEC_{50} at the β_2-adrenoceptor - pEC_{50} at the β_1-adrenoceptor.

2.2.2 Intrinsic activity in non-overexpressed HEK293 cells

The results described so far were obtained from HEK293 cells overexpressing β_2-

or β_1-AR. To study the efficacies of the compounds, it is necessary to conduct experiments on cells expressing the β_2-AR at levels resembling those in airway smooth muscles. The non-overexpressed HEK293 cells expressing endogenous human β_2-AR was used for this purpose. The intrinsic activities of the compounds were determined and compared against that of isoprenaline which was defined as 100%. Compounds with high activities in the cell-based assay, for instance **18a**, **18c-f** and **(R)-18a-f**, were chosen for testing. Salmeterol and olodaterol were used as reference drugs (**Table 2**). The intrinsic activities of salmeterol (28.76%) and olodaterol (30.60%) were found to be lower than that of isoprenaline, so as the other compounds tested. Compounds **18f**, **(R)-18e** and **(R)-18f** showed the highest intrinsic activities with values of 26.48%, 21.61% and 28.68%, respectively. Therefore, in non-overexpressed HEK293 cells, all the test compounds behaved as partial β_2-AR agonists.

Table 2

Intrinsic activities of selected compounds **18a, 18c-f** and **(R)-18a-f,** salmeterol, olodaterol and isoprenaline on HEK293 cells endogenously expressing human β_2-adrenoceptors

Compound	Intrinsic activity (*vs*. isoprenaline %)
18a	32.91
18c	28.79
18d	38.90
18e	33.98
18f	26.48

(R)-18a	33.58
(R)-18b	27.87
(R)-18c	32.82
(R)-18d	32.48
(R)-18e	21.61
(R)-18f	28.68
salmeterol	28.76
olodaterol	30.60
isoprenaline	100

2.2.3 Tracheal muscle relaxant activities

To predict the bronchodilator effects of the compounds *in vivo*, we examined the smooth muscle relaxant effects of the compounds on isolated guinea pig tracheal strips. Compounds **18a-f** and **(R)-18a-f** with high activities in the cell-based assay were chosen for the study. Relaxant responses were expressed as percentages of the maximal contractile effect induced by histamine. Concentration-relaxation curves were constructed (**Fig. 3-5**). E_{max} represents the maximal effect attainable from the compounds. Potency (pD_2) represents the negative logarithm of the concentration of the compounds at which the relaxant effect equals to 50% of the drug's own maximal effect. The pD_2 values were determined by non-linear regression analysis using GraphPad Prism, version 5.0. Comparison between sets of data was performed by paired Student's test with statistical software SPSS 20.0 and $P<0.05$ was considered significant. The E_{max} values for **18b**, **18c**, **18e**, **(R)-18a** and **(R)-18e** were at the same level and substantially less than those of isoprenaline, salmeterol and olodaterol (**Fig.**

3). The pD_2 values of **18c** and **18e** were 6.28 and 8.08, respectively, which were comparable to those of isoprenaline (6.00) and olodaterol (7.50) (**Table 3**). The E_{max} values of **18a**, **18f**, **(R)-18b** and **(R)-18f** approached those of isoprenaline, salmeterol and olodaterol (**Fig. 4**). The pD_2 values of **18a**, **18f** and **(R)-18f** were similar to that of isoprenaline but less than those of salmeterol and olodaterol. Compound **(R)-18b** had a pD_2 value of 8.04. The E_{max} values of **18d**, **(R)-18c** and **(R)-18d** were similar or higher than those of isoprenaline, salmeterol and olodaterol (**Fig. 5**). Compound **(R)-18c** had a pD_2 value of 7.78 and an E_{max} value of 219.43%. These results suggest that **18d**, **(R)-18c** and **(R)-18d** are full β_2-AR agonists in this assay, and **18a-c, 18e, 18f, (R)-18a, (R)-18b, (R)-18e** and **(R)-18f** are partial agonists.

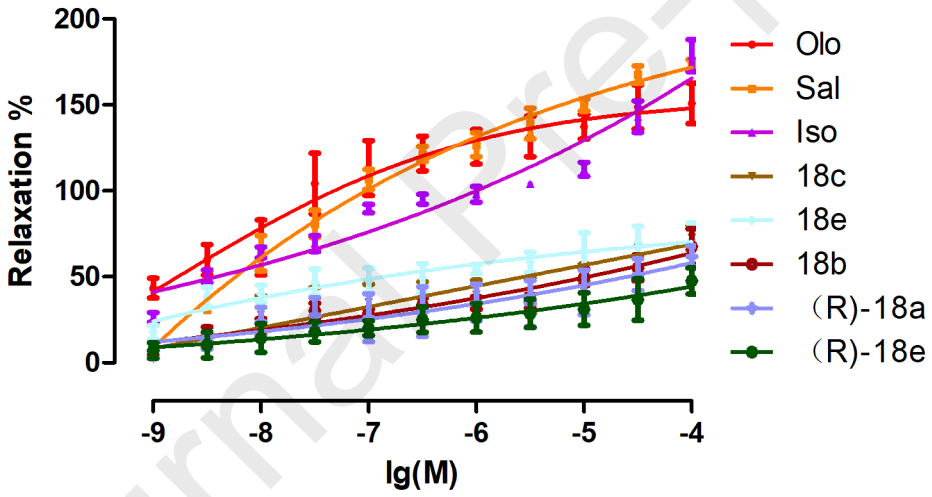


Fig. 3 Concentration–relaxation curves of olodaterol (Olo), salmeterol (Sal), isoprenaline (Iso), **18c**, **18e**, **18b**, **(R)-18a** and **(R)-18e** on contracted guinea pig trachea. Data are expressed as relaxation percentages of the maximum contraction induced with histamine (5×10^{-5} M). Error bar represents S.D. (n=4).

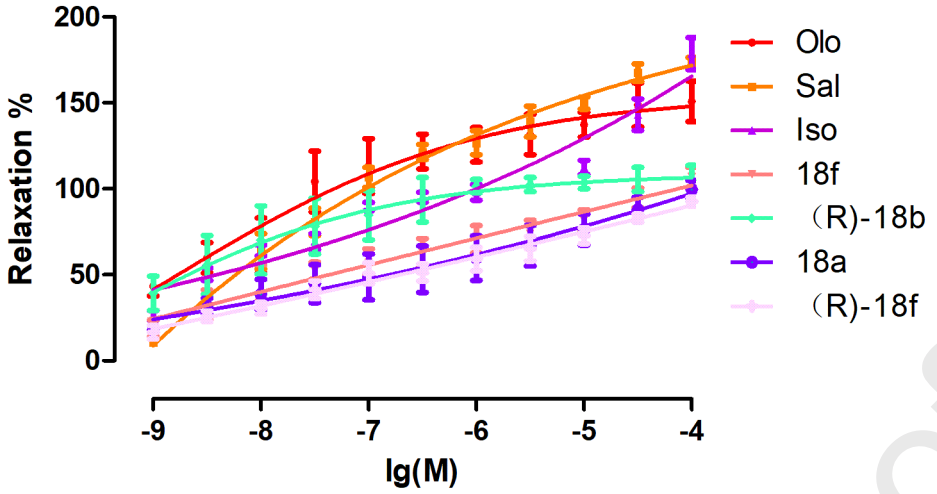


Fig. 4 Concentration–relaxation curves of olodaterol (Olo), salmeterol (Sal), isoprenaline (Iso), **18f**, **(R)-18b**, **18a** and **(R)-18f** on contracted guinea-pig trachea. Data are expressed as relaxation percentages of the maximum contraction induced with histamine (5×10^{-5} M). Error bar represents S.D. (n=4).

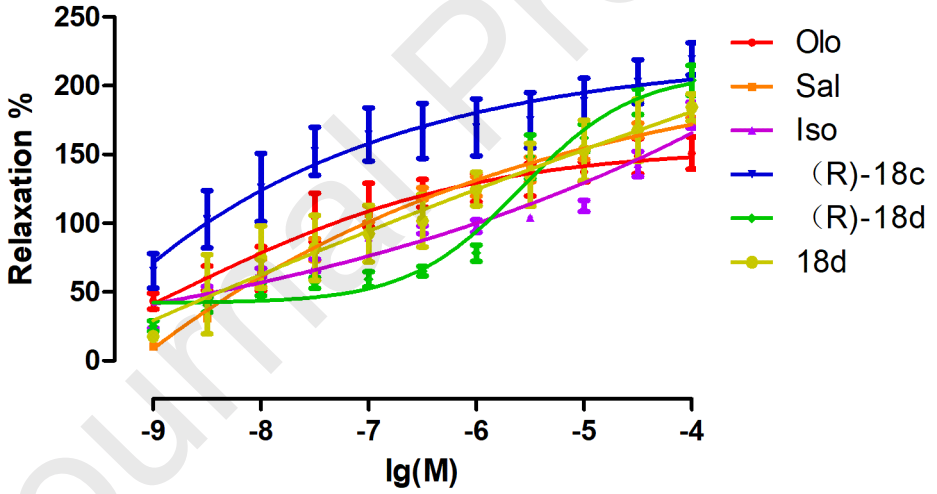


Fig.5 Concentration–relaxation curves of olodaterol (Olo), salmeterol (Sal), isoprenaline (Iso), **(R)-18c**, **(R)-18d** and **18d** on contracted guinea pig trachea. Data are expressed as relaxation percentages of the maximum contraction induced with histamine (5×10^{-5} M). Error bar represents S.D. (n=4).

Table 3

E_{max} and pD_2 of the smooth muscle relaxant responses of selected compounds **18a-f** and **(R)-18a-f,** isoprenaline, salmeterol and olodaterol.

Compound	pD_2	E_{max} (%)
18a	6.14±0.32	99.57±5.24
18b	5.76±0.25	67.48±10.37
18c	6.28±0.29	72.74±7.05
18d	6.56±0.34	184.28±9.80
18e	8.08±0.32*&	71.47±9.78
18f	6.46±0.29	106.86±5.71
(R)-18a	5.92±0.26	61.84±6.14
(R)-18b	8.04±0.54*&	105.07±8.60
(R)-18c	7.78±0.48*	219.43±11.74*&#
(R)-18d	5.56±0.83	203.79±10.92#
(R)-18e	5.61±0.26	47.67±7.49
(R)-18f	6.50±0.35	92.60±2.58
isoprenaline	6.00±0.29	178.71±9.37
salmeterol	7.23±0.51	173.61±2.86
olodaterol	7.50±0.61	150.92±11.76

The pharmacodynamic parameters were derived from non-linear regression analysis of data from the experiments. *$p < 0.05$, compared with that of isoprenaline; &$p < 0.05$, compared with that of salmeterol; #$p < 0.05$, compared with that of olodaterol.

2.2.4 *In vitro* study of onset time and duration of action of compounds

Compounds producing strong relaxant effects on isolated tracheal strips, for instance **18d**, **(R)-18c** and **(R)-18d**, were selected for further testing. The onset times and the duration of action of olodaterol, salmeterol, isoprenaline, **18d**, **(R)-18c** and **(R)-18d**

were determined graphically from the relaxation-time profiles (**Fig. 6**) and the recovery-time profiles (**Fig. 7**), respectively (**Table 4**). The onset time is explained by the Ot_{50} value which is the duration from application of the compound till attainment of 50% of the E_{max} of the compound. Duration of action (DoA) is defined as the duration from withdrawal of the compound producing a relaxant response till the relaxant effect reduces by half. Compound **18d** had an Ot_{50} value about two folds larger than that of isoprenaline (3.10 min), the largest among the compounds tested. The Ot_{50} value of **(R)-18d** was 3.73 min, which was smaller than that of salmeterol. Compound **(R)-18c** had the smallest Ot_{50} value (3.50 min) among the compounds tested which was close to that of isoprenaline. The Ot_{50} values of the three compounds were larger than that of olodaterol, and their DoAs were longer than 12 h. Therefore, the three compounds are expected to have equally fast action onset *in vivo* and behave as long-acting bronchodilators.

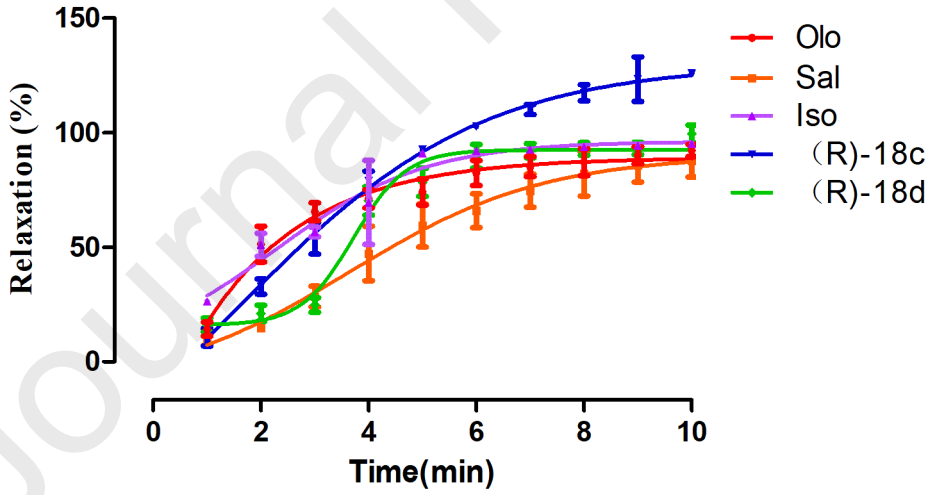


Fig. 6 Relaxation-time profiles of olodaterol (Olo), salmeterol (Sal), isoprenaline (Iso), **(R)-18c** and **(R)-18d** on guinea pig tracheal muscles pre-contracted with histamine. Data are expressed as relaxation percentages of Iso (mean ± S.D., n=4).

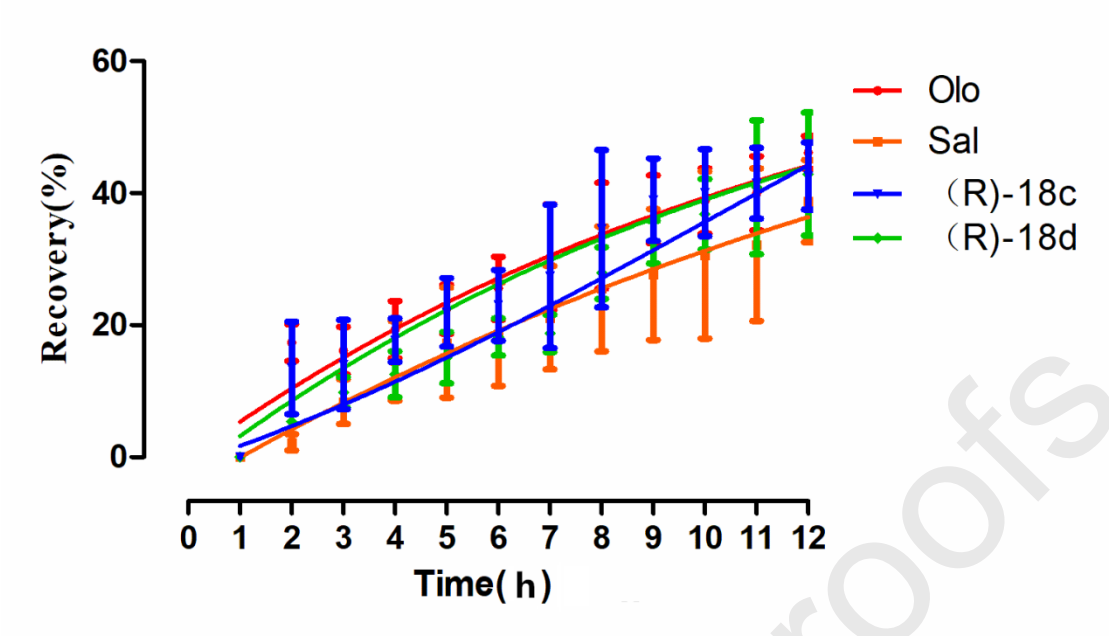


Fig. 7 Recovery-time profiles of olodaterol (Olo), salmeterol (Sal), **(R)-18c** and **(R)-18d**. Guinea pig tracheal muscles were pre-contracted with 5×10^{-5} M of histamine and relaxed by addition of the test compounds. Recording of the recovery process began upon withdrawal of the test compounds. Data are expressed as recovery percentages of the test compounds (mean ± S.D., n=4).

Table 4

Onset time (Ot_{50}) and duration of action (DoA) of the test compounds.

Compound	Ot_{50} (min)	DoA (h)
18d	5.86±0.21	> 12
(R)-18c	3.50±0.38	> 12
(R)-18d	3.73±0.89	> 12
isoprenaline	3.10±0.42	-
salmeterol	4.23±0.34	> 12
olodaterol	2.31±0.49	> 12

The pharmacodynamic parameters of the tested compounds were determined on guinea pig tracheal muscles pre-contracted with 5×10^{-5} M

of histamine by non-linear regression analysis of the data in Fig. 6 and Fig. 7 using GraphPad Prism version 5.0. Data are expressed as means ± S.D. (n=4).

2.3 Molecular modeling

In the subsequent molecular modeling study, flexible structures of the compounds were applied to automated docking simulations. The crystallographic structure of the β_2-AR co-crystallized with hydroxybenzyl isoproterenol (**HBI**) (PDB: 4LDL) [22] was used as the template for docking. Docking was performed using Glide in Schrödinger Maestro. Compounds **(R)-18a**, **(R)-18c**, **(R)-18f** with high biological activities were chosen for the study. The binding pose of **HBI** in **Fig. 8A** shows that interactions with the β_2-AR involved hydrogen bonding of the two hydroxyl groups on the catechol head with residues THR118 and SER207, which are considered necessary for β_2-AR activation.[22] Within the common 8-(2-amino-1-hydroxyethyl)-6-hydroxy-1,4-benzoxazine-3(4*H*)-one moiety of olodaterol, **(R)-18a**, **(R)-18c** and **(R)-18f**, the oxygen atom of the amide also interacted with these two amino acid residues (**Fig. 8B-E**). Besides, the oxygen atom, the nitrogen of the amide and the hydroxyl group in this moiety also formed hydrogen bonds with ASP113, SER203 and ASN293, respectively. These interactions may explain the superior activities of this series of compounds. The 2-amino-1-phenylethanol structure is regarded as the backbone of classical β_2-AR agonists because the interactions between the charged secondary amine group and the β-hydroxyl group with ASN312 and ASP113 are indispensable for anchorage of the agonist in the ligand-binding pocket of β_2-AR. The docking simulations reveal that the hydroxyl group and the secondary amine group of **HBI**, olodaterol, **(R)-18a** and **(R)-18c** interacted with ASN312 and ASP113. But for **(R)-18f**, the secondary amine group interacted with ASP113 and TYR316, and the β-hydroxyl group interacted with TYR308. This maybe an alternative way to activate the receptor. The difference in the lengths of carbon chains in the tail groups of the compounds could explain why the

para-hydroxyphenyl group of **HBI** interacted with CYS191 in extracellular loop 2 while the respective benzene moiety of **(R)-18c** and olodaterol interacted with PHE193 through π-σ interactions. Similarly, the *meta*-hydroxyphenyl group of **(R)-18f** formed a hydrogen bond with ASP192. In contrast, no interaction was found between the terminal phenyl group of **(R)-18a** and any amino acid residue in this region. The identical interaction patterns of olodaterol and **(R)-18c** may explain the high and very similar activities of the two compounds in various assays. Interestingly, for **(R)-18f** compared to **18f**, there was an increase in β_2-mediated activity but a decrease in β_1-mediated activity. According to the modeling results, **(R)-18f** has a slightly different interaction pattern with β_2-adrenoceptor as compared to **(R)-18a** and **(R)-18c**. The meta-hydroxyphenyl group of **(R)-18f** formed a hydrogen bond with ASP192 which is not present in the interaction patterns of **(R)-18a** and **(R)-18c**. This hydrogen bonding with ASP192 also appeared in the interaction pattern of olodaterol with the β_2-adrenoceptor, and **(R)-18f** were comparable to olodaterol in terms of activity and selectivity. Therefore, the interaction with ASP192 may be specific to the β_2-adrenoceptor but not to the β_1-adrenoceptor and important for β_2-agonistic activity. The superior activity and selectivity of **(R)-18f** as compared to **18f** also implies that this interaction plays an important role on β2-agonistic activity and β_2/β_1-selectivity. **(R)-18a** and **(R)-18f** with slightly different interaction patterns were similarly active as olodaterol and **(R)-18c** in the cell-based cAMP assay but somewhat less active in the tracheal muscle relaxation assay. Together, the molecular modeling results suggest that while the 8-(2-amino-1-hydroxyethyl)-6-hydroxy-1,4-benzoxazine-3(4*H*)-one head group in this series of compounds contributes to a high receptor binding affinity, a high activity also relies on proper binding interactions of the 2-amino-1-phenylethanol core and the tail group with the receptor.

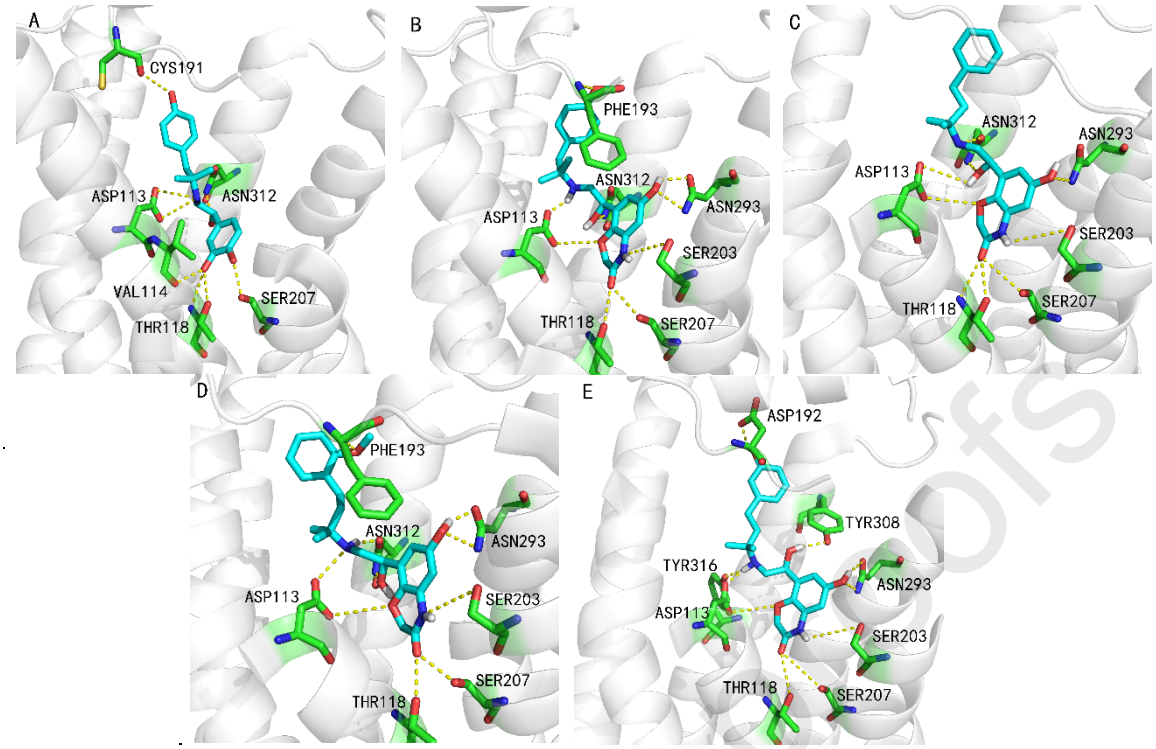


Fig. 8 Binding modes of **HBI** (A), olodaterol (B), compound **(R)-18a** (C), compound **(R)-18c** (D) and compound **(R)-18f** (E).

2.4 General discussion

We have previously synthesized and tested a series of 5-(2-amino-1-hydroxyethyl)-8-hydroxyquinolin-2(1*H*)-one derivatives for β_2-AR stimulatory effects and have identified two compounds **9g** (Ref. 18) and **(R)-18c** (Ref. 18) with high potency and β_2/β_1-selectivity.[18] In the present study, compounds with the 8-(2-amino-1-hydroxyethyl)-6-hydroxy-1,4-benzoxazine-3(4*H*)-one head group were studied. Compound **(R)-18c** in this series has been found to possess a high activity with a selectivity towards the β_2-AR, a strong smooth muscle relaxant effect, a rapid onset of action and a long duration of action. Molecular modeling reveals that compound **(R)-18c** (present study) has 10 interactions with the β_2-AR molecule (9 hydrogen bonds or electrostatic interactions and 1 π-σ interaction). Compound **9g** (Ref. 18) has 9 hydrogen

bonds or electrostatic interactions, and compound **(R)-18c** (Ref. 18) has 9 hydrogen bonds or electrostatic interactions and 1 π-σ interaction.[18] Their activities in stimulating the β_2-AR have been found to be 24 pM, 36 pM and 21 pM, respectively[18]. Thus, activities of the compounds are proportional to the number of interactions with the β_2-AR molecule. However, compound **(R)-18c** possesses higher β_1-adrenoceptor activity and this influences its β_2/β_1-selectivity. In considering the high β_2-AR agonistic activities of compounds **(R)-18a-f**, the 8-(2-amino-1-hydroxyethyl)-6-hydroxy-1,4-benzoxazine-3(4*H*)-one scaffold was a promising structure for further development. Another class of β_2-AR agonists synthesized in our laboratory include trantinterol[23] and its derivatives[24,25], which contain a non-classical 2-amino-2-phenylethanol core structure. This class of compounds are characterized by high β_2/β_1-selectivity but low β_2-AR agonistic activity (pEC50 values: 6 - 9) as compared with compounds containing the 2-amino-1-phenylethanol structure (pEC_{50} values: 8 - 11).[18,24,25]

3. Conclusion

In summary, several potent β_2-AR agonists have been identified from 8-(2-amino-1-hydroxyethyl)-6-hydroxy-1,4-benzoxazine-3(4*H*)-one derivatives. Among these compounds, compound **(R)-18c** with an EC_{50} value of 24 pM possesses the highest β_2-AR agonistic activity. It also has a strong airway smooth muscle relaxant effect with a rapid onset of action (3.50 min) and a long duration of action (>12 h) in isolated guinea pig trachea. In pharmacological assays, compound **(R)-18c** exhibits similar efficacy and potency as compared with olodaterol though with a lower β_2/β_1-

selectivity. Molecular modeling reveals that **(R)-18c** assumes an optimal receptor binding pattern same as olodaterol. These findings provide a theoretical basis for further optimization of β_2-AR agonists.

4. Experimental section

4.1. Chemistry

All reagents and solvents were purchased through commercial sources and were used without further purification. Anhydrous solvents were prepared by distillation over a silk of sodium. Reaction products were monitored by TLC (Silica gel60 GF254, Merck) and visualized under UV light. If necessary, crude products were purified on silica gel (200-300 mesh) by column chromatography. The purity of all test compounds was more than 95%. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker ARX-400-MHz or Bruker ARX-600-MHz spectrometer. High resolution mass spectrometry (HRMS) was performed on a Bruker Micromass Time of Flight mass spectrometer equipped with an electrospray ionization (ESI) probe. Melting points were measured by a Buchi B-540 melting point meter.

*4.1.1. General procedures for the synthesis of compounds **10a-l**.*

t-BuOK (5.66 g, 50.0 mmol) was added to a stirred solution of anhydrous THF containing an appropriate benzaldehyde derivative (**7a-l**) (5.00 g, 42.0 mmol) and 2-diethoxyphosphorylacetonitrile (8.10 g, 46.0 mmol) at 0°C for 30 min. Then, the reaction was maintained at 25°C for a further 30 min. The mixture was extracted with ethyl acetate and the combined organics were washed with brine, dried over sodium

sulfate, and concentrated to give compounds **8a-l**. To a solution of **8a-l** in ethyl acetate, 5% Pd/C (0.2 equiv.) was added. The suspension was filtered and evaporated. Then, the mixture was maintained at 30°C under 1 atm of hydrogen for 1 h. Removal of the solvent by rotary evaporation gave **9a-l**. Under nitrogen protection, BH_3·THF (2 mol/L, 20.7 mL, 41.4 mmol) was added slowly to a solution of **9a-l** (4.12 g, 27.6 mmol) in anhydrous THF at room temperature. Then, the reaction was refluxed at 60°C for 2 h. The mixture was extracted with ethyl acetate and the combined organics were washed with brine and dried over sodium sulfate, and concentrated to give compounds **10a-l**.

*4.1.2. General procedures for the synthesis of compounds **14a-h**.*

Compounds **14a-h** were synthesized according to reported methods.[18]

4.1.3. 6-(benzyloxy)-8-(2,2-dihydroxyacetyl)-2H-benzo[b][1,4]oxazin-3(4H)-one (***16***)

Intermediate **15** (20.00 g, 67.2 mmol) was suspended in DMSO (120 mL) and 48% hydrobromic acid (40.78 g, 201.6 mmol) was added. Then the suspension was stirred for 1.5 h at 80°C. The resulting yellow solution was cooled to 25°C and extracted three times with ethyl acetate. The combined organics were washed with brine, dried over sodium sulfate, and concentrated in a rotary evaporator to give a white solid **16**.

*4.1.4. General procedures for the synthesis of compounds **17-1 - 17-4**.*

The purchased amines (1.1 equiv.) was added into intermediate **16** (1.0 equiv.) dissolved in DMSO and the reaction was stirred at 35°C for more than 4 h. Then, sodium borohydride (1.0 equiv.) was added with a suitable amount of methanol and the reaction was stirred for a further 1 h at 0°C. After removal of the methanol by rotary

evaporation, the concentrate was purified by column chromatography (silica gel, CH_2Cl_2-MeOH 50:1) to give a white solid. The white solid dissolved in MeOH and added ethyl acetate-HCl, stirred at room temperature for 2 h, the suspension was filtered and yield white solid. The desired fraction was dissolved in methanol and mixed with 5% Pd/C (0.2 equiv.). The reaction was maintained at 30°C under 1 atm of hydrogen for 1 h. The suspension was filtered and evaporated to afford compounds **17-1 - 17-4**.

*4.1.4.1. 6-hydroxy-8-(1-hydroxy-2-(phenethylamino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17-1**).*

White solid; mp: 148.1-150.4 °C; yield 96 %; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.67 (s, 1H), 9.36 (s, 1H), 9.15 (s, 1H), 8.85 (s, 1H), 7.36 (t, J = 7.4 Hz, 2H), 7.28 (d, J = 7.5 Hz, 3H), 6.56 (s, 1H), 6.38 (s, 1H), 6.13 (d, J = 3.4 Hz, 1H), 5.14 (d, J = 9.8 Hz, 1H), 4.55 – 4.47 (m, 2H), 3.23 (m, 3H), 3.04 (m, 3H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.8, 153.0, 137.8, 132.8, 130.4, 130.0, 129.4, 129.2, 129.1, 128.2, 127.2, 107.0, 102.7, 67.4, 63.3, 52.3, 48.6, 31.8. Calcd. for $C_{18}H_{20}N_2O_4$ $[M+H]^+$ 329.1423; found 329.1449.

*4.1.4.2. 8-(2-((4-fluorophenethyl)amino)-1-hydroxyethyl)-6-hydroxy-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17-2**).*

White solid; mp: 148.1-149.7 °C; yield 97 %; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.65 (s, 1H), 9.34 (s, 1H), 9.14 (s, 1H), 8.81 (s, 1H), 7.33 – 7.28 (m, 2H), 7.17 (t, J = 8.6 Hz, 2H), 6.54 (d, J = 2.1 Hz, 1H), 6.37 (s, 1H), 6.12 (s, 1H), 5.12 (d, J = 9.6 Hz, 1H), 4.52 – 4.45 (m, 2H), 3.14 (dd, J = 22.0, 6.5 Hz, 3H), 2.96 (ddd, J = 33.6, 17.9, 8.6 Hz, 3H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.8, 162.4, 160.8, 153.0, 133.9, 132.7, 131.0,

130.4, 128.2, 115.9, 115.7, 107.0, 102.7, 67.4, 63.3, 52.3, 48.5, 30.9. Calcd. for $C_{18}H_{19}FN_2O_4$ $[M+H]^+$ 347.1329; found 347.1413.

*4.1.4.3. 6-hydroxy-8-(1-hydroxy-2-((4-methoxyphenethyl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17-3**)*

White solid; mp: 229.9-230.2 °C; yield 95 %; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.65 (s, 1H), 9.34 (s, 1H), 9.10 (s, 1H), 8.78 (s, 1H), 7.17 (d, J = 8.6 Hz, 2H), 6.89 (d, J = 8.6 Hz, 2H), 6.53 (d, J = 2.7 Hz, 1H), 6.36 (d, J = 2.7 Hz, 1H), 6.11 (d, J = 4.0 Hz, 1H), 5.14 – 5.09 (m, 1H), 4.49 (q, J = 15.0 Hz, 2H), 3.73 (s, 3H), 3.11 (s, 3H), 2.91 (pd, J = 13.3, 6.2 Hz, 3H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.8, 158.5, 153.0, 132.7, 130.4, 130.1, 130.1, 129.5, 128.2, 114.5, 114.5, 107.0, 102.7, 67.4, 63.3, 55.5, 52.3, 48.8, 30.9. Calcd. for $C_{19}H_{22}FN_2O_5$ $[M+H]^+$ 359.1529; found 359.1610.

*4.1.4.4. 6-hydroxy-8-(1-hydroxy-2-((3-phenylpropyl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17-4**)*

White solid; mp: 203.7-205.0 °C; yield 97 %; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H), 9.28 (s, 1H), 8.82 (s, 1H), 8.62 (s, 1H), 7.27 (t, J = 7.5 Hz, 2H), 7.17 (dd, J = 15.3, 7.5 Hz, 3H), 6.50 (d, J = 2.0 Hz, 1H), 6.34 (s, 1H), 6.03 (s, 1H), 5.06 (d, J = 9.8 Hz, 1H), 4.44 (t, J = 8.9 Hz, 2H), 3.02 (s, 1H), 2.95 – 2.79 (m, 3H), 2.57 (t, J = 6.8 Hz, 2H), 1.60 (d, J = 3.5 Hz, 4H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.7, 153.0, 142.1, 132.7, 130.4, 128.7, 128.7, 128.2, 126.4, 126.2, 107.1, 102.7, 67.4, 63.3, 52.3, 47.3, 35.0, 28.2, 25.2. Calcd. for $C_{20}H_{24}N_2O_4$ $[M+H]^+$ 357.1736; found 357.1812.

*4.1.5. General procedures for the synthesis of compounds **17a-l**.*

An appropriate amine (**10a-l**) (1.1 equiv.) was added into intermediate **16** (1.0 equiv.) dissolved in DMSO and the reaction was stirred at 35°C for more than 4 h. Then, sodium borohydride (1.0 equiv.) was added with a suitable amount of methanol and the reaction was stirred for a further 1 h at 0°C. After removal of the methanol by rotary evaporation, the concentrate was purified by column chromatography (silica gel, CH_2Cl_2-MeOH 50:1) to give a white solid. The white solid dissolved in MeOH and added ethyl acetate-HCl, stirred at room temperature for 2 h, the suspension was filtered and yield white solid. The desired fraction was dissolved in methanol and mixed with 5% Pd/C (0.2 equiv.). The reaction was maintained at 30°C under 1 atm of hydrogen for 1 h. The suspension was filtered and evaporated to afford compounds **17a-l**.

*4.1.5.1. 6-hydroxy-8-(1-hydroxy-2-((3-phenylpropyl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17a**).*

White solid; mp: 148.1-150.4 °C; yield 96%; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.64 (s, 1H), 9.33 (s, 1H), 8.99 (s, 1H), 8.76 (s, 1H), 7.31 (t, J = 7.4 Hz, 2H), 7.26 – 7.16 (m, 3H), 6.52 (s, 1H), 6.36 (s, 1H), 6.07 (s, 1H), 5.09 (s, 1H), 4.48 (q, J = 15.0 Hz, 2H), 3.06 (d, J = 12.2 Hz, 1H), 2.94 (t, J = 7.7 Hz, 2H), 2.87 (t, J = 11.2 Hz, 1H), 2.64 (t, J = 7.6 Hz, 2H), 1.96 (d, J = 7.3 Hz, 2H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.8, 153.0, 141.2, 132.7, 130.4, 128.9, 128.9, 128.7, 128.7, 128.2, 126.5, 107.1, 102.6, 67.4, 63.3, 52.3, 47.1, 32.4, 27.3. Calcd. for $C_{19}H_{22}N_2O_4$ $[M+H]^+$ 343.1580; found 343.1651.

*4.1.5.2. 8-(2-((3-(4-fluorophenyl)propyl)amino)-1-hydroxyethyl)-6-hydroxy-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17b**).*

White solid; mp: 148.1-149.7 °C; yield 97%; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.64 (s, 1H), 9.33 (s, 1H), 9.00 (s, 1H), 8.74 (s, 1H), 7.29 – 7.23 (m, 2H), 7.13 (dd, J = 12.3, 5.4 Hz, 2H), 6.52 (d, J = 2.7 Hz, 1H), 6.36 (d, J = 2.8 Hz, 1H), 6.07 (d, J = 3.9 Hz, 1H), 5.09 (dd, J = 6.8, 3.3 Hz, 1H), 4.48 (q, J = 15.0 Hz, 2H), 3.06 (d, J = 11.9 Hz, 1H), 2.92 (t, J = 7.7 Hz, 2H), 2.89 – 2.85 (m, 1H), 2.63 (t, J = 7.6 Hz, 2H), 1.98 – 1.90 (m, 2H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.8, 162.0, 160.4, 153.0, 137.3, 130.5, 130.5, 130.4, 130.4, 128.2, 115.6, 107.1, 102.6, 67.4, 63.3, 52.3, 47.0, 31.5, 27.4. Calcd. for $C_{19}H_{21}FN_2O_4$ $[M+H]^+$ 361.1485; found 361.1567.

*4.1.5.3. 6-hydroxy-8-(1-hydroxy-2-((3-(4-hydroxyphenyl)propyl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17c**).*

White solid; mp: 239.7-240.8 °C; yield 98%; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.64 (s, 1H), 9.32 (s, 1H), 9.25 (s, 1H), 8.92 (s, 1H), 8.69 (s, 1H), 6.99 (d, J = 8.4 Hz, 2H), 6.69 (d, J = 8.4 Hz, 2H), 6.52 (d, J = 2.6 Hz, 1H), 6.35 (d, J = 2.8 Hz, 1H), 6.06 (d, J = 3.6 Hz, 1H), 5.10 – 5.06 (m, 1H), 4.48 (q, J = 15.0 Hz, 2H), 3.05 (s, 1H), 2.93 – 2.83 (m, 3H), 2.52 (d, J = 9.2 Hz, 2H), 1.89 (dt, J = 12.5, 6.9 Hz, 2H).^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.8, 156.0, 153.0, 132.7, 131.1, 130.4, 130.4, 129.5, 128.2, 115.6, 115.6, 107.0, 102.6, 67.4, 63.3, 52.3, 47.1, 31.6, 27.6. Calcd. for $C_{19}H_{22}N_2O_5$ $[M+H]^+$ 359.1529; found 359.1602.

*4.1.5.4. 6-hydroxy-8-(1-hydroxy-2-((3-(2-hydroxyphenyl)propyl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17d**).*

White solid; mp: 212.1-213.0 °C; yield 96%; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.64 (s, 1H), 9.32 (s, 1H), 9.25 (s, 1H), 8.92 (s, 1H), 8.69 (s, 1H), 6.99 (d, J = 8.4 Hz, 2H), 6.69 (d, J = 8.4 Hz, 2H), 6.52 (d, J = 2.6 Hz, 1H), 6.35 (d, J = 2.8 Hz, 1H), 6.06 (d, J = 3.6 Hz, 1H), 5.10 – 5.06 (m, 1H), 4.48 (q, J = 15.0 Hz, 2H), 3.05 (s, 1H), 2.93 – 2.83

(m, 3H), 2.52 (d, J = 9.2 Hz, 2H), 1.89 (dt, J = 12.5, 6.9 Hz, 2H).^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.8, 156.0, 153.0, 132.7, 132.7, 131.1, 130.4, 130.2, 129.5, 128.2, 115.6, 107.0, 102.6, 67.4, 63.3, 52.3, 47.1, 31.6, 27.6. Calcd. for $C_{19}H_{22}N_2O_5$ $[M+H]^+$ 359.1529; found 359.1608.

*4.1.5.5. 6-hydroxy-8-(1-hydroxy-2-((3-(3-hydroxyphenyl)propyl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17e**).*

White solid; mp: 224.5-225.1 °C; yield 97%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H), 9.32 (s, 1H), 9.27 (s, 1H), 8.91 (s, 1H), 8.68 (s, 1H), 7.06 (t, J = 7.9 Hz, 1H), 6.59 (t, J = 5.0 Hz, 3H), 6.50 (d, J = 2.7 Hz, 1H), 6.33 (d, J = 2.8 Hz, 1H), 6.03 (s, 1H), 5.06 (d, J = 9.6 Hz, 1H), 4.52 – 4.40 (m, 2H), 3.09 – 2.99 (m, 1H), 2.95 – 2.79 (m, 3H), 2.52 (t, J = 7.6 Hz, 2H), 1.96 – 1.82 (m, 2H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.8, 157.9, 153.0, 142.5, 132.8, 130.4, 129.7, 128.2, 119.2, 115.6, 113.5, 107.0, 102.6, 67.4, 63.3, 52.3, 47.1, 32.4, 27.2. Calcd. for $C_{19}H_{22}N_2O_5$ $[M+H]^+$ 359.1529; found 359.1606.

*4.1.5.6. 6-hydroxy-8-(1-hydroxy-2-((3-(4-methoxyphenyl)propyl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17f**).*

White solid; mp: 207.6-208.1 °C; yield 94%; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.62 (s, 1H), 9.31 (s, 1H), 8.98 (s, 1H), 8.71 (s, 1H), 7.11 (d, J = 8.5 Hz, 2H), 6.85 (d, J = 8.6 Hz, 2H), 6.50 (d, J = 2.7 Hz, 1H), 6.34 (d, J = 2.8 Hz, 1H), 6.05 (d, J = 3.8 Hz, 1H), 5.07 (d, J = 10.0 Hz, 1H), 4.46 (q, J = 15.0 Hz, 2H), 3.70 (s, 3H), 3.06 – 3.00 (m, 1H), 2.94 – 2.80 (m, 3H), 2.55 (t, J = 7.6 Hz, 2H), 1.90 (qd, J = 13.4, 7.1 Hz, 2H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.8, 158.0, 153.0, 133.0, 132.7, 130.4, 129.8, 129.6, 128.2, 114.4, 114.2, 107.1, 102.6, 67.4, 63.3, 55.4, 52.3, 47.1, 31.5, 27.5. Calcd. for $C_{20}H_{24}N_2O_5$ $[M+H]^+$ 373.1685; found 373.1762.

*4.1.5.7. 6-hydroxy-8-(1-hydroxy-2-((3-(2-methoxyphenyl)propyl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17g**).*

White solid; mp: 212.0-212.4 °C; yield 95%; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.60 (s, 1H), 9.28 (s, 1H), 8.84 (s, 1H), 8.67 (s, 1H), 7.19 – 7.15 (m, 1H), 7.13 – 7.09 (m, 1H), 6.93 (d, J = 8.1 Hz, 1H), 6.85 (t, J = 7.3 Hz, 1H), 6.48 (d, J = 2.7 Hz, 1H), 6.31 (d, J = 2.8 Hz, 1H), 6.02 (d, J = 3.8 Hz, 1H), 5.04 (d, J = 10.1 Hz, 1H), 4.43 (q, J = 15.0 Hz, 2H), 3.74 (s, 3H), 3.01 (d, J = 11.1 Hz, 1H), 2.88 (t, J = 7.9 Hz, 2H), 2.85 – 2.80 (m, 1H), 2.55 (t, J = 7.6 Hz, 2H), 1.86 (ddd, J = 16.8, 13.2, 5.5 Hz, 2H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.8, 157.4, 153.0, 132.7, 130.4, 130.0, 128., 128.25, 128.0, 120.8, 111.2, 107.0, 102.6, 67.4, 63.3, 55.7, 52.3, 47.3, 27.0, 25.9. Calcd. for $C_{20}H_{24}N_2O_5$ $[M+H]^+$ 373.1685; found 373.1762.

*4.1.5.8. 6-hydroxy-8-(1-hydroxy-2-((3-(3-methoxyphenyl)propyl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17h**).*

White solid; mp: 213.2-213.6 °C; yield 97%; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.62 (s, 1H), 9.31 (s, 1H), 8.98 (s, 1H), 8.72 (s, 1H), 7.20 (t, J = 8.0 Hz, 1H), 6.82 – 6.72 (m, 3H), 6.50 (d, J = 2.7 Hz, 1H), 6.34 (d, J = 2.7 Hz, 1H), 6.05 (d, J = 4.0 Hz, 1H), 5.07 (d, J = 10.0 Hz, 1H), 4.45 (q, J = 15.0 Hz, 2H), 3.72 (s, 3H), 3.04 (d, J = 12.2 Hz, 1H), 2.87 (dt, J = 22.7, 9.4 Hz, 3H), 2.59 (t, J = 7.6 Hz, 2H), 1.98 – 1.90 (m, 2H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.8, 159.8, 153.0, 142.7, 132.7, 130.4, 129.9, 128.2, 120.9, 114.4, 111.8, 107.1, 102.6, 67.4, 63.3, 55.4, 52.3, 47.1, 32.4, 27.1. Calcd. for $C_{20}H_{24}N_2O_5$ $[M+H]^+$ 373.1685; found 373.1761.

*4.1.5.9. 6-hydroxy-8-(1-hydroxy-2-((3-(p-tolyl)propyl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17i**).*

White solid; mp: 211.6-211.9 °C; yield 93%; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.65 (s, 1H), 9.35 (s, 1H), 9.07 (s, 1H), 8.78 (s, 1H), 7.10 (s, 4H), 6.52 (d, J = 2.1 Hz, 1H), 6.37 (d, J = 2.3 Hz, 1H), 6.08 (d, J = 3.4 Hz, 1H), 5.10 (d, J = 9.5 Hz, 1H), 4.47 (q, J = 15.0 Hz, 2H), 3.05 (s, 1H), 2.89 (d, J = 31.3 Hz, 3H), 2.59 (t, J = 7.4 Hz, 2H), 2.26 (s,

3H), 1.94 (dd, J = 15.5, 7.7 Hz, 2H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.8, 153.0, 138.0, 135.4, 132.7, 130.4, 129.4, 129.2, 128.6, 128.4, 128.2, 107.1, 102.7, 67.4, 63.3, 52.3, 47.1, 32.0, 27.3, 21.0. Calcd. for $C_{20}H_{24}N_2O_4$ $[M+H]^+$ 357.1736; found 357.1811.

*4.1.5.10. 6-hydroxy-8-(1-hydroxy-2-((3-(m-tolyl)propyl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17j**).*

White solid; mp: 214.4-215.0 °C; yield 96%; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 10.64 (s, 1H), 9.31 (s, 1H), 8.93 (s, 1H), 8.69 (s, 1H), 7.19 (t, J = 7.4 Hz, 1H), 7.06 – 6.96 (m, 3H), 6.52 (d, J = 2.5 Hz, 1H), 6.35 (d, J = 2.6 Hz, 1H), 6.06 (d, J = 3.8 Hz, 1H), 5.08 (d, J = 9.9 Hz, 1H), 4.48 (q, J = 15.0 Hz, 2H), 3.05 (s, 1H), 2.90 (d, J = 30.6 Hz, 3H), 2.59 (t, J = 7.6 Hz, 2H), 2.28 (s, 3H), 1.98 – 1.90 (m, 2H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 165.8, 160.4, 153.0, 137.9, 137.3, 132.7, 130.4, 129.4, 128.8, 128.2, 127.1, 125.7, 107.0, 102.6, 67.4, 63.3, 52.3, 47.1, 32.3, 27.3. Calcd. for $C_{20}H_{24}N_2O_4$ $[M+H]^+$ 357.1736; found 357.1817.

*4.1.5.11. 6-hydroxy-8-(1-hydroxy-2-((3-(3-(trifluoromethyl)phenyl)propyl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17k**).*

White solid; mp: 214.4-214.9 °C; yield 94%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.62 (s, 1H), 9.29 (s, 1H), 8.92 (s, 1H), 8.68 (s, 1H), 7.58 (d, J = 14.8 Hz, 4H), 6.52 (s, 1H), 6.35 (s, 1H), 6.05 (s, 1H), 5.08 (d, J = 8.8 Hz, 1H), 4.54 – 4.42 (m, 2H), 3.07 (s, 1H), 2.95 (s, 3H), 2.76 (t, J = 7.0 Hz, 2H), 1.99 (s, 2H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.8, 153.1, 142.6, 133.0, 132.8, 130.4, 129.9, 129.5, 128.3, 125.5, 123.5, 107.0, 102.6, 67.4, 63.3, 60.2, 52.3, 46.9, 32.0, 27.1. Calcd. for $C_{20}H_{21}F_3N_2O_4$ $[M+H]^+$ 411.1453; found 411.1533.

*4.1.5.12. 8-(2-((3-(3,5-difluorophenyl)propyl)amino)-1-hydroxyethyl)-6-hydroxy-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**17l**).*

White solid; mp: 214.4-214.9 °C; yield 98%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H), 9.28 (s, 1H), 8.98 (s, 1H), 8.70 (s, 1H), 7.04 (tt, J = 9.5, 2.3 Hz, 1H), 7.00 – 6.94 (m, 2H), 6.50 (d, J = 2.7 Hz, 1H), 6.33 (d, J = 2.8 Hz, 1H), 6.03 (d, J = 4.0 Hz, 1H), 5.07 (dd, J = 6.7, 3.4 Hz, 1H), 4.50 – 4.41 (m, 2H), 3.05 (d, J = 11.8 Hz, 1H), 2.94 – 2.82 (m, 3H), 2.67 (t, J = 7.5 Hz, 2H), 2.01 – 1.89 (m, 2H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.7, 164.0, 161.7, 161.5, 153.0, 145.9, 132.8, 130.4, 128.2, 112.0, 111.8, 107.1, 102.7, 102.0, 67.4, 63.3, 52.3, 46.8, 31.9, 26.5. Calcd. for $C_{20}H_{20}F_3N_2O_4$ $[M+H]^+$ 379.1391; found 379.1472.

*4.1.6. The synthesis of compounds **18a-h**.*

To a stirred solution of intermediate **16** (1.0 equiv.), different substituted amines side chains **14a-h** (1.0 equiv.) in DMSO were added and stirred at room temperature for 1.5 h. Then appropriate methanol and sodium borohydride (1.0 equiv.) were added into the reaction mixture stirred on an ice-bath for 2 h. Removal of the solvent gave a residue that was subsequently purified by column chromatography (silica gel, CH_2Cl_2-MeOH 30:1 as an eluent) to give the white solid. A solution of the white solid in methanol and 10% Pd/C (0.1 equiv.) was added. The reaction mixture was stirred at room temperature at 1 atmosphere of hydrogen for 2 h. The resulting mixture was filtered and concentrated to give **18a-h** as a white solid.

*4.1.6.1 6-hydroxy-8-(1-hydroxy-2-((2-methyl-4-phenylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one (**18a**).*

White solid; mp: 155.6-156.2 °C; yield 95%; ^{1}H-NMR (400 MHz, MeOD-d_6) δ 7.16 (dd, J = 16.4, 7.0 Hz, 4H), 7.11 – 7.06 (m, 1H), 6.61 (d, J = 2.7 Hz, 1H), 6.27 (d, J = 2.8 Hz, 1H), 5.09 (dd, J = 10.1, 2.3 Hz, 1H), 4.42 (t, J = 13.2 Hz, 2H), 3.16 (dd, J =

12.5, 2.5 Hz, 1H), 2.86 (dd, J = 12.4, 10.2 Hz, 1H), 2.60 (ddd, J = 12.3, 9.7, 6.4 Hz, 2H), 1.87 (t, J = 8.7 Hz, 2H), 1.35 (s, 6H). ^{13}C-NMR (101 MHz, $CDCl_3$-d_6) δ 170.3, 156.8, 144.6, 136.9, 133.7, 132.4, 132.2, 131.8, 131.6, 131.4, 129.9, 110.7, 106.4, 70.8, 68.0, 63.5, 50.5, 43.4, 33.3, 26.1, 26.0. Calcd. for $C_{21}H_{26}N_2O_4$ $[M+H]^+$ 371.1893; found 371.1970.

*4.1.6.2 6-hydroxy-8-(1-hydroxy-2-((4-(4-methoxyphenyl)-2-methylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**18b**).*

White solid; mp: 223.9-224.4 °C; yield 97%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.61 (s, 1H), 9.28 (s, 1H), 8.90 (s, 1H), 8.59 (s, 1H), 7.12 (d, J = 8.5 Hz, 2H), 6.83 (d, J = 8.6 Hz, 2H), 6.55 (d, J = 2.7 Hz, 1H), 6.34 (d, J = 2.7 Hz, 1H), 6.06 (s, 1H), 5.10 (d, J = 9.9 Hz, 1H), 4.46 (q, J = 15.0 Hz, 2H), 3.70 (s, 3H), 3.05 (t, J = 10.4 Hz, 1H), 2.78 (dd, J = 19.0, 9.4 Hz, 1H), 2.61 – 2.50 (m, 2H), 1.93 – 1.79 (m, 2H), 1.32 (s, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.7, 158.0, 153.0, 133.5, 132.8, 130.5, 129.6, 129.4, 128.2, 114.5, 114.3, 107.1, 102.6, 67.4, 64.0, 60.2, 59.6, 55.4, 47.2, 28.8, 23.0, 21.2. Calcd. for $C_{22}H_{28}N_2O_5$ $[M+H]^+$ 401.1998; found 401.2069.

*4.1.6.3 6-hydroxy-8-(1-hydroxy-2-((4-(2-methoxyphenyl)-2-methylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**18c**).*

White solid; mp: 228.7-229.1 °C; yield 97%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H), 9.26 (s, 1H), 8.71 (s, 1H), 8.49 (s, 1H), 7.26 – 7.06 (m, 2H), 6.93 (d, J = 7.6 Hz, 1H), 6.85 (td, J = 7.4, 1.0 Hz, 1H), 6.55 (d, J = 2.7 Hz, 1H), 6.33 (d, J = 2.8 Hz, 1H), 6.01 (s, 1H), 5.08 (d, J = 9.4 Hz, 1H), 4.46 (q, J = 15.0 Hz, 2H), 3.71 (s, 3H), 3.05 (d, J = 12.2 Hz, 1H), 2.86 – 2.74 (m, 1H), 2.54 (dd, J = 16.9, 8.0 Hz, 2H), 1.76 (dd, J = 9.7, 7.2 Hz, 2H), 1.32 (d, J = 1.2 Hz, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 170.8, 165.8, 157.4, 153.0, 133.0, 130.1, 129.4, 128.4, 128.0, 120.8, 120.7, 111.1, 110.9, 107.1, 102.5, 67.4, 60.3, 55.7, 24.5, 23.1, 21.1, 14.5. Calcd. for $C_{22}H_{28}N_2O_5$ $[M+H]^+$ 401.1998;

found 401.2078.

*4.1.6.4 6-hydroxy-8-(1-hydroxy-2-((4-(3-methoxyphenyl)-2-methylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**18d**).*

White solid; mp: 220.9-222.0 °C; yield 96%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.57 (s, 1H), 9.21 (s, 1H), 8.68 (d, J = 101.1 Hz, 1H), 7.18 (t, J = 8.0 Hz, 1H), 6.74 (dt, J = 10.3, 4.0 Hz, 3H), 6.53 (d, J = 2.8 Hz, 1H), 6.31 (d, J = 2.7 Hz, 1H), 5.91 (s, 1H), 5.01 (s, 1H), 4.45 (q, J = 15.0 Hz, 2H), 3.71 (s, 3H), 2.94 (s, 1H), 2.71 (s, 1H), 2.60 – 2.50 (m, 2H), 1.79 (s, 2H), 1.25 (s, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 166.1, 165.8, 159.7, 159.4, 153.1, 132.8, 129.8, 129.6, 128.2, 121.0, 120.9, 114.5, 114.3, 111.8, 107.3, 102.4, 67.4, 60.3, 55.6, 29.8, 21.1, 14.5. Calcd. for $C_{22}H_{28}N_2O_5$ $[M+H]^+$ 401.1998; found 401.2080.

*4.1.6.5 6-hydroxy-8-(1-hydroxy-2-((4-(4-hydroxyphenyl)-2-methylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**18e**).*

White solid; mp: 167.1-167.8 °C; yield 93%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H), 9.27 (s, 1H), 9.19 (s, 1H), 8.78 (s, 1H), 8.54 (s, 1H), 6.98 (d, J = 8.4 Hz, 2H), 6.66 (d, J = 8.4 Hz, 2H), 6.54 (d, J = 2.7 Hz, 1H), 6.34 (d, J = 2.8 Hz, 1H), 6.04 (s, 1H), 5.09 (d, J = 9.6 Hz, 1H), 4.46 (dd, J = 30.5, 15.0 Hz, 2H), 3.05 (t, J = 10.6 Hz, 1H), 2.78 (dd, J = 19.1, 9.4 Hz, 1H), 2.44 (s, 2H), 1.89 – 1.73 (m, 2H), 1.31 (s, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.7, 156.0, 153.0, 132.8, 131.6, 130.5, 129.4, 128.2, 115.6, 115.4, 107.1, 102.6, 67.4, 64.0, 60.2, 59.6, 47.0, 28.8, 23.0, 21.2, 14.5. Calcd. for $C_{21}H_{26}N_2O_5$ $[M+H]^+$ 387.1842; found 387.1919.

*4.1.6.6 6-hydroxy-8-(1-hydroxy-2-((4-(3-hydroxyphenyl)-2-methylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**18f**).*

White solid; mp: 231.7-232.1 °C; yield 96%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.67 (s, 1H), 9.40 (s, 1H), 9.35 (s, 1H), 9.06 (s, 1H), 8.72 (s, 1H), 7.09 (t, J = 7.8 Hz, 1H),

6.72 – 6.59 (m, 4H), 6.42 (d, J = 2.5 Hz, 1H), 6.13 (s, 1H), 5.17 (d, J = 9.6 Hz, 1H), 4.51 (q, J = 15.0 Hz, 2H), 3.10 (d, J = 11.4 Hz, 1H), 2.84 (t, J = 10.1 Hz, 1H), 2.57 (d, J = 7.7 Hz, 2H), 1.98 – 1.86 (m, 2H), 1.38 (s, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.8, 157.9, 153.0, 142.5, 132.7, 130.4, 129.7, 128.2, 119.2, 115.6, 113.5, 107.0, 102.6, 67.4, 63.3, 60.2, 59.5, 52.3, 47.1, 32.4, 27.2. Calcd. for $C_{21}H_{26}N_2O_5$ $[M+H]^+$ 387.1842; found 387.1909.

*4.1.6.7 6-hydroxy-8-(1-hydroxy-2-((2-methyl-4,4-diphenylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**18g**).*

White solid; mp: 194.3-194.9 °C; yield 97%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H), 9.27 (s, 1H), 8.89 (s, 1H), 8.62 (s, 1H), 7.40 (d, J = 7.3 Hz, 4H), 7.25 (td, J = 7.7, 1.7 Hz, 4H), 7.13 (t, J = 7.3 Hz, 2H), 6.54 (d, J = 2.7 Hz, 1H), 6.34 (d, J = 2.8 Hz, 1H), 6.06 (d, J = 3.9 Hz, 1H), 5.09 (d, J = 9.5 Hz, 1H), 4.43 (dd, J = 33.7, 15.0 Hz, 2H), 4.22 (t, J = 6.6 Hz, 1H), 3.05 (t, J = 10.0 Hz, 1H), 2.86 – 2.73 (m, 1H), 2.53 (t, J = 6.8 Hz, 2H), 1.11 (d, J = 8.7 Hz, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 170.8, 165.8, 152.9, 146.1, 146.0, 132.7, 130.5, 128.9, 128.7, 128.3, 128.2, 127.9, 127.9, 126.6, 107.1, 102.6, 67.3, 64.0, 60.5, 60.2, 46.4, 46.2, 41.9, 23.6, 23.4, 21.2, 14.5. Calcd. for $C_{27}H_{30}N_2O_4$ $[M+H]^+$ 447.2206; found 447.2285.

*4.1.6.8 6-hydroxy-8-(2-((4,4-bis(4-fluorophenyl)-2-methylbutan-2-yl)amino)-1-hydroxyethyl)-6-hydroxy-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**18h**).*

White solid; mp: 199.8-200.2 °C; yield 94%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H), 9.26 (s, 1H), 8.79 (s, 1H), 8.55 (s, 1H), 7.42 (dd, J = 8.1, 5.6 Hz, 4H), 7.09 (td, J = 8.9, 2.7 Hz, 4H), 6.53 (d, J = 2.7 Hz, 1H), 6.33 (d, J = 2.8 Hz, 1H), 6.04 (s, 1H), 5.07 (d, J = 9.9 Hz, 1H), 4.51 – 4.38 (m, 2H), 4.29 (t, J = 6.6 Hz, 1H), 3.04 (t, J = 10.4 Hz, 1H), 2.83 – 2.73 (m, 1H), 2.51 (d, J = 6.9 Hz, 2H), 1.10 (d, J = 10.4 Hz, 6H).^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.8, 162.0, 160.2, 152.8, 142.1, 133.0, 130.7, 130.5,

129.7, 129.7, 129.6, 129.6, 129.6, 128.2, 115.8, 115.7, 115.6, 107.3, 102.5, 67.4, 64.0, 60.5, 46.9, 44.4, 42.1, 23.6, 23.4. Calcd. for $C_{27}H_{28}F_2N_2O_4$ $[M+H]^+$ 483.2017; found 483.2097.

*4.1.7. 8-(2-bromoacetyl)-6-hydroxy-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**19**).*

Intermediate **15** (10.00 g, 34.0 mmol) was dissolved in 1,4-dioxane (167 mL) and methanol (9 mL). Then, tetrabutylammonium tribromide (34.0 mmol) was added and the reaction was stirred for 2 h at room temperature. After that, water (250 mL) was added and stirred for 1 h at 0°C. The mixture was filtered through a Buchner funnel and the residue was washed by water (10 mL) for three times to give a white solid.

*4.1.8. (R)-6-hydroxy-8-(oxiran-2-yl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**20**).*

Under nitrogen protection, intermediate **19** (5.00 g, 13.3 mmol) was dissolved in anhydrous THF (50 mL), (-)-DIP-Cl (16.4 mL, 29.2 mmol) was dropped slowly at 0°C for more than 30 min. The reaction was maintained at 0°C for 1 h. Then 2 mol/L NaOH (50 mL) was added followed by stirring for 2 h at 0°C. The resultant solution was extracted three times with ethyl acetate. The combined organics were washed with brine, dried over sodium sulfate, and then concentrated in a rotary evaporator to give a white solid.

*4.1.9. General procedures for the synthesis of compounds **(R)-18a-h**.*

Intermediate **16** (1.0 equiv.) and an appropriate amine (**14a-h**) (1.0 equiv.) were dissolved in an appropriate amount of isopropanol. The reaction was maintained at 90°C for 15 h. After removal of the isopropanol by rotary evaporation, the concentrate was purified by column chromatography (silica gel, CH_2Cl_2-MeOH 50:1) to give a

white solid. The white solid dissolved in MeOH and added ethyl acetate-HCl, stirred at room temperature for 2 h, the suspension was filtered and yield white solid. The desired fraction was dissolved in methanol and mixed with 5% Pd/C (0.2 equiv.). The reaction was maintained at 30°C under 1 atm of hydrogen for 1 h. The suspension was filtered and evaporated to afford a white solid ***(R)-18a-h***.

*4.1.9.1. (R)-6-hydroxy-8-(1-hydroxy-2-((2-methyl-4-phenylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**(R)-18a**).*

White solid; mp: 154.6-155..1°C; yield 95%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.59 (s, 1H), 9.24 (s, 1H), 8.65 (d, J = 52.9 Hz, 2H), 7.27 (t, J = 7.4 Hz, 2H), 7.18 (dd, J = 16.3, 7.6 Hz, 3H), 6.54 (d, J = 2.7 Hz, 1H), 6.33 (d, J = 2.8 Hz, 1H), 5.99 (s, 1H), 5.06 (d, J = 9.0 Hz, 1H), 4.45 (q, J = 15.0 Hz, 2H), 3.01 (d, J = 11.3 Hz, 1H), 2.75 (t, J = 10.7 Hz, 1H), 2.59 (dd, J = 9.0, 6.4 Hz, 2H), 1.84 (s, 2H), 1.30 (s, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.8, 153.0, 142.0, 132.8, 132.6, 128.8, 128.7, 128.6, 128.4, 128.2, 126.4, 107.0, 102.5, 67.4, 64.3, 60.2, 47.3, 29.8, 23.4, 21.2, 14.5. Calcd. for $C_{21}H_{26}N_2O_4$ $[M+H]^+$ 371.1893; found 371.1972.

*4.1.9.2. (R)-6-hydroxy-8-(1-hydroxy-2-((4-(4-methoxyphenyl)-2-methylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**(R)-18b**).*

White solid; mp: 198.4-199.2°C; yield 94%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H), 9.26 (s, 1H), 8.78 (s, 1H), 8.54 (s, 1H), 7.12 (d, J = 8.6 Hz, 2H), 6.89 – 6.76 (m, 2H), 6.54 (d, J = 2.7 Hz, 1H), 6.33 (d, J = 2.8 Hz, 1H), 6.04 (s, 1H), 5.08 (d, J = 10.0 Hz, 1H), 4.46 (dd, J = 30.1, 15.0 Hz, 2H), 3.70 (s, 3H), 3.11 – 3.00 (m, 1H), 2.79 (d, J = 10.5 Hz, 1H), 2.58 – 2.50 (m, 2H), 1.84 (t, J = 8.5 Hz, 2H), 1.31 (s, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.7, 158.0, 153.0, 133.5, 132.8, 130.5, 129.6, 129.4, 128.2, 114.5, 114.3, 107.1, 102.6, 67.4, 64.0, 60.2, 55.4, 47.2, 28.8, 23.1, 21.2, 14.5.

Calcd. for $C_{22}H_{28}N_2O_5$ $[M+H]^+$ 401.1998; found 401.2081.

*4.1.9.3. (R)-6-hydroxy-8-(1-hydroxy-2-((4-(2-methoxyphenyl)-2-methylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**(R)-18c**).*

White solid; mp: 205.3-206.3°C; yield 95%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.63 (s, 1H), 9.30 (s, 1H), 8.85 (s, 1H), 8.59 (s, 1H), 7.18 (dd, J = 17.7, 7.6 Hz, 2H), 6.95 (d, J = 8.1 Hz, 1H), 6.87 (t, J = 7.4 Hz, 1H), 6.57 (d, J = 2.6 Hz, 1H), 6.37 (d, J = 2.6 Hz, 1H), 6.07 (s, 1H), 5.13 (d, J = 9.6 Hz, 1H), 4.48 (q, J = 15.0 Hz, 2H), 3.73 (s, 3H), 3.08 (t, J = 10.4 Hz, 1H), 2.83 (dd, J = 19.1, 9.3 Hz, 1H), 2.57 (dd, J = 16.4, 7.6 Hz, 2H), 1.80 (t, J = 8.4 Hz, 2H), 1.36 (s, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.7, 157.3, 153.0, 132.8, 130.6, 129.9, 129.4, 128.2, 127.9, 120.8, 111.2, 107.1, 102.6, 67.4, 63.9, 59.6, 55.6, 47.1, 37.7, 24.5, 23.1, 23.1. Calcd. for $C_{22}H_{28}N_2O_5$ $[M+H]^+$ 401.1998; found 401.2075.

*4.1.9.4. (R)-6-hydroxy-8-(1-hydroxy-2-((4-(3-methoxyphenyl)-2-methylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**(R)-18d**).*

White solid; mp: 205.3-206.3°C; yield 95%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.62 (s, 1H), 9.28 (s, 1H), 8.82 (s, 1H), 8.57 (s, 1H), 7.21 (t, J = 8.0 Hz, 1H), 6.84 – 6.74 (m, 3H), 6.57 (d, J = 2.7 Hz, 1H), 6.36 (d, J = 2.8 Hz, 1H), 6.07 (d, J = 3.9 Hz, 1H), 5.11 (d, J = 9.8 Hz, 1H), 4.49 (dd, J = 31.9, 15.0 Hz, 2H), 3.73 (s, 3H), 3.08 (t, J = 10.3 Hz, 1H), 2.81 (dd, J = 19.1, 9.4 Hz, 1H), 2.60 (dd, J = 10.0, 6.7 Hz, 2H), 1.90 (t, J = 8.6 Hz, 2H), 1.35 (s, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.8, 159.8, 153.0, 143.3, 132.8, 130.5, 129.9, 128.2, 120.9, 114.5, 111.8, 107.1, 102.6, 67.4, 64.0, 59.6, 55.4, 47.0, 29.7, 23.1, 23.0, 14.5. Calcd. for $C_{22}H_{28}N_2O_5$ $[M+H]^+$ 401.1998; found 401.2078.

*4.1.9.5. (R)-6-hydroxy-8-(1-hydroxy-2-((4-(4-hydroxyphenyl)-2-methylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**(R)-18e**).*

White solid; mp: 212.5-213.2°C; yield 96%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.60

(s, 1H), 9.26 (s, 1H), 9.18 (s, 1H), 8.78 (s, 1H), 8.53 (s, 1H), 6.98 (d, J = 8.5 Hz, 2H), 6.71 – 6.64 (m, 2H), 6.54 (d, J = 2.7 Hz, 1H), 6.34 (d, J = 2.8 Hz, 1H), 6.04 (d, J = 3.9 Hz, 1H), 5.09 (d, J = 10.1 Hz, 1H), 4.46 (dd, J = 30.5, 15.0 Hz, 2H), 3.05 (t, J = 10.5 Hz, 1H), 2.78 (dd, J = 18.9, 9.3 Hz, 1H), 2.46 (s, 2H), 1.86 – 1.77 (m, 2H), 1.31 (s, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 169.3, 164.3, 154.5, 151.5, 131.3, 130.2, 129.1, 128.0, 126.8, 114.2, 105.7, 101.2, 66.0, 62.6, 58.7, 58.1, 47.0, 27.4, 21.6, 19.8, 13.1. Calcd. for $C_{21}H_{26}N_2O_5$ $[M+H]^+$ 387.1842; found 387.1917.

*4.1.9.6. (R)-6-hydroxy-8-(1-hydroxy-2-((4-(3-hydroxyphenyl)-2-methylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**(R)-18f**).*

White solid; mp: 237.5-238.4°C; yield 96%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.62 (s, 1H), 9.31 (d, J = 15.1 Hz, 2H), 8.86 (s, 1H), 8.60 (s, 1H), 7.08 (t, J = 7.9 Hz, 1H), 6.67 – 6.55 (m, 4H), 6.37 (d, J = 2.8 Hz, 1H), 6.08 (d, J = 3.9 Hz, 1H), 5.13 (d, J = 9.9 Hz, 1H), 4.50 (dd, J = 30.8, 15.0 Hz, 2H), 3.08 (t, J = 10.1 Hz, 1H), 2.81 (dd, J = 19.0, 9.5 Hz, 1H), 2.53 (s, 2H), 1.94 – 1.83 (m, 2H), 1.35 (s, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.7, 157.9, 153.0, 143.1, 132.8, 130.5, 129.7, 128.2, 119.2, 115.6, 113.4, 107.1, 102.6, 67.4, 64.0, 60.2, 59.6, 47.1, 29.7, 23.0, 21.2, 14.5. Calcd. for $C_{22}H_{26}N_2O_5$ $[M+H]^+$ 387.1842; found 387.1917.

*4.1.9.7. (R)-6-hydroxy-8-(1-hydroxy-2-((2-methyl-4,4-diphenylbutan-2-yl)amino)ethyl)-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**(R)-18g**).*

White solid; mp: 221.3-222.1°C; yield 97%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.62 (s, 1H), 9.29 (s, 1H), 8.97 (s, 1H), 8.67 (s, 1H), 7.42 (d, J = 7.3 Hz, 4H), 7.27 (td, J = 7.7, 1.8 Hz, 4H), 7.15 (t, J = 7.3 Hz, 2H), 6.56 (d, J = 2.7 Hz, 1H), 6.36 (d, J = 2.8 Hz, 1H), 6.09 (d, J = 4.1 Hz, 1H), 5.12 (d, J = 9.9 Hz, 1H), 4.45 (dd, J = 33.5, 15.0 Hz, 2H), 4.25 (t, J = 6.6 Hz, 1H), 3.07 (t, J = 10.1 Hz, 1H), 2.81 (dd, J = 18.8, 9.3 Hz, 1H), 2.56 (t, J = 6.7 Hz, 2H), 1.13 (d, J = 8.7 Hz, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.8,

152.9, 146.1, 146.0, 132.7, 130.5, 128.9, 128.7, 128.5, 128.3, 128.2, 127.9, 127.6, 127.4, 127.2, 126.6, 107.1, 102.6, 67.3, 64.0, 60.5, 49.0, 46.9, 46.2, 42.0, 23.6, 23.4. Calcd. for $C_{27}H_{30}N_2O_4$ $[M+H]^+$ 447.2206; found 447.2281.

*4.1.9.8. (R)-8-(2-((4,4-bis(4-fluorophenyl)-2-methylbutan-2-yl)amino)-1-hydroxyethyl)-6-hydroxy-2H-benzo[b][1,4]oxazin-3(4H)-one hydrochloride (**(R)-18h**).*

White solid; mp: 232.1-232.5°C; yield 93%; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 10.62 (s, 1H), 9.29 (s, 1H), 8.91 (s, 1H), 8.63 (s, 1H), 7.45 (dd, J = 8.3, 5.6 Hz, 4H), 7.11 (td, J = 8.9, 2.7 Hz, 4H), 6.56 (d, J = 2.7 Hz, 1H), 6.36 (d, J = 2.8 Hz, 1H), 6.08 (d, J = 4.0 Hz, 1H), 5.11 (d, J = 10.0 Hz, 1H), 4.46 (dd, J = 32.5, 15.0 Hz, 2H), 4.33 (t, J = 6.7 Hz, 1H), 3.06 (t, J = 10.2 Hz, 1H), 2.81 (dd, J = 19.1, 9.4 Hz, 1H), 2.56 (d, J = 6.7 Hz, 2H), 1.13 (d, J = 10.4 Hz, 6H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ 165.7, 162.3, 159.9, 152.9, 142.2, 142.1, 132.8, 130.5, 129.7, 129.6, 129.4, 128.2, 116.0, 115.8, 115.6, 115.4, 115.2, 107.1, 102.6, 67.4, 64.0, 60.4, 46.9, 44.4, 42.2, 23.6, 23.4. Calcd. for $C_{27}H_{28}F_2N_2O_4$ $[M+H]^+$ 483.2017; found 483.2097.

4.2 Biological tests

4.2.1 Cellular cAMP assay

The cellular cAMP assay was performed according to a previously reported method.[18]

4.2.2 Isolated guinea pig trachea relaxation assay

The isolated guinea pig trachea relaxation assay was performed according to a previously reported method.[18]

4.3 Molecular modeling

Molecular modeling was performed according to a previously reported method.[18]

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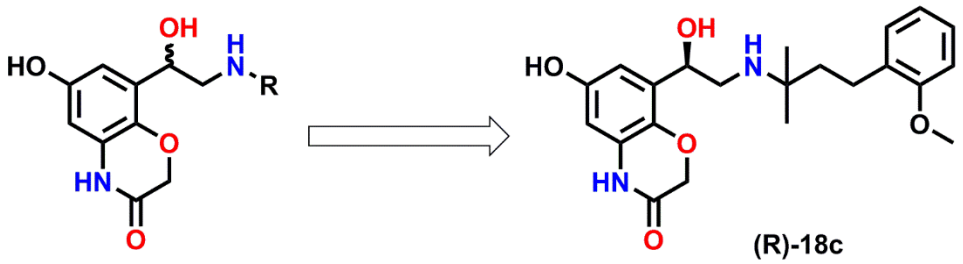
Highlights

- (**R**)**-18a (R)-18c** and (**R**)**-18f** exhibited the most potent with EC_{50} values of 9.1 pM 24 pM and 16 pM, respectively.
- (**R**)**-18f** exhibited the highest β_2/β_1-selectivity.
- **(R)-18c** showed the best bronchodilator effects and the duration of action is more than 12h (long duration of action) and the onset time is 3.50 min (fast onset of action) *in vitro*.
- **(R)-18c** formed tight binding with β_2-AR co-crystallized on the basis of the molecular modeling result.
- **(R)-18c** were worthy of further investigation as a lead compound for the development of β_2-adrenoceptor agonists.

Graphical abstract



(R)-18c

β_2 pEC_{50} = 10.62
Ot_{50} = 3.50 min
DoA = more than 12 h

Conflict of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.