Design, synthesis and pharmacological evaluation of tricyclic derivatives as selective RXFP4 agonists

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ABSTRACT
Relaxin family peptide receptors (RXFPs) are the potential therapeutic targets for neuroscience, cardiovascular, and metabolic indications. Among them, RXFP3 and RXFP4 (formerly known as GPR100 or GPCR142) are homologous class A G protein-coupled receptors with short N-terminal domain. Ligands of RXFP3 or RXFP4 are only limited to endogenous peptides and their analogues, and no natural product or synthetic agonists have been reported to date except for a scaffold of indole-containing derivatives as dual agonists of RXFP3 and RXFP4. In this study, a new scaffold of tricyclic derivatives represented by compound 7a was disclosed as a selective RXFP4 agonist after a high-throughput screening campaign against a diverse library of 52,000 synthetic and natural compounds. Two rounds of structural modification around this scaffold were performed focusing on three parts: 2-chlorophenyl group, 4-hydroxylphenyl group and its skeleton including cyclohexane-1,3-dione and 1,2,4-triazole group. Compound 14b with a new skeleton of 7,9-dihydro-4H-thiopyrano[3,4-d][1,2,4]triazolo[1,5-a]pyrimidin-8(5H)-one was thus obtained. The enantiomers of 7a and 14b were also resolved with their 9-(S)-conformer favoring RXFP4 agonism.
Compared with 7a, compound 9-(S)-14b exhibited 2.3-fold higher efficacy and better selectivity for RXFP4 (selective ratio of RXFP4 vs. RXFP3 for 9-(S)-14b and 7a were 26.9 and 13.9, respectively).

**Graphical abstract**

**KEYWORDS**

Synthesis, structure-activity relationship, relaxin family peptide receptor 4, selective agonist, molecular docking.

**Glossary**

HTRF Homogeneous time-resolved fluorescence  
HTS High-throughput screening  
SAR Structure-activity relationship  
RXFPs1-4 Relaxin family peptide receptors 1-4  
GPCRs G protein-coupled receptors

**Highlights**

A series of tricyclic derivatives were synthesized via Biginelli cyclocondensation. The analogues were screened for their biological activities using a HTRF assay that measures the inhibition of forskolin-stimulated cAMP accumulation in human RXFP4-overexpressing CHO cells. The specificity of the analogues for RXFP4 was also examined in human RXFP3-overexpressing CHO-K1 cells and human RXFP1-overexpressing 293T cells. Compared with 7a, compound 9-(S)-14b behaved as a RXFP4 agonist and exhibited 2.3-fold higher efficacy and better selectivity for RXFP4 vs. RXFP3. A relatively high LibDock fitness score of 111.977 was obtained for the docking of 14b
with RXFP4, and several functions were involved including two hydrogen bonds, Pi-Pi stack and Pi-cation individually, and one additional Pi-sulfur function, which may explain the efficacy difference between 14b and 7a.

1. Introduction

Relaxin family is a group of peptide hormones that perform a variety of biological functions after activation of the relaxin family peptide receptors 1-4 (RXFPs 1-4) [1, 2], such as reproduction regulation, stress responses, food intake and glucose homeostasis, etc. These associated activities enable RXFPs to be the potential therapeutic targets for neurological, cardiovascular and metabolic disorders [3-9]. Among them, RXFP3 and RXFP4 (formerly known as GPR100 or GPCR142) are homologous class A G protein-coupled receptors (GPCRs) with a short N-terminal domain. RXFP4 is predominantly expressed in the colon and rectum with implications of insulin secretion, appetite and regulation of colon motility [1, 10]. The cognate ligands of RXFP3 and RXFP4 are relaxin-3 and insulin-like peptide 5 (INSL5), respectively. Relaxin-3 also activates RXFP1 and RXFP4 in vitro [8, 11]. Relaxin-3 interacts with both the LRR domain and ECL2 of the TM domain of RXFP1 to produce the full binding and cAMP signaling profile [2]. R3/I5, a chimeric peptide, contains the B chain of relaxin-3 and the A chain of INSL5 and activates RXFP3 and RXFP4 at almost equal potency [2]. INSL5 is a two-chain, three-disulfide-bonded peptide and mainly expressed in the colorectum and enteric nervous system together with glucagon-like peptide 1 (GLP-1) and peptide YY. Binding of INSL5 to RXFP4 increases GTPγS activity, inhibits forskolin-stimulated cyclic adenosine monophosphate (cAMP) accumulation and elevates phosphorylation of ERK1/2, p38MAPK, Akt Ser473, Akt Thr308 and S6 ribosomal protein in CHO-RXFP4 cells [1, 12]. INSL5 also suppresses glucose-stimulated insulin secretion and Ca2+ mobilization in MIN6 insulinoma cells and forskolin-stimulated cAMP accumulation in NCI-H716 enteroendocrine cells [12]. Despite these attractive properties, ligands of RXFP3 or RXFP4 are only limited to endogenous peptides and their analogues, and no natural product or synthetic agonists have been reported to date except for a scaffold of indole-containing derivatives disclosed by DeChristopher and colleagues as dual agonists of RXFP3 and RXFP4 (shown as compound 1 in Figure 1) [13].

In an attempt to discover non-peptidic small molecules as selective RXFP4 agonists, a high-throughput screening (HTS) campaign against a diverse library of 52,000 synthetic and natural compounds was carried out using a homogeneous time-
resolved fluorescence (HTRF) assay that measures the inhibition of forskolin-stimulated cAMP accumulation in human RXFP3- and RXFP4-overexpressing CHO cells. This led to the discovery of a new scaffold, 5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (represented by 7a), as selective RXFP4 agonist as demonstrated by its activities in pCRE activation, ERK1/2 phosphorylation, intracellular calcium mobilization and β-arrestin recruitment [14]. In this paper, we described in detail their chemical synthesis, structure-activity relationship (SAR) analysis, molecular docking and subsequent bioactivity evaluation.

**Figure 1. Structures of compounds 1 and 7a.**

2. Results and Discussion

2.1. **SAR-based design and chemical synthesis**

Based on SAR information, rational design around the scaffold of 5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (represented by 7a) was conducted focusing on five parts: 2-chlorophenyl group, 4-hydroxylphenyl group, its skeleton including cyclohexane-1,3-dione and 1,2,4-triazole group, and chiral resolution (Figure 2).
Figure 2. Rational design of RXFP4 agonists.

The general synthetic route is described as follows. Substituted methyl benzoate 2 or benzoyl chloride 3 was used as the starting material, which underwent hydrazinolysis to obtain benzohydrazide derivatives 4. The key intermediate 1,2,4-triazole 6 was synthesized by reacting compound 4 with S-methylisothiourea sulfate which was cyclized in the following step using p-toluenesulfonic acid as a catalyst. Biginelli cyclocondensation [15-17] was then conducted under the catalysis of acetic acid with the triazole derivative 6, cyclohexane-1,3-dione and 4-hydroxybenzaldehyde to obtain the final product 7 (Scheme 1). Compound 7a-m were then designed and synthesized for the purpose of finding the suitable substitutes on the phenyl group by involving the electron-donating group (7h-i), electron-withdrawing group (7a-g and 7j-l), the effect of steric-hindrance (7f-g) or the substituted position (7a-c and 7j-l) to improve their binding to RXFP4.
Scheme 1. Synthetic route for compounds 7a-m. (i) 2 and NH₂NH₂·H₂O in EtOH, reflux or 3 and NH₂NH₂·H₂O in DCM, r.t. overnight; (ii) 4 and S-methylisothiourea sulfate in dioxane and 2N NaOH, reflux, two steps yield: 70-98%; (iii) 5 and p-TsOH in water and dioxane, reflux, yield: 75-97%; (iv) HOAc in EtOH, reflux, yield: 30-61%.

Subsequent SAR studies around 4-hydroxylphenyl and cyclohexane-1,3-dione were carried out using a similar procedure, except that different substituted benzaldehyde 8 and 1,3-cycloalkandione 10 were employed to obtain derivatives 9 and 11, respectively. Compounds 9a-i were thus designed and synthesized for the purpose of examining the necessity of 9-aromatic nucleus (9b), restriction of the number and position for 4-hydroxyl group (9a and 9c), effect of steric hindrance (9d and 9h) and possibility of introduction of other heteroatom instead of oxygen (9d-e and 9g-i).

Compounds 11a-d were synthesized for the purpose of increasing the steric hindrance (11a-b) by addition of di-methyl group and inserting heteroatom such as oxygen (11c) and sulphur (11d), aiming at finding other possible scaffolds except for 5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one, e.g., compounds 7 and 9.

Further SAR analysis was made around the skeleton of 1,2,4-triazole part with 4H-1,2,4-triazol-3-amine (12a) and tetrazole (12b) instead of 5-(2-chlorophenyl)-4H-1,2,4-triazol-3-amine 6a to examine if the 2-chlorophenyl group is required for RXFP4 binding. Compounds 13a and 13b were thus synthesized. Additionally, a derivative 13c
was synthesized with only two nitrogen atoms involved as compared with compound 7a to see whether the number of nitrogen atom has any impact on its bioactivity (Scheme 2).

Scheme 2. Synthetic route for compounds 9a-i, 11a-d and 13a-c. (i) HOAc in EtOH, reflux, yields for 9: 36-59%; 11: 57-67%; 13: 69-76%.

2.2. Chiral resolution

The enantiomers of compound 7a were resolved by CHIRALPAKIC (IC00CD-NA012) column (0.46 cm × 15 cm) on Shimadzu LC-20AT HPLC eluting with dichloromethane/ethanol = 90/10 (v/v) at a flow rate of 1.0 mL/min (35°C). Two peaks were separately collected at tR = 2.090 min (isomer 1) and tR = 2.409 min (isomer 2) and the enantiomeric excess (e.e.) value of each product was above 98% (Figure 3A). The white block-shaped single crystal of isomer 1 was acquired with orthorhombic crystal system and its X-ray diffraction data were collected on a Bruker D8 VENTURE single-crystal diffractometer. The absolute configuration of isomer 1 was determined as 9-(R)-7a. Its molecular structure was made up of one aromatic heterocycle, two aromatic rings and one aliphatic ring. The 2-chlorophenyl ring was almost coplanar with the middle tricycle (Figure 3B). The crystallographic data was shown in Table S1-
6 (Supplementary Information). CCDC2004638 contains the supplementary crystallographic data for compound 9-(R)-7a and could be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Figure 3. Chiral resolution of compound 7a. A, The condition of chiral resolution and its chromatography; B, Crystal structure of 9-(R)-7a with the atom labelling.

2.3. cAMP accumulation and SAR analysis
RXFP4 agonist activities of compounds 7, 9, 11 and 13 were evaluated with a homogeneous time-resolved fluorescence (HTRF) assay that measures the inhibition of forskolin-stimulated cAMP accumulation in human RXFP4-overexpressing CHO cells. The specificity of these compounds for RXFP4 was also examined in CHO-K1 cells stably expressing the human RXFP3, which is the most closely related receptor to RXFP4, and RXFP1, which could be activated by the same endogenous peptide relaxin-
3. Agonist activity was expressed as % INSL5 in hRXFP4-CHO-K1 cells, % R3/I5 in hRXFP3-CHO-K1 cells or % relaxin-3 in hRXFP1-293T cells (Table 1, Figures S1 and S3).

Compound 7a was capable of inhibiting cAMP accumulation in hRXFP4 overexpressing CHO-K1 cells, while exhibiting little or no agonist activity in hRXFP3-overexpressing CHO-K1 cells and hRXFP1-overexpressing 293T cells (EC_{50} = 9.3±2.9 μM for RXFP4, Efficacy = 58.5±14.7% for RXFP4 and 4.2±2.8% for RXFP3, respectively), indicating that this agonism was selective (Figures 4A, 4B and S3). Compound 7a also activated RXFP4-mediated signaling pathways including ERK1/2 phosphorylation and β-arrestin 1/2 recruitment [14]. Chiral resolution of compound 7a resulted in a couple of enantiomers 9-(S)-7a and 9-(R)-7a with its 9-(S)-conformer displaying full agonism as INSL5 (Efficacy = 106.6±9.9% and 22.3±9.3% for RXFP4 and RXFP3, respectively), while its 9-(R)-conformer was inactive in both RXFP4- and RXFP3-overexpressing cells.
Figure 4. Inhibition of forskolin-stimulated cAMP accumulation by test compounds in CHO-K1 cells overexpressing hRXFP4 or hRXFP3 (A-F). Each compound was tested in duplicate and each experiment was repeated for three times. Agonist activity was expressed as % INSL5 in hRXFP4-CHO-K1 cells or % R3/I5 in hRXFP3-CHO-K1 cells. For each concentration, the value of 665/615 was calculated, and normalized to the corresponding maximum value obtained for INSL5 in hRXFP4-CHO-K1 cells and for R3/I5 in hRXFP3-CHO-K1 cells. Normalized values were plotted vs. ligand concentration using GraphPad PRISM 8 and are expressed as means ± SEM. Cpd, compound.

It follows that shifting the position of 2-chloro group (strategy 1 in Figure 2) from ortho- to that of meta- or para- resulted in a total loss of activity (7a vs. 7b and 7c). The size of atomic radius (7d: R₁ = F; 7a: R₁ = Cl; 7e: R₁ = Br; 7f: R₁ = I) also affected bioactivity with the tendency that the bigger the atomic radius, the higher the agonist effect, i.e., 2-iodine substituted analogue 7f showed 96.2±9.1% efficacy of INSL5, while its potency was also elevated by nearly 4.9-fold compared to 7a (EC₅₀ = 1.9±0.3 μM for 7f and EC₅₀ = 9.3±2.9 μM for 7a). Similar phenomenon was observed for 7g (2-CF₃) that has a bigger steric hindrance than 7a (2-Cl) and exhibited almost an equal efficacy (93.3±11.2%) to 7f. Dual chloro-substituted analogues, such as 2,3-di-Cl (7j), 2,4-di-Cl (7k) and 2,5-di-Cl (7l) were designed for examining the optimal substituted position of chloro group. The results showed that compounds with 2,3-di-Cl (7j) or 2,4-di-Cl (7k) maintained about 63%-78% efficacy while 2,5-di-Cl (7l) displayed only 1/3 efficacy compared to 7a. Removal of this 2-chloro group led to nearly 60% decrease in agonist activity (7m vs. 7a). The above observations indicate that 2-position on the phenyl group is essential.

The inhibitory effects on forskolin-stimulated cAMP accumulation in compounds with modifications around 4-hydroxyl phenyl part (strategy 2 in Figure 2, 9a-i) suggest that this part is crucial for selective binding to RXFP4. Change of 4-OH group to 3-OH
and 9-aromatic nucleus to cyclohexyl group led to 70% reduction and a total loss of efficacy, respectively (9a and 9b vs. 7a). 3,4-Dihydroxyl substituted derivative (9c) only retained 65% efficacy as 9a. Of note is that increase of steric hindrance such as 4-morpholinophenyl (9d) and 4-(pyrrolidin-1-yl)phenyl (9h) caused a weak agonist effect on RXFP3 (Efficacy = 18.3±6.2% for 9h), indicating that 4-phenyl position may be a key site that defines receptor selectivity. Replacement of 4-OH group with 4-SH could maintain its RXFP4 agonistic activity, however, the potency was nearly decreased by 2-fold (9i vs. 7a).

Subsequent studies on the cyclohexane-1,3-dione skeleton was performed through inserting a dimethyl group thereby increasing the steric hindrance, i.e., 11a and 11b synthesized by Biginelli cyclocondensation of 5,5-dimethylcyclohexane-1,3-dione (10a)/4,4-dimethylcyclohexane-1,3-dione (10b) with 3-amine-5-(2-chlorophenyl)-4H-1,2,4-triazol (6a) and 4-hydroxybenzaldehyde. A sharp reduction in agonism was observed (Efficacy = 9.9±1.2% for 11a and 18.3±3.5% for 11b, respectively), suggesting that cyclohexane-1,3-dione skeleton is not tolerant to structural modification.

Next, we tried to insert heteroatom to the cyclohexane-1,3-dione skeleton, such as oxygen for 11c and sulphur for 11d. In comparison with cyclohexane-1,3-dione skeleton (7a), 2H-thiopyran-3,5(4H,6H)-dione skeleton (11d) still retained the agonist effect while 2H-pyran-3,5(4H,6H)-dione skeleton (11c) significantly decreased its agonist activity (Efficacy = 10.5±2.4% for 11c and 60.8±7.6% for 11d, respectively).

For the 1,2,4-triazole skeleton, replacing its triazole group (7a) with pyrazole (13c) and keeping other substitutes unchanged resulted in complete loss of activity. Removal of 2-chlorophenyl from this skeleton (13a vs. 7a) or substitution of 1,2,4-triazole with tetrazole (13b vs. 7a) caused a marked decline in agonism, indicating that 3-phenyl-1,2,4-triazole skeleton is an important functional group.

Table 1. Inhibition of forskolin-stimulated cAMP accumulation by synthetic compounds in CHO-K1 cells stably overexpressing hRXFP4 or hRXFP3.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>RXFP4 EC50 (μM)b</th>
<th>RXFP3 EC50 (μM)b</th>
<th>RXFP4 Efficacy (%)c</th>
<th>RXFP3 Efficacy (%)c</th>
<th>Cpd</th>
<th>RXFP4 EC50 (μM)b</th>
<th>RXFP3 EC50 (μM)b</th>
<th>RXFP4 Efficacy (%)c</th>
<th>RXFP3 Efficacy (%)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>7a</td>
<td>9.3±2.9</td>
<td>N.D.</td>
<td>58.5±14.7</td>
<td>4.2±2.8</td>
<td>9d</td>
<td>50.2±10.5</td>
<td>N.D.</td>
<td>33.9±8.1</td>
<td>6.93e</td>
</tr>
<tr>
<td>9-R-7a</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>9e</td>
<td>N.D.</td>
<td>N.D.</td>
<td>7.0±1.6</td>
<td>N.A.</td>
</tr>
<tr>
<td>9-S-7a</td>
<td>6.8±1.3</td>
<td>230.2±148.0</td>
<td>106.6±9.9</td>
<td>22.3±9.3</td>
<td>9f</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.A.</td>
<td>8.2e</td>
</tr>
<tr>
<td>7b</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.A.</td>
<td>N.A.</td>
<td>9g</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.A.</td>
<td>10.4e</td>
</tr>
<tr>
<td>7c</td>
<td>N.D.</td>
<td>N.D.</td>
<td>6.5±0.4</td>
<td>N.A.</td>
<td>9h</td>
<td>N.D.</td>
<td>97.2±11.0</td>
<td>N.A.</td>
<td>18.3±6.2</td>
</tr>
<tr>
<td>7d</td>
<td>11.8±6.2</td>
<td>N.D.</td>
<td>17.6±4.7</td>
<td>N.A.</td>
<td>9i</td>
<td>18.7±3.5</td>
<td>N.D.</td>
<td>52.3±12.3</td>
<td>N.A.</td>
</tr>
<tr>
<td>7e</td>
<td>7.1±3.4</td>
<td>N.D.</td>
<td>78.2±5.3</td>
<td>5.34e</td>
<td>11a</td>
<td>19.3±12.3</td>
<td>N.D.</td>
<td>9.9±1.2</td>
<td>N.A.</td>
</tr>
<tr>
<td>7f</td>
<td>1.9±0.3</td>
<td>30.7e</td>
<td>96.2±9.1</td>
<td>13.6e</td>
<td>11b</td>
<td>6.5±2.3</td>
<td>N.D.</td>
<td>18.3±3.5</td>
<td>N.A.</td>
</tr>
<tr>
<td>7g</td>
<td>26.5±9.6</td>
<td>141.9e</td>
<td>93.3±11.2</td>
<td>13.6e</td>
<td>11c</td>
<td>35.2±12.3</td>
<td>N.D.</td>
<td>10.5±2.4</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

*Note: *Efficacy values are reported as mean±SEM.*
Each compound was tested in duplicate and each experiment was repeated at least for three times. EC<sub>50</sub> values are presented as means ± SEM. Agonist activity is expressed as % INSL5 in hRXFP4-CHO-K1 cells or % R3/I5 in hRXFP3-CHO-K1 cells. The HCl salt of compound 9h was used in the test. Compound was tested only once. Cpd, compound; N.D., not detectable; N.A., not active.

### 2.4. Physicochemical properties

Physicochemical properties of synthesized compounds were calculated according to both Lipinski’s rule of five and Veber’s rule through selecting appropriate molecules based on size, molecular weight (MW), number of hydrogen bond donors (nHBD) and acceptors (nHBA), molecular octanol/water partition coefficient (MolLogP), number of rotatable bonds (nRotB) and molecular polar surface area (MolPSA). This was carried out using Molsoft online software (http://molsoft.com/mprop/) and Molinspiration cheminformatics software (https://www.molinspiration.com/). Other parameters like molecular water solubility (MolLogS), molecular volume (MolVol) and drug-likeness score were also assessed in silico (Table 2). A positive compound is determined when (i) nHBD is ≤5; (ii) nHBA is ≤10; (iii) MW is ≤500; (iv) MolLogP is < 5; (v) nRotB is ≤10; and (vi) PSA is ≤140 Å<sup>2</sup> or nHBD + nHBA ≤12. Our analyses revealed that most of these synthesized compounds conform to the two rules, indicative of optimal membrane permeability, sound bioavailability and acceptable druggability (Table 2).

### Table 2. Calculated physicochemical properties of synthesized compounds.

<table>
<thead>
<tr>
<th>Cpd</th>
<th>MolLogS</th>
<th>MolLogP</th>
<th>MW</th>
<th>nHBD</th>
<th>nHBA</th>
<th>nSC</th>
<th>nRotB</th>
<th>MolVol (Å&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>MolPSA (Å&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Drug-likeness model score</th>
</tr>
</thead>
<tbody>
<tr>
<td>7a</td>
<td>-5.69</td>
<td>4.08</td>
<td>392.1</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>372.53</td>
<td>67.96</td>
<td>1.21</td>
</tr>
<tr>
<td>7b</td>
<td>-6.05</td>
<td>4.2</td>
<td>392.1</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>374.56</td>
<td>67.96</td>
<td>0.7</td>
</tr>
<tr>
<td>7c</td>
<td>-6.07</td>
<td>4.2</td>
<td>392.1</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>374.48</td>
<td>67.96</td>
<td>0.74</td>
</tr>
<tr>
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<td>-5.73</td>
<td>3.63</td>
<td>376.13</td>
<td>2</td>
<td>4</td>
<td>1</td>
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<td>362.08</td>
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<tr>
<td>7e</td>
<td>-6.00</td>
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<td>436.05</td>
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<td>2</td>
<td>378.22</td>
<td>67.96</td>
<td>0.88</td>
</tr>
</tbody>
</table>
All the calculations were carried out online. MolLogS, molecular water solubility in Log (moles/L); MolLogP, molecular octanol/water partition coefficient; nHBD, number of hydrogen bond donors; nHBA, number of hydrogen bond acceptors; nSC, number of stereo centers; nRotB, number of rotatable bonds; MolVol, molecular volume; MolPSA, molecular polar surface area. Drug-likeness score predicts an overall drug-likeness (druggability) using Molsoft's chemical fingerprints. The training set for this mode consists of 5,000 known drugs from WDI (positives) and 100,000 carefully selected non-drug-like compounds (negatives).

2.5. Further optimization

Based on the above SAR studies, several conclusions could be drawn: 2-Br, 2-I and 2-
CF₃ substitutes are superior to 2-Cl (7e, 7f and 7g vs. 7a, strategy 1 in Figure 2); 4-OH phenyl is an essential group for receptor selectivity (strategy 2 in Figure 2); the skeleton of 5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (7a) could be changed to 7,9-dihydro-4H-thiopyran-[3,4-d][1,2,4]triazolo[1,5-a]pyrimidin-8(5H)-one (11d) without affecting the agonist effect on RXFP4 (strategy 3 in Figure 2); and 3-phenyl-1,2,4-triazole skeleton is an key functional group for RXFP4 binding (strategy 4 in Figure 2). We then conducted the 2nd round structural optimization for the purpose of combining these proponent functional groups. Compounds 14a-c were thus made by Biginelli cyclocondensation of 5-(2-bromophenyl)-4H-1,2,4-triazol-3-amine (6e), or 5-(2-iodophenyl)-4H-1,2,4-triazol-3-amine (6f), or 5-(2-(trifluoromethyl)phenyl)-4H-1,2,4-triazol-3-amine (6g), cyclohexane-1,3-dione/2H-thiopyran-3,5(4H, 6H)-dione and 4-hydroxybenzaldehyde under the catalysis of acetic acid (Scheme 3). They were subsequently examined with cAMP accumulation assay. Similar phenomenon was observed for strategy 1 (Figure 2) that bigger steric hindrance was beneficial to its RXFP4 agonism as compared with compounds 14a and 14b (Efficacy = 76.0±12.3 for 14a and 113.9±13.9 for 14b, respectively). Of these three compounds, 14b exhibited the highest efficacy, close to that of the isomer 9-(S)-7a with better selective ratio for RXFP4 than RXFP3 (18.4 and 4.8 for 14b and 9-(S)-7a, respectively; Table 1). Also, compound 14b showed no agonistic effect on RXFP1 (Figure S3). In addition, the solubility of 14b was ameliorated compared to 7a (MolLog S = -4.02 and -5.69 for 14b and 7a, respectively; Table 2). Next, the enantiomers of compound 14b were resolved by CHIRALPAK IC column (0.46 cm × 15 cm) on Shimadzu LC-2010 HPLC eluting with ethanol with a flow rate of 1.0 mL/min at 25°C. Two peaks were separately collected at the tR = 4.568 min (9-R) and tR = 5.981 min (9-S) and the enantiomeric excess (e.e.) value of each product was above 99% (Figure 5). Like compound 7a, the conformer 9-(S)-14b presented a superior RXFP4 agonism over that of 9-R (Efficacy = 134.4±5.2% and 27.8±8.9% for 9-S and 9-R, respectively), accompanied by an improved RXFP4 selective ratio vs. RXFP3 (from 4.8 for 9-(S)-7a to 26.9 for 9-(S)-14b). Compound 14c exhibited agonist effects on both RXFP4 and RXFP3, which may result from the cytotoxicity (Figure S2).
Scheme 3. Synthetic route for compounds 14a-c. (i) HOAc in EtOH, reflux, yield: 41-51%.

Figure 5. Chiral resolution of compound 14b. The condition of chiral resolution and its chromatography.

2.6. Receptor binding
Representative analogues 9-(S)-7a, 7e, 7g, 14a, 14b and 9-(S)-14b that exhibited better agonistic effects in cAMP accumulation assay were selected for the following competitive binding assay in CHO-K1 cells stably expressing human RXFP4 with europium-labeled Eu(A)-R3/I5 as control. The 9-(R) conformers of 7a and 14b were also tested in the binding assay as comparison. The results showed that 9-(S)-7a and 9-(S)-14b displayed superior binding affinity to their corresponding 9-(R)-conformers. However, their displacement curves were not paralleled with that of peptide R3/I5, indicating that only parts of the binding site for R3/I5 were competitively bound by the ligands. It was noted that 9-(R)-7a showed an increased binding effect which might be caused by the cell toxicity at high concentration (100 μM).
Figure 6. Competitive binding assay of selected compounds with hRXFP4 performed in CHO-K1 cells stably expressing the receptor RXFP4. Europium-labeled Eu(A)-R3/I5 was used in the presence of increasing amounts of compounds. Each compound was measured in triplicate, and each experiment was repeated independently for three times. Data were analyzed using GraphPad PRISM 8 (GraphPad Inc., San Diego, CA) and expressed as means ± SEM.

2.7. Docking studies

Molecular docking was conducted using the Dock Ligands module of LibDock genetic algorithm program in BIOVIA Discovery Studio 2016 (Accelrys Software, San Diego, USA). Homology models of hRXFP4 and hRXFP3 (SWISS-MODEL: Q8TDU9 and Q9NSD7, respectively), modeled on the template of agonist-bound apelin receptor (PDB code: 5VBL), were used because the apelin receptor is also determined with agonist. The sequence identity between RXFP3/RXFP4 and apelin receptor is 32.1% and 28.2%, respectively. The proteins were prepared before docking and then cavity searching was performed to find the orthosteric binding site, which showed the best pocket score. Next, ligands were prepared using energy minimization by CHARMM forcefield until RMS gradient of 0.01 was reached. Compounds 7a and 14b were docked into the hRXFP4 orthosteric binding site constructed by residues L118\textsuperscript{3.29}, T176\textsuperscript{4.60}, R208\textsuperscript{5.42}, F291\textsuperscript{7.35}, Q205\textsuperscript{5.39}, T266\textsuperscript{6.55}, G269\textsuperscript{6.58}, V265\textsuperscript{6.54}, Q287\textsuperscript{7.31}, K273\textsuperscript{6.62}, Y284\textsuperscript{7.28}, T288\textsuperscript{7.32}, L201\textsuperscript{5.35}, P196\textsuperscript{5.30}, L193\textsuperscript{ECL2}, L192\textsuperscript{ECL2}, L190\textsuperscript{ECL2} and Y204\textsuperscript{5.38}, as well as the hRXFP3 orthosteric binding site constructed by residues T346\textsuperscript{6.55}, Y369\textsuperscript{7.33}, L345\textsuperscript{5.45}, L365\textsuperscript{7.29}, C366\textsuperscript{7.30}, S349\textsuperscript{6.58}, Y267\textsuperscript{5.38}, L264\textsuperscript{5.35}, I350\textsuperscript{6.59}, K353\textsuperscript{6.62}, F262\textsuperscript{ECL2}, W263\textsuperscript{5.34}, R250\textsuperscript{ECL2} and F251\textsuperscript{ECL2} (superscripts indicate Ballesteros-Weinstein numbering for GPCRs, [Ballesteros and Weinstein, 1995]) with the top 10 poses presented and scored while keeping other options in their default values (Figures 7 and S4). LibDock fitness scores of 114.064 and 111.977 with peptide RXFP4, and 111.545 and 111.749 with peptide RXFP3 for 7a and 14b were then obtained respectively. After
binding to RXFP4, the phenolic hydroxyl group and nitrogen atom on the 1,2,4-triazole interacted via hydrogen bonds with the carbonyl group of L193<sup>ECL2</sup> and the terminal amide of Q205<sup>3.39</sup>, separately. Two aromatic rings (2-Cl/I-Ph and 1,2,4-triazole) formed Pi-Pi stack with F291<sup>7.35</sup>. The aromatic rings (2-Cl/I-Ph) also formed Pi-cation function with the terminal amino group of K273<sup>6.62</sup>. In addition, the sulfur atom on the thiopyrano ring of compound 14b interacted with the phenolic ring of Y204<sup>5.38</sup>, which may explain the efficacy difference between 14b and 7a. As comparison, docking studies of 7a and 14b with RXFP3 indicated that no hydrogen bond was formed between the peptide and ligands except for Pi-Pi stack, Pi-cation and Pi-sulfur functions. This may be interpreted as the selectivity of ligands for RXFP4 vs. RXFP3.
Figure 7. A, Molecular docking of compounds 7a and 14b with hRXFP4 (SWISS-MODEL: Q8TDU9) at the orthosteric binding site. Compounds 7a and 14b were displayed as cyan and pink sticks, respectively, while the amino acids of RXFP4 are shown as grey cartoon or sticks; B, Sequence alignment among RXFP3 (SWISS-MODEL: Q9NSD7), RXFP4 and apelin receptor (PDB code: 5VBL).

3. Conclusions
A new scaffold of tricyclic derivatives represented by 7a as non-peptidic selective RXFP4 agonist was disclosed after HTS, capable of suppressing forskolin-stimulated cAMP production in hRXFP4-overexpressing CHO-K1 cells as opposed to hRXFP3 and hRXFP1. A pair of enantiomers (9-R and 9-S) was resolved and their structures were confirmed by X-ray crystallography. Medicinal chemistry efforts in modification of 7a was then performed focusing on three parts: 2-chlorophenyl group, 4-hydroxyphenyl group and its skeleton including cyclohexane-1,3-dione and 1,2,4-triazole group. Initial optimization revealed that 2-bromophenyl, 2-iodophenyl and 2-trifluoromethylphenyl substitutes are superior to 2-chlorophenyl, 4-hydroxyphenyl is an essential group for receptor selectivity, the skeleton of 5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one could be changed to 7,9-dihydro-4H-
thiopyrano[3,4-d][1,2,4]triazolo[1,5-a]pyrimidin-8(5H)-one without loss of agonist activity on RXFP4 and 3-phenyl-1,2,4-triazole skeleton is a key functional group for RXFP4 binding. Based on this, our follow-up optimization resulted in 9-(S)-14b with 2.3-fold higher efficacy and better selectivity (selective ratio of RXFP4 vs. RXFP3 for 9-(S)-14b and 7a were 26.9 and 13.9, respectively). Subsequent molecular docking was carried out to elucidate a possible reason of this selectivity for RXFP4 vs. RXFP3. Competitive binding assay of representative compounds were performed which further demonstrated 9-(S)-14b as the most potent RXFP4 agonist with a pKi value of 5.86 ± 0.15.

4. Experimental protocols

4.1. Chemistry

Reagents are of commercial grade and were used as received unless otherwise noted. The structures of all new compounds are consistent with their 1H, 13C NMR and mass spectra, and are judged to be ≥95% pure by HPLC. NMR spectra were recorded on Bruker AN-400, AVANCE III 500 and Varian Inova 600 spectrometers. Chemical shifts were reported in parts per million (ppm), with the solvent resonance as the internal standard (CD3OD 3.31 ppm, CDCl3 7.26 ppm and DMSO-d6 2.50 ppm for 1H NMR; CD3OD 49.15 ppm, CDCl3 77.23 ppm and DMSO-d6 39.52 ppm for 13C NMR). Low resolution mass spectral data (electrospray ionization) were acquired on a Finnigan LCQ-DECA mass spectrometer. High resolution mass spectral data were collected on Agilent G6520 Q-TOF mass spectrometer. Samples were analyzed for purity on a HP1100 series equipped with a Zorbax SB-C18 column (5 μm, 4.6 mm × 250 mm). Purities of final compounds were determined using a 5 μL injection with quantitation by AUC at 210 and 254 nm (Agilent diode array detector). X-ray diffraction was recorded on a Bruker D8 VENTURE single-crystal diffractometer. Specific optical rotation was determined on Autopol VI-Rudolph polarimeter. All the melting points of synthesized compounds were measured on WRS-1B digital melting point apparatus. The procedures for compounds 4-5 were included in Supplementary Information.

4.1.1. Synthesis of 3-Amine-5-(2-chlorophenyl)-4H-1,2,4-triazol (6a)

N-(2-Chlorobenzamido)-guanidine (5a, 997.7 mg, 4.71 mmol, 1 eq) and p-toluenesulfonic acid monohydrate (116.2 mg, 0.61 mmol, 0.13 eq) were dissolved in a mixture of water (28 mL) and dioxane (14 mL). The resulted solution was refluxed overnight. After cooling, it was filtered and the solution was evaporated in vacuo into a small amount, which was placed at room temperature (RT) for 1 h. The product was
precipitated and then filtered as white powder (1.33 g, yield: 95.0 %). m.p. 222-223°C
195.0439. ¹H NMR (CD₃OD, 400 MHz) 7.37 (t, J = 7.6 Hz, 1H, phenyl C₄-H), 7.41
(dt, J = 1.6 Hz, J = 7.2 Hz, 1H, phenyl C₅-H), 7.50 (dd, J = 1.2 Hz, J = 7.6 Hz, 1H,
phenyl C₃-H), 7.64 (dd, J = 2.0 Hz, J = 7.6 Hz, 1H, phenyl C₆-H). ¹³C NMR (CD₃OD,
125 MHz) 128.1 (phenyl C₅), 131.4 (phenyl C₆), 131.7 (phenyl C₃), 132.6 (phenyl C₄,₅),
134.2 (phenyl C₁), 155.9 (CNH₂), 157.2 (CN₁N₃).
The procedures for compounds 6b-m are the same as 6a.

4.1.2. 5-(3-Chlorophenyl)-4H-1,2,4-triazol-3-amine (6b)
Yield: 76.2 %, white powder, m.p. 200-202°C. LR-ESI: 195.1 [M+H]⁺. HR-ESI m/z calcd for
C₈H₈ClN₄ [M+H]⁺ 195.0437, found 195.0436. ¹H NMR (CD₃OD, 400 MHz)
7.40 (m, 2H, phenyl C₄,₅-H), 7.82 (d, J = 6.0 Hz, 1H, phenyl C₆-H), 7.91 (s, 1H, phenyl
C₂-H). ¹³C NMR (CD₃OD, 100 MHz) 125.5 (phenyl C₆), 127.1 (phenyl C₂), 130.2
(phenyl C₄), 131.3 (phenyl C₅), 134.2 (phenyl C₁), 135.7 (phenyl C₃), 159.1 (CNH₂),
159.7 (CN₁N₃).

4.1.3. 5-(4-Chlorophenyl)-4H-1,2,4-triazol-3-amine (6c)
Yield: 74.5 %, white powder, m.p. 227-229°C. LR-ESI: 195.1 [M+H]⁺. HR-ESI m/z calcd for
C₈H₈ClN₄ [M+H]⁺ 195.0437, found 195.0436. ¹H NMR (CD₃OD, 400 MHz)
7.42 (d, J = 8.4 Hz, 2H, phenyl C₃,₅-H), 7.88 (d, J = 8.4 Hz, 2H, phenyl C₂,₆-H). ¹³C NMR
(CD₃OD, 100 MHz) 128.7 (phenyl C₂,₆), 129.9 (phenyl C₃,₅), 132.1 (phenyl C₁),
136.2 (phenyl C₄), 158.8 (CNH₂), 159.3 (CN₁N₃).

4.1.4. 5-(2-Fluorophenyl)-4H-1,2,4-triazol-3-amine (6d)
Yield: 90.0 %, white powder, m.p. 188-189°C. LR-ESI: 179.0 [M+H]⁺. HR-ESI m/z calcd for
C₈H₈FN₄ [M+H]⁺ 179.0733, found 179.0732. ¹H NMR (CD₃OD, 400 MHz)
7.23 (m, 2H, phenyl C₄,₅-H), 7.44 (m, 1H, phenyl C₆-H), 7.87 (t, J = 7.6 Hz, 1H, phenyl
C₃-H). ¹³C NMR (CD₃OD, 125 MHz) 117.3 (phenyl C₃), 119.6 (phenyl C₁), 125.5
(phenyl C₅), 131.0 (phenyl C₄), 132.3 (phenyl C₆), 160.6 (CNH₂), 162.6 (phenyl C₂,
CN₁N₃).

4.1.5. 5-(2-Bromophenyl)-4H-1,2,4-triazol-3-amine (6e)
Yield: 87.5 %, m.p. 227-228°C. LR-ESI: 238.8 [M+H]⁺. HR-ESI m/z calcd for
C₈H₈BrN₄ [M+H]⁺ 238.9932, found 238.9932. ¹H NMR (CD₃OD, 400 MHz) 7.33 (t, J =
7.6 Hz, 1H, phenyl C₅-H), 7.42 (t, J = 7.6 Hz, 1H, phenyl C₄-H), 7.57 (d, J = 7.6 Hz,
1H, phenyl C₃-H), 7.69 (d, J = 8.0 Hz, 1-H, phenyl C₃-H). ¹³C NMR (CD₃OD, 125 MHz)
123.5 (phenyl C₂), 128.6 (phenyl C₃), 132.8 (phenyl C₄,₅), 134.6 (phenyl C₃), 137.8
(phenyl C₁), 157.2 (CNH₂), 158.5 (CN₁N₃).

4.1.6. 5-(2-Iodophenyl)-4H-1, 2, 4-triazol-3-amine (6f)
Yield: 97.0 %, m.p. 240-242°C. LR-ESI: 287.0 [M+H]+. HR-ESI m/z calcd for C₈H₈IN₄ [M+H]+ 286.9794, found 286.9795. ¹H NMR (CD₃OD, 400 MHz) 7.10 (t, J = 7.2 Hz, 1H, phenyl C₅-H), 7.28 (d, J = 8.4 Hz, 1H, phenyl C₆-H), 7.47 (d, J = 7.6 Hz, 1H, phenyl C₄-H), 7.74 (d, J = 8.0 Hz, 1H, phenyl C₃-H).

4.1.7. 5-(2-Trifluoromethylphenyl)-4H-1, 2, 4-triazol-3-amine (6g)
Yield: 81.6 %, m.p. 165-167°C. LR-ESI: 228.9 [M+H]+. HR-ESI m/z calcd for C₉H₈F₃N₄ [M+H]+ 229.0701, found 229.0702. ¹H NMR (CD₃OD, 400 MHz) 7.63 (m, 2H, phenyl C₄,5-H), 7.68 (d, J = 6.8 Hz, 1H, phenyl C₃-H), 7.80 (d, J = 7.6 Hz, 1H, phenyl C₆-H). ¹³C NMR (CD₃OD, 125 MHz) 127.5 (CF₃), 130.3 (phenyl C₂), 130.5 (phenyl C₁), 133.0 (phenyl C₃,4), 133.1 (phenyl C₅,6), 157.8 (CNH₂), 158.8 (CN₁N₃).

4.1.8. 5-(3-Methoxyphenyl)-4H-1,2,4-triazol-3-amine (6h)
Yield: 79.1 %, white powder, m.p. 223-225°C. LR-ESI: 191.1 [M+H]+. HR-ESI m/z calcd for C₉H₁₁N₄O [M+H]+ 191.0933, found 191.0930. ¹H NMR (CD₃OD, 400 MHz) 3.84 (s, 3H, OCH₃), 6.96 (d, J = 8.4 Hz, 1H, phenyl C₄-H), 7.32 (t, J = 8.4 Hz, 1H, phenyl C₅-H), 7.48 (m, 2H, phenyl C₂,6-H). ¹³C NMR (CD₃OD, 125 MHz) 55.9 (OCH₃), 112.4 (phenyl C₂), 116.5 (phenyl C₄), 119.6 (phenyl C₆), 130.8 (phenyl C₅), 133.1 (phenyl C₁), 159.9 (CNH₂), 161.5 (2C, OCH₃, CN₁N₃).

4.1.9. 5-(4-Methoxyphenyl)-4H-1,2,4-triazol-3-amine (6i)
Yield: 87.4 %, white powder, m.p. 224-226°C. LR-ESI: 191.2 [M+H]+. HR-ESI m/z calcd for C₉H₁₁N₄O [M+H]+ 191.0933, found 191.0930. ¹H NMR (CD₃OD, 400 MHz) 3.83 (s, 3H, OCH₃), 6.97 (d, J = 8.0 Hz, 2H, phenyl C₃,5-H), 7.82 (d, J = 8.5 Hz, 2H, phenyl C₂,6-H). ¹³C NMR (CD₃OD, 125 MHz) 56.0 (OCH₃), 115.2 (2C, phenyl C₃,5), 124.6 (phenyl C₁), 128.8 (2C, phenyl C₂,6), 149.1 (CNH₂), 152.3 (CN₁N₃), 159.4 (phenyl C₄).

4.1.10. 5-(2, 3-Dichlorophenyl)-4H-1, 2, 4-triazol-3-amine (6j)
Yield: 82.1%, white powder, m.p. 243-245°C. LR-ESI: 229.0 [M+H]+. HR-ESI m/z calcd for C₈H₇Cl₂N₄ [M+H]+ 229.0046, found 229.0048. ¹H NMR (CD₃OD, 400 MHz) 7.36 (t, J = 7.6 Hz, 1H, phenyl C₅-H), 7.57 (d, J = 7.6 Hz, 1H, phenyl C₆-H), 7.61 (d, J = 8.0 Hz, 1H, phenyl C₄-H). ¹³C NMR (CD₃OD, 125 MHz) 128.8 (phenyl C₆), 131.1 (2C, phenyl C₄,5), 132.4 (phenyl C₂), 132.7 (phenyl C₃), 135.0 (phenyl C₁), 158.6 (CNH₂), 158.9 (CN₁N₃).

4.1.11. 5-(2, 4-Dichlorophenyl)-4H-1, 2, 4-triazol-3-amine (6k)
Yield: 82.1%, white powder, m.p. 243-245°C. LR-ESI: 229.0 [M+H]+. HR-ESI m/z calcd for C₈H₇Cl₂N₄ [M+H]+ 229.0046, found 229.0048. ¹H NMR (CD₃OD, 400 MHz) 7.36 (t, J = 7.6 Hz, 1H, phenyl C₅-H), 7.57 (d, J = 7.6 Hz, 1H, phenyl C₆-H), 7.61 (d, J = 8.0 Hz, 1H, phenyl C₄-H). ¹³C NMR (CD₃OD, 125 MHz) 128.8 (phenyl C₆), 131.1 (2C, phenyl C₄,5), 132.4 (phenyl C₂), 132.7 (phenyl C₃), 135.0 (phenyl C₁), 158.6 (CNH₂), 158.9 (CN₁N₃).
Yield: 89.8 %, white powder, m.p. 242-243°C. LR-ESI: 229.0 [M+H]+. HR-ESI m/z calcd for C₈H₇Cl₂N₄ [M+H]+ 229.0048, found 229.0046. ¹H NMR (CD₃OD, 400MHz) 7.41 (dd, J₁ = 1.6 Hz, J₂ = 8.4 Hz, 1H, phenyl C₅-H), 7.58 (s, 1H, phenyl C₃-H), 7.66 (d, J₁ = 8.4 Hz, 1H, phenyl C₆-H). ¹³C NMR (CD₃OD, 125 MHz) 128.4 (phenyl C₅), 131.2 (phenyl C₆), 133.6 (phenyl C₃), 135.1(2C, phenyl C₂,₄), 136.7 (phenyl C₁), 158.4 (CNH₂), 159.3 (CN₁N₃).

4.1.12. 5-(2,5-Dichlorophenyl)-4H-1,2,4-triazol-3-amine (6l)
Yield: 87.0 %, white powder, m.p. 268-269°C. LR-ESI: 229.1 [M+H]+. HR-ESI m/z calcd for C₈H₇Cl₂N₄ [M+H]+ 229.0048, found 229.0047. ¹H NMR (CD₃OD, 400 MHz) 7.42 (d, J₁ = 8.8 Hz, 1H, phenyl C₃-H), 7.49 (d, J₂ = 8.8 Hz, 1H, phenyl C₄-H), 7.70 (s, 1H, phenyl C₆-H).

4.1.13. 5-Phenyl-4H-1,2,4-triazol-3-amine (6m)
Yield: 90.0 %, white powder, m.p. 187-188°C. LR-ESI: 161.1 [M+H]+. HR-ESI m/z calcd for C₈H₉N₄ [M+H]+ 161.0827, found 161.0827. ¹H NMR (CD₃OD, 400 MHz) 7.40 (m, 3H, phenyl C₃,₄,₅-H), 7.89 (d, J = 6.8 Hz, 2H, phenyl C₂,₆-H).

Acetic acid (600 μL) was added to the solution of 5-(2-chloro)phenyl-4H-1,2,4-triazol-3-amine (6a, 4 g, 20.6 mmol, 1 eq), cyclohexane-1,3-dione (2.3 g, 20.6 mmol, 1 eq) and 4-hydroxybenzaldehyde (2.5 g, 20.6 mmol, 1 eq) in EtOH (130 mL). The reaction was refluxed overnight. After cooling, the solvent was removed in vacuo and the residue was separated on the Biogate® SNAP Cartridge Sil-100g column eluting with 0-10% methanol/chloromethane to obtain the product as yellowish powder (1.8 g, yield: 32.3 %). m.p. 297-298°C. LR-ESI: 391.2 [M-H]. HR-ESI m/z calcd for C₂₁H₁₆ClN₄O₂ [M-H]: 391.0962, found 391.0968. ¹H NMR (CD₃OD, 500 MHz) 2.05 (m, 1H, C₆-H), 2.10 (m, 1H, C₅-H), 2.40 (m, 2H, C₃-H), 2.75 (m, 2H, C₇-H), 6.34 (s, 1H, C₉-H), 6.70 (d, J₁ = 8.5 Hz, 2H, 4-OH phenyl C₃,₅-H), 7.14 (d, J₂ = 9.0 Hz, 2H, 4-OH phenyl C₂,₆-H), 7.33 (dt, J₁ = 1.0 Hz, J₂ = 7.5 Hz, 1H, 2-Cl phenyl C₅-H), 7.38 (dt, J₁ = 1.0 Hz, J₂ = 7.5 Hz, 1H, 2-Cl phenyl C₄-H), 7.47 (dd, J₁ = 1.0 Hz, J₂ = 7.5 Hz, 1H, 2-Cl phenyl C₃-H), 7.63 (dd, J₁ = 2.0 Hz, J₂ = 8.0 Hz, 1H, 2-Cl phenyl C₆-H). ¹³C NMR
(CD$_3$OD, 125 MHz) 22.3 (C$_5$), 28.0 (C$_7$), 37.7 (C$_3$), 59.4 (C$_9$), 109.8 (C$_{8a}$), 116.3 (2C, 4-OH phenyl C$_{3,5}$), 128.0 (2-Cl phenyl C$_3$), 129.6 (2C, 4-OH phenyl C$_{2,6}$), 131.5 (2C, 2-Cl phenyl C$_{1,3}$), 131.8 (2-Cl phenyl C$_4$), 132.7 (2-Cl phenyl C$_6$), 133.6 (4-OH phenyl C$_1$), 134.3 (2-Cl phenyl C$_2$), 148.8 (C$_{5a}$), 154.5 (CN$_2$N$_4$), 158.6 (4-OH phenyl C$_4$), 160.2 (CN$_1$N$_3$), 197.2 (CO).

Compounds 7b-m, 9a-i, 11a-d, 13a-c and 14a-c were prepared using similar procedures as compound 7a.

4.1.15. 2-(3-Chlorophenyl)-9-(4-hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (7b)

Yield: 43.3%, yellowish powder. m.p. 297-299°C. LR-ESI: 391.1 [M-H]$. HR-ESI m/z calcd for C$_{22}$H$_{16}$ClN$_4$O$_2$ [M-H]$^-$ 391.0962, found 391.0962. $^1$H NMR (CD$_3$OD, 500 MHz) 2.03 (m, 1H, C$_6$-H), 2.11 (m, 1H, C$_6$-H), 2.40 (m, 2H, C$_5$-H), 2.78 (m, 2H, C$_7$-H), 6.30 (s, 1H, C$_9$-H), 6.71 (d, $J$ = 8.5 Hz, 2H, 4-OH phenyl C$_{3,5}$-H), 7.14 (d, $J$ = 8.5 Hz, 2H, 4-OH phenyl C$_{2,6}$-H), 7.37 (m, 2H, 3-Cl phenyl C$_{4,5}$-H), 7.86 (m, 1H, 3-Cl phenyl C$_6$-H), 7.93 (s, 1H, 3-Cl phenyl C$_2$-H). $^{13}$C NMR (CD$_3$OD, 125 MHz) 22.3 (C$_6$), 28.0 (C$_5$), 37.7 (C$_7$), 56.0 (C$_9$), 109.8 (C$_{8a}$), 116.3 (2C, 4-OH phenyl C$_{3,5}$), 125.6 (3-Cl phenyl C$_6$), 124.9 (3-Cl phenyl C$_5$), 127.2 (3-Cl phenyl C$_7$), 129.6 (2C, 4-OH phenyl C$_{2,6}$), 130.4 (3-Cl phenyl C$_4$), 131.3 (3-Cl phenyl C$_5$), 133.7 (4-OH phenyl C$_1$), 134.1 (3-Cl phenyl C$_1$), 135.7 (3-Cl phenyl C$_3$), 151.4 (C$_{5a}$), 154.5 (CN$_2$N$_4$), 159.2 (4-OH phenyl C$_4$), 160.4 (CN$_1$N$_3$), 197.3 (CO).

4.1.16. 2-(4-Chlorophenyl)-9-(4-hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (7c)

Yield: 33.4%, yellowish powder. m.p. 297-298°C. LR-ESI: 391.2 [M-H]$. HR-ESI m/z calcd for C$_{22}$H$_{16}$ClN$_4$O$_2$ [M-H]$^-$ 391.0962, found 391.0954. $^1$H NMR (CD$_3$OD, 400 MHz) 2.03 (m, 1H, C$_6$-H), 2.10 (m, 1H, C$_6$-H), 2.40 (m, 2H, C$_5$-H), 2.76 (m, 2H, C$_7$-H), 6.30 (s, 1H, C$_9$-H), 6.70 (d, $J$ = 8.4 Hz, 2H, 4-OH phenyl C$_{3,5}$-H), 7.12 (d, $J$ = 8.4 Hz, 2H, 4-OH phenyl C$_{2,6}$-H), 7.39 (d, $J$ = 8.4 Hz, 2H, 4-Cl phenyl C$_{2,6}$-H). $^{13}$C NMR (CD$_3$OD, 125 MHz) 22.2 (C$_6$), 28.0 (C$_5$), 37.7 (C$_7$), 59.5 (C$_9$), 109.8 (C$_{8a}$), 116.3 (2C, 4-OH phenyl C$_{3,5}$), 129.6 (2C, 4-OH phenyl C$_{2,6}$), 129.9 (2C, 4-Cl phenyl C$_2$), 132.7 (2-Cl phenyl C$_4$), 134.1 (3-Cl phenyl C$_1$), 135.7 (3-Cl phenyl C$_3$), 151.4 (C$_{5a}$), 154.5 (CN$_2$N$_4$), 159.2 (4-OH phenyl C$_4$), 160.4 (CN$_1$N$_3$), 197.3 (CO).

4.1.17. 2-(2-Fluorophenyl)-9-(4-hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (7d)

Yield: 33.4%, yellowish powder. m.p. 297-298°C. LR-ESI: 391.2 [M-H]$. HR-ESI m/z calcd for C$_{22}$H$_{16}$ClN$_4$O$_2$ [M-H]$^-$ 391.0962, found 391.0954. $^1$H NMR (CD$_3$OD, 400 MHz) 2.03 (m, 1H, C$_6$-H), 2.10 (m, 1H, C$_6$-H), 2.40 (m, 2H, C$_5$-H), 2.76 (m, 2H, C$_7$-H), 6.29 (s, 1H, C$_9$-H), 6.70 (d, $J$ = 8.4 Hz, 2H, 4-OH phenyl C$_{3,5}$-H), 7.12 (d, $J$ = 8.4 Hz, 2H, 4-OH phenyl C$_{2,6}$-H), 7.39 (d, $J$ = 8.4 Hz, 2H, 4-Cl phenyl C$_{2,6}$-H). $^{13}$C NMR (CD$_3$OD, 125 MHz) 22.2 (C$_6$), 28.0 (C$_5$), 37.7 (C$_7$), 59.5 (C$_9$), 109.8 (C$_{8a}$), 116.3 (2C, 4-OH phenyl C$_{3,5}$), 129.6 (2C, 4-OH phenyl C$_{2,6}$), 129.9 (2C, 4-Cl phenyl C$_2$), 132.7 (2-Cl phenyl C$_4$), 134.1 (3-Cl phenyl C$_1$), 135.7 (3-Cl phenyl C$_3$), 151.4 (C$_{5a}$), 154.5 (CN$_2$N$_4$), 159.2 (4-OH phenyl C$_4$), 160.4 (CN$_1$N$_3$), 197.3 (CO).
Yield: 52.8 %, yellowish powder, m.p. 263-264°C. LR-ESI: 375.2 [M-H]. HR-ESI m/z calcd for C_{21}H_{16}FN_{2}O_{2} [M-H]: 375.1257, found 375.1256. \(^1\)H NMR (CD_{3}OD, 400 MHz) 2.09 (m, 2H, C\textsubscript{6}-H), 2.40 (m, 2H, C\textsubscript{5}-H), 2.77 (m, 2H, C\textsubscript{7}-H), 6.34 (s, 1H, C\textsubscript{9}-H), 6.70 (d, J = 8.0 Hz, 2H, 4-OH phenyl C\textsubscript{3,5}-H), 7.14 (d, J = 8.4 Hz, 2H, 4-OH phenyl C\textsubscript{2,6}-H), 7.20 (m, 2H, 2-F phenyl C\textsubscript{5,6}-H), 7.41 (m, 1H, 2-F phenyl C\textsubscript{4}-H), 7.88 (t, J = 7.6 Hz, 1H, 2-F phenyl C\textsubscript{3}-H). \(^{13}\)C NMR (CD_{3}OD, 125 MHz) 22.3 (C\textsubscript{6}), 28.0 (C\textsubscript{5}), 37.7 (C\textsubscript{7}), 59.4 (C\textsubscript{9}), 110.0 (C\textsubscript{8a}), 116.3 (2C, 4-OH phenyl C\textsubscript{3,5}), 117.4 (2-F phenyl C\textsubscript{3}), 120.1 (2-F phenyl C\textsubscript{1}), 125.4 (2-F phenyl C\textsubscript{3}), 129.6 (2C, 4-OH phenyl C\textsubscript{2,6}), 131.4 (2-F phenyl C\textsubscript{6}), 132.3 (2-F phenyl C\textsubscript{4}), 133.7 (4-OH phenyl C\textsubscript{1}), 149.0 (C\textsubscript{5a}), 154.4 (CN\textsubscript{2}N\textsubscript{4}), 158.6 (4-OH phenyl C\textsubscript{4}), 160.7 (2-F phenyl C\textsubscript{2}), 162.7 (CN\textsubscript{1}N\textsubscript{3}), 197.2 (CO).

4.1.18. 2-(2-Bromophenyl)-9-(4-hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (7e)

Yield: 55.3 %, yellowish powder, m.p. >300°C. LR-ESI: 435.1 [M-H], 437.1. HR-ESI m/z calcd for C_{21}H_{16}BrN_{2}O_{2} [M-H]: 435.0457, found 435.0463. \(^1\)H NMR (CD_{3}OD, 400 MHz) 2.03 (m, 2H, C\textsubscript{6}-H), 2.37 (m, 2H, C\textsubscript{5}-H), 2.69 (m, 2H, C\textsubscript{7}-H), 6.33 (s, 1H, C\textsubscript{9}-H), 6.71 (d, J = 8.0 Hz, 2H, 4-OH phenyl C\textsubscript{3,5}-H), 7.14 (d, J = 8.0 Hz, 2H, 4-OH phenyl C\textsubscript{2,6}-H), 7.28 (t, J = 7.6 Hz, 1H, 2-Br phenyl C\textsubscript{4}-H), 7.36 (t, J = 7.2 Hz, 1H, 2-Br phenyl C\textsubscript{5}-H), 7.55 (d, J = 7.6 Hz, 1H, 2-Br phenyl C\textsubscript{3}-H), 7.64 (d, J = 7.6 Hz, 1H, 2-Br phenyl C\textsubscript{6}-H). \(^{13}\)C NMR (CD_{3}OD, 125 MHz) 22.2 (C\textsubscript{6}), 28.0 (C\textsubscript{5}), 37.7 (C\textsubscript{7}), 59.3 (C\textsubscript{9}), 109.7 (C\textsubscript{8a}), 116.3 (2C, 4-OH phenyl C\textsubscript{3,5}), 123.4 (2-Br phenyl C\textsubscript{2}), 128.5 (2-Br phenyl C\textsubscript{3}), 129.6 (2C, 4-OH phenyl C\textsubscript{2,6}), 132.0 (2-Br phenyl C\textsubscript{6}), 132.8 (2-Br phenyl C\textsubscript{3}), 133.5 (4-OH phenyl C\textsubscript{1}), 133.7 (2-Br phenyl C\textsubscript{1}), 134.7 (2-Br phenyl C\textsubscript{3}), 148.8 (C\textsubscript{5a}), 154.7 (CN\textsubscript{2}N\textsubscript{4}), 158.6 (4-OH phenyl C\textsubscript{4}), 161.2 (CN\textsubscript{1}N\textsubscript{3}), 197.2 (CO).

4.1.19. 2-(2-Iodophenyl)-9-(4-hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (7f)

Yield: 50.0 %, yellowish powder, m.p. >300°C. LR-ESI: 483.0 [M-H]. HR-ESI m/z calcd for C_{21}H_{16}IN_{4}O_{2} [M-H]: 483.0318, found 483.0321. \(^1\)H NMR (CD_{3}OD, 400 MHz) 2.05 (m, 2H, C\textsubscript{6}-H), 2.38 (m, 2H, C\textsubscript{5}-H), 2.69 (m, 2H, C\textsubscript{7}-H), 6.32 (s, 1H, C\textsubscript{9}-H), 6.70 (d, J = 8.0 Hz, 2H, 4-OH phenyl C\textsubscript{3,5}-H), 7.14 (d, J = 7.6 Hz, 2H, 4-OH phenyl C\textsubscript{2,6}-H), 7.39 (m, 2H, 2-I phenyl C\textsubscript{5,6}-H), 7.47 (d, J = 7.6 Hz, 1H, 2-I phenyl C\textsubscript{4}-H), 7.93 (d, J = 8.0 Hz, 1H, 2-I phenyl C\textsubscript{3}-H). \(^{13}\)C NMR (CD_{3}OD, 125 MHz) 22.2 (C\textsubscript{6}), 28.0 (C\textsubscript{5}), 37.7 (C\textsubscript{7}), 59.4 (C\textsubscript{9}), 109.9 (C\textsubscript{8a}), 116.9 (2C, 4-OH phenyl C\textsubscript{3,5}), 128.2 (2-I phenyl C\textsubscript{3}), 129.2 (2-I phenyl C\textsubscript{6}), 130.6 (2C, 4-OH phenyl C\textsubscript{2,6}), 130.9 (2-I phenyl C\textsubscript{4}), 132.0 (4-OH
phenyl C₁), 132.3 (2-I phenyl C₃), 133.6 (2-I phenyl C₁), 133.8 (2-I phenyl C₂), 149.2 (C₅a), 154.5 (CN₂N₄), 158.5 (4-OH phenyl C₄), 161.8 (CN₁N₃), 197.3 (CO).

4.1.20. 2-(2-Trifluoromethylphenyl)-9-(4-hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b] quinazolin-8(4H)-one (7g)

Yield: 57.1 %, yellowish powder, m.p. >300°C. LR-ESI: 425.2 [M-H]. HR-ESI m/z calcd for C₂₂H₁₆F₃N₄O₂ [M-H]⁻ 425.1225, found 425.1221. ¹H NMR (CD₃OD, 400 MHz) 2.10 (m, 2H, C₆-H), 2.41 (m, 2H, C₅-H), 2.77 (m, 2H, C₇-H), 6.33 (s, 1H, C₉-H), 6.71 (d, J = 8.0 Hz, 2H, 4-OH phenyl C₃,5-H), 7.12 (d, J = 7.6 Hz, 2H, 4-OH phenyl C₂,6-H), 7.63 (m, 3H, 2-CF₃ phenyl C₃,4,5-H), 7.77 (d, J = 7.2 Hz, 1H, 2-CF₃ phenyl C₆-H). ¹³C NMR (CD₃OD, 125 MHz) 22.2 (C₆), 28.0 (C₅), 37.7 (C₇), 59.3 (C₉), 109.8 (C₈a), 116.2 (2C, 4-OH phenyl C₃,5), 124.4 (CF₃), 127.6 (2-CF₃ phenyl C₃), 129.5 (2C, 4-OH phenyl C₂,6), 130.3 (2-CF₃ phenyl C₂), 130.5 (4-OH phenyl C₁), 130.9 (2-CF₃ phenyl C₄), 133.0 (2-CF₃ phenyl C₆), 133.1 (2-CF₃ phenyl C₅), 133.5 (2-CF₃ phenyl C₁), 148.9 (C₈a), 154.6 (CN₂N₄), 158.5 (4-OH phenyl C₄), 160.4 (CN₁N₃), 197.3 (CO).

4.1.21. 2-(3-Methoxyphenyl)-9-(4-hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b] quinazolin-8(4H)-one (7h)

Yield: 52.8 %, white powder, m.p. 291-292°C. LR-ESI: 387.3 [M-H]. HR-ESI m/z calcd for C₂₂H₁₉N₄O₃ [M-H]⁻ 387.1457, found 387.1455. ¹H NMR (CD₃OD, 400 MHz) 2.09 (m, 2H, C₆-H), 2.39 (m, 2H, C₅-H), 2.77 (m, 2H, C₇-H), 3.82 (s, 3H, OCH₃), 6.30 (s, 1H, C₉-H), 6.70 (d, J = 8.4 Hz, 2H, 4-OH phenyl C₃,5-H), 6.94 (dd, J = 2.0 Hz, J = 8.8 Hz, 1H, 3-OCH₃ phenyl C₅-H), 7.13 (d, J = 8.4 Hz, 2H, 4-OH phenyl C₂,6-H), 7.29 (t, J = 8.0 Hz, 1H, 3-OCH₃ phenyl C₅-H), 7.50 (d, J = 2.8 Hz, 1H, 3-OCH₃ phenyl C₂-H), 7.52 (d, J = 8.8 Hz, 1H, 3-OCH₃ phenyl C₀-H). ¹³C NMR (CD₃OD, 125 MHz) 22.3 (C₆), 28.0 (C₅), 37.7 (C₇), 55.9 (OCH₃), 59.4 (C₉), 110.0 (C₈a), 112.4 (3-OCH₃ phenyl C₂), 116.3 (2C, 4-OH phenyl C₃,5), 116.7 (3-OCH₃ phenyl C₄), 119.8 (3-OCH₃ phenyl C₆), 129.6 (2C, 4-OH phenyl C₂,6), 130.8 (3-OCH₃ phenyl C₅), 133.3 (4-OH phenyl C₁), 133.8 (3-OCH₃ phenyl C₁), 149.2 (C₈a), 154.4 (CN₂N₄), 158.6 (4-OH phenyl C₄), 161.5 (CN₁N₃), 161.7 (3-OCH₃ phenyl C₃), 197.2 (CO).

4.1.22. 9-(4-Hydroxyphenyl)-2-(4-methoxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b] quinazolin-8(4H)-one (7i)

Yield: 30.1%, yellowish powder. LR-ESI: 387.2 [M-H]. HR-ESI m/z calcd for C₂₂H₁₉N₄O₃ [M-H]⁻ 387.1457, found 387.1457. ¹H NMR (CD₃OD, 400 MHz) 2.09 (m, 2H, C₆-H), 2.39 (m, 2H, C₅-H), 2.77 (m, 2H, C₇-H), 3.81 (s, 3H, OCH₃), 6.28 (s, 1H, C₉-H), 6.70 (d, J = 8.4 Hz, 2H, 4-OH phenyl C₃,5-H), 6.94 (d, J = 8.8 Hz, 2H, 4-OCH₃
phenyl C$_{3,5}$-H), 7.12 (d, $J = 8.4$ Hz, 2H, 4-OH phenyl C$_{2,6}$-H), 7.85 (d, $J = 9.2$ Hz, 2H, 4-OCH$_3$ phenyl C$_{2,6}$-H).

4.1.23. 2-(2,3-Dichlorophenyl)-9-(4-hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b] quinazolin-8(4H)-one (7j)

Yield: 50.6 %, yellowish powder, m.p. 280-281°C. LR-ESI: 425.1 [M-H]: HR-ESI m/z calcd for C$_{21}$H$_{15}$Cl$_2$N$_4$O$_2$ [M-H]$^-$ 425.0572, found 425.0574. $^1$H NMR (CD$_3$OD, 400 MHz) 2.06 (m, 1H, C$_6$-H), 2.12 (m, 1H, C$_6$-H), 2.41 (m, 2H, C$_5$-H), 2.77 (m, 2H, C$_7$-H), 6.34 (s, 1H, C$_9$-H), 6.71 (d, $J = 8.8$ Hz, 2H, 4-OH phenyl C$_{3,5}$-H), 7.14 (d, $J = 8.8$ Hz, 2H, 4-OH phenyl C$_{2,6}$-H), 7.33 (t, $J = 8.0$ Hz, 1H, 2,3-di Cl phenyl C$_5$-H), 7.57 (d, $J = 8.0$ Hz, 1H, 2,3-di Cl phenyl C$_6$-H), 7.59 (d, $J = 8.0$ Hz, 1H, 2,3-di Cl phenyl C$_4$-H). $^{13}$C NMR (CD$_3$OD, 125 MHz) 22.3 (C$_6$), 28.0 (C$_5$), 37.7 (C$_7$), 59.4 (C$_8$), 109.8 (C$_{8a}$), 116.3 (2C, 4-OH phenyl C$_{3,5}$), 128.8 (2,3-di Cl phenyl C$_6$), 129.6 (2C, 4-OH phenyl C$_{2,6}$), 131.2 (2,3-di Cl phenyl C$_3$), 132.5 (2,3-di Cl phenyl C$_4$), 133.5 (2,3-di Cl phenyl C$_2$), 133.9 (4-OH phenyl C$_4$), 135.0 (2,3-di Cl phenyl C$_3$), 135.1 (2,3-di Cl phenyl C$_1$), 148.8 (C$_{8a}$), 154.6 (CN$_2$N$_3$), 158.6 (4-OH phenyl C$_4$), 159.7 (CN$_1$N$_3$), 197.3 (CO).

4.1.24. 2-(2,4-Dichlorophenyl)-9-(4-hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b] quinazolin-8(4H)-one (7k)

Yield: 55.9 %, yellowish powder, m.p. 281-283°C. LR-ESI: 425.2 [M-H]: HR-ESI m/z calcd for C$_{21}$H$_{15}$Cl$_2$N$_4$O$_2$ [M-H]$^-$ 425.0572, found 425.0580. $^1$H NMR (CD$_3$OD, 500 MHz) 2.03 (m, 1H, C$_6$-H), 2.09 (m, 1H, C$_6$-H), 2.39 (m, 2H, C$_5$-H), 2.75 (m, 2H, C$_7$-H), 6.33 (s, 1H, C$_9$-H), 6.70 (d, $J = 8.5$ Hz, 2H, 4-OH phenyl C$_{3,5}$-H), 7.13 (d, $J = 8.5$ Hz, 2H, 4-OH phenyl C$_{2,6}$-H), 7.36 (dd, $J = 2.5$ Hz, $J = 8.5$ Hz, 1H, 2,4-di Cl phenyl C$_5$-H), 7.52 (d, $J = 2.0$ Hz, 1H, 2,4-di Cl phenyl C$_3$-H), 7.67 (d, $J = 8.5$ Hz, 1H, 2,4-di Cl phenyl C$_6$-H). $^{13}$C NMR (CD$_3$OD, 125 MHz) 22.2 (C$_6$), 28.0 (C$_5$), 37.7 (C$_7$), 59.4 (C$_8$), 109.7 (C$_{8a}$), 116.2 (2C, 4-OH phenyl C$_{3,5}$), 128.3 (2,4-di Cl phenyl C$_3$), 129.6 (2C, 4-OH phenyl C$_{2,6}$), 130.2 (2,4-di Cl phenyl C$_2$), 131.3 (2,4-di Cl phenyl C$_6$), 133.6 (2,4-di Cl phenyl C$_4$), 135.0 (4-OH phenyl C$_1$), 136.8 (2,4-di Cl phenyl C$_1$), 148.8 (C$_{8a}$), 154.6 (CN$_2$N$_3$), 158.6 (4-OH phenyl C$_4$), 159.3 (CN$_1$N$_3$), 197.2 (CO).

4.1.25. 2-(2,5-Dichlorophenyl)-9-(4-hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b] quinazolin-8(4H)-one (7l)

Yield: 61.4 %, yellowish powder, 283-284°C. LR-ESI: 425.1 [M-H]: HR-ESI m/z calcd for C$_{21}$H$_{15}$Cl$_2$N$_4$O$_2$ [M-H]$^-$ 425.0572, found 425.0578. $^1$H NMR (DMSO-d$_6$, 400 MHz) 1.96 (m, 2H, C$_6$-H), 2.28 (m, 2H, C$_5$-H), 2.68 (m, 2H, C$_7$-H), 6.21 (s, 1H, C$_9$-H), 6.66 (d, $J = 7.6$ Hz, 2H, 4-OH phenyl C$_{3,5}$-H), 7.07 (d, $J = 8.0$ Hz, 2H, 4-OH phenyl C$_{2,6}$-H),
7.49 (d, J = 8.8 Hz, 1H, 2,5-di Cl phenyl C₄-H), 7.57 (d, J = 8.8 Hz, 1H, 2,5-di Cl phenyl C₃-H), 7.76 (brs, 1H, 2,5-di Cl phenyl C₆-H), 9.40 (s, OH), 11.26 (brs, NH). ¹³C NMR (DMSO-d₆, 125 MHz) 20.6 (C₆), 26.4 (C₅), 36.3 (C₇), 57.3 (C₉), 107.0 (C₈α), 115.0 (2C, 4-OH phenyl C₃,5), 128.2 (2C, 4-OH phenyl C₂,6), 129.9 (2,5-di Cl phenyl C₆), 130.0 (2C, 4-OH phenyl C₂,6), 132.4 (2,5-di Cl phenyl C₃), 131.2 (2,5-di Cl phenyl C₂), 131.6 (2,5-di Cl phenyl C₅), 131.7 (C₅α), 134.3 (2,5-di Cl phenyl C₃), 147.1 (2,5-di Cl phenyl C₁), 152.1 (CN N₄), 156.5 (4-OH phenyl C₁), 157.0 (CN N₃), 193.2 (CO).

4.1.26. 2-(Phenyl)-9-(4-hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (7m)
Yield: 60.4 %, yellowish powder, m.p. 260-262°C. LR-ESI: 357.2 [M-H]. HR-ESI m/z calcd for C₂₁H₁₇N₄O₂ [M-H] 357.1352, found 357.1354. ¹H NMR (CD₃OD, 400 MHz) 2.07 (m, 2H, C₆-H), 2.39 (m, 2H, C₅-H), 2.77 (m, 2H, C₇-H), 6.29 (s, 1H, C₉-H), 6.71 (d, J = 8.4 Hz, 2H, 4-OH phenyl C₃,5-H), 7.14 (d, J = 8.4 Hz, 2H, 4-OH phenyl C₂,6-H), 7.38 (m, 3H, phenyl C₃,4,5-H), 7.92 (m, 2H, phenyl C₂,6-H). ¹³C NMR (CD₃OD, 125 MHz) 22.2 (C₆), 30.8 (C₅), 37.7 (C₇), 55.8 (C₉), 109.8 (C₈α), 116.2 (2C, 4-OH phenyl C₃,5), 127.3 (2C, phenyl C₂,6), 129.5 (4-OH phenyl C₄), 129.6 (2C, phenyl C₃,5), 129.7 (2C, 4-OH phenyl C₂,6), 130.1 (phenyl C₆), 133.8 (C₅α), 134.4 (phenyl C₁), 153.2 (CN N₄), 154.6 (4-OH phenyl C₆), 158.5 (CN N₃), 197.3 (CO).

4.1.27. 2-(2-Chlorophenyl)-9-(3-hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (9a)
Yield: 43.6 %, yellowish powder, m.p. 280-282°C. LR-ESI: 391.3 [M-H]. HR-ESI m/z calcd for C₂₁H₁₆ClN₄O₂ [M-H] 391.0962, found 391.0960. ¹H NMR (CD₃OD, 400 MHz) 2.11 (m, 1H, C₆-H), 2.20 (t, J = 7.2 Hz, 1H, C₆-H), 2.42 (m, 2H, C₅-H), 2.78 (m, 2H, C₇-H), 6.36 (s, 1H, C₉-H), 6.67 (d, J = 8.4 Hz, 1H, 3-OH phenyl C₄-H), 6.75 (s, 1H, 3-OH phenyl C₂-H), 6.78 (d, J = 7.6 Hz, 1H, 3-OH phenyl C₆-H), 7.11 (t, J = 8.0 Hz, 1H, 3-OH phenyl C₂-H), 7.34 (t, J = 7.6 Hz, 1H, 2-Cl phenyl C₄-H), 7.39 (t, J = 7.6 Hz, 1H, 2-Cl phenyl C₅-H), 7.47 (d, J = 7.6 Hz, 1H, 2-Cl phenyl C₃-H), 7.65 (d, J = 6.8 Hz, 1H, 2-Cl phenyl C₆-H). ¹³C NMR (CD₃OD, 125 MHz) 22.1 (C₆), 28.0 (C₅), 37.7 (C₇), 59.6 (C₉), 109.6 (C₈α), 115.2 (3-OH phenyl C₄), 116.2 (3-OH phenyl C₂), 119.5 (3-OH phenyl C₆), 128.0 (2-Cl phenyl C₅), 130.6 (2-Cl phenyl C₆), 131.5 (2-Cl phenyl C₃), 131.8 (3-OH phenyl C₅), 132.7 (2-Cl phenyl C₄), 134.3 (2-Cl phenyl C₂), 138.6 (2-Cl phenyl C₁), 139.0 (C₅α), 143.8 (3-OH phenyl C₁), 148.9 (CN N₄), 154.8 (3-OH phenyl C₃), 158.8 (CN N₃), 197.2 (CO).

4.1.28. 2-(2-Chlorophenyl)-9-cyclohexyl-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (7m)
b|quinazolin-8(4H)-one (9b)

Yield: 59.2%, white powder. LR-ESI: 381.2 [M-H]. HR-ESI m/z calced for C_{21}H_{22}ClN_{4}O [M-H]: 381.1482, found 381.1485. \textsuperscript{1}H NMR (CD_{3}OD, 400 MHz) 1.02 (m, 1H, cyclohexyl C\textsubscript{1}-H), 1.17 (m, 2H, cyclohexyl C\textsubscript{2,6}-H), 1.66 (m, 8H, cyclohexyl), 2.07 (m, 2H, C\textsubscript{6}-H), 2.45 (m, 2H, C\textsubscript{5}-H), 2.66 (brs, 2H, C\textsubscript{7}-H), 5.30 (s, 1H, C\textsubscript{9}-H), 7.39 (m, 2H, 2-Cl phenyl C\textsubscript{4,5}-H), 7.50 (d, J = 7.2 Hz, 1H, 2-Cl phenyl C\textsubscript{3}-H), 7.72 (d, J = 6.8 Hz, 1H, 2-Cl phenyl C\textsubscript{6}-H). \textsuperscript{13}C NMR (CD_{3}OD, 125 MHz) 22.2 (C\textsubscript{6}), 27.4 (cyclohexyl C\textsubscript{3}), 27.5 (cyclohexyl C\textsubscript{5}), 27.6 (cyclohexyl C\textsubscript{4}), 27.8 (cyclohexyl C\textsubscript{2}), 27.9 (cyclohexyl C\textsubscript{6}), 32.4 (C\textsubscript{5}), 37.8 (C\textsubscript{7}), 46.7 (cyclohexyl C\textsubscript{1}), 60.6 (C\textsubscript{9}), 108.1 (C\textsubscript{8a}), 128.0 (2-Cl phenyl C\textsubscript{3}), 131.5 (2-Cl phenyl C\textsubscript{6}), 131.6 (2-Cl phenyl C\textsubscript{2}), 131.7 (2-Cl phenyl C\textsubscript{5}), 132.6 (2-Cl phenyl C\textsubscript{4}), 149.9 (C\textsubscript{5a}), 156.4 (CN\textsubscript{2}N\textsubscript{4}), 159.3 (CN\textsubscript{1}N\textsubscript{3}), 197.6 (CO).

4.1.29. 2-(2-Chlorophenyl)-9-(3,4-dihydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (9c)

Yield: 61.3 %, yellowish powder, m.p. 298-300°C. LR-ESI: 407.1 [M-H]. HR-ESI m/z calcd for C_{21}H_{16}ClN_{4}O_{3} [M-H]: 407.0911, found 407.0916. \textsuperscript{1}H NMR (CD_{3}OD, 400 MHz) 2.04 (m, 2H, C\textsubscript{6}-H), 2.40 (brs, 2H, C\textsubscript{5}-H), 2.74 (brs, 2H, C\textsubscript{7}-H), 6.28 (s, 1H, C\textsubscript{9}-H), 6.66 (d, J = 8.4 Hz, 1H, 3,4-di OH phenyl C\textsubscript{6}-H), 6.70 (d, J = 8.4 Hz, 1H, 3,4-di OH phenyl C\textsubscript{5}-H), 6.76 (s, 1H, 3,4-di OH phenyl C\textsubscript{2}-H), 7.33 (t, J = 7.2 Hz, 1H, 2-Cl phenyl C\textsubscript{4}-H), 7.38 (t, J = 7.2 Hz, 1H, 2-Cl phenyl C\textsubscript{5}-H), 7.46 (d, J = 8.0 Hz, 1H, 2-Cl phenyl C\textsubscript{6}-H), 7.64 (d, J = 7.2 Hz, 1H, 2-Cl phenyl C\textsubscript{3}-H). \textsuperscript{13}C NMR (CD_{3}OD, 125 MHz) 22.2 (C\textsubscript{6}), 28.0 (C\textsubscript{5}), 37.7 (C\textsubscript{7}), 59.3 (C\textsubscript{9}), 109.8 (C\textsubscript{8a}), 115.5 (3,4-di OH phenyl C\textsubscript{5}), 116.2 (3,4-di OH phenyl C\textsubscript{2}), 120.0 (3,4-di OH phenyl C\textsubscript{6}), 128.0 (2-Cl phenyl C\textsubscript{5}), 131.5 (2-Cl phenyl C\textsubscript{6}), 131.8 (2-Cl phenyl C\textsubscript{2}), 132.7 (2-Cl phenyl C\textsubscript{3}), 134.2 (3,4-di OH phenyl C\textsubscript{4}), 146.3 (3,4-di OH phenyl C\textsubscript{4}), 146.4 (3,4-di OH phenyl C\textsubscript{2}), 148.7 (C\textsubscript{5a}), 154.5 (CN_{2}N_{4}), 160.1 (CN_{1}N_{3}), 197.3 (CO).

4.1.30. 2-(2-Chlorophenyl)-9-(4-morpholinophenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (9d)

Yield: 35.8 %, yellowish powder, m.p. 298-300°C. LR-ESI: 460.2 [M-H]. HR-ESI m/z calcd for C_{25}H_{23}ClN_{5}O_{2} [M-H]: 460.1540, found 460.1543. \textsuperscript{1}H NMR (CD_{3}OD, 400 MHz) 2.07 (m, 2H, C\textsubscript{6}-H), 2.41 (m, 2H, C\textsubscript{5}-H), 2.75 (m, 2H, C\textsubscript{7}-H), 3.09 (t, J = 5.2 Hz, 4H, morpholino NCH\textsubscript{2}), 3.78 (t, J = 4.8 Hz, 4H, morpholino OCH\textsubscript{2}), 6.36 (s, 1H, C\textsubscript{9}-H), 6.88 (d, J = 8.8 Hz, 2H, phenyl C\textsubscript{3,5}-H), 7.20 (d, J = 8.8 Hz, 2H, phenyl C\textsubscript{2,6}-H), 7.33 (dt, J = 7.2 Hz, J = 1.6 Hz, 1H, 2-Cl phenyl C\textsubscript{4}-H), 7.40 (dt, J = 8.0 Hz, J = 2.0 Hz, 2-Cl phenyl C\textsubscript{6}-H).
Hz, 1H, 2-Cl phenyl C₅-H), 7.46 (dd, J = 7.6 Hz, J = 1.2 Hz, 1H, 2-Cl phenyl C₅-H), 7.64 (dd, J = 7.6 Hz, J = 2.0 Hz, 1H, 2-Cl phenyl C₆-H). ¹³C NMR (CD₃OD, 125 MHz) 22.2 (C₆), 28.0 (C₅), 37.7 (C₇), 50.7 (2C, morpholino NCH₂), 59.3 (C₉), 68.1 (2C, morpholino OCH₂), 109.7 (C₈a), 116.8 (2C, phenyl C₃,5), 128.0 (2-Cl phenyl C₅), 129.2 (2C, phenyl C₂,₆), 131.5 (phenyl C₁), 131.6 (2-Cl phenyl C₆), 131.8 (2-Cl phenyl C₃), 132.6 (2-Cl phenyl C₄), 134.0 (2-Cl phenyl C₂), 134.3 (2-Cl phenyl C₁), 148.8 (C₈a), 152.8 (phenyl C₄), 154.5 (CN₂N₄), 160.2 (CN₁N₃), 197.2 (CO).

4.1.31. N-(4-(2-(2-Chlorophenyl)-8-oxo-4,5,6,7,8,9-hexahydro-[1,2,4]triazolo[5,1-b]quinazolin-9-yl) phenyl)acetamide (9e)
Yield: 55.8 %, yellowish powder, m.p. 290-291°C. LR-ESI: 432.2 [M-H]⁻. HR-ESI m/z calcd for C₂₃H₁₉ClN₅O₂ [M-H]⁻ 432.1233, found 432.1237. ¹H NMR (CD₃OD, 400 MHz) 2.08 (m, 2H, C₆-H), 2.09 (s, 3H, AcNH), 2.40 (m, 2H, C₅-H), 2.75 (m, 2H, C₇-H), 6.39 (s, 1H, C₉-H), 7.26 (d, J = 8.8 Hz, 2H, 4-AcNH phenyl C₂,₅-H), 7.38 (dt, J = 7.6 Hz, J = 1.6 Hz, 1H, 2-Cl phenyl C₄-H), 7.38 (dt, J = 7.2 Hz, J = 2.0 Hz, 1H, 2-Cl phenyl C₅-H), 7.48 (d, J = 8.8 Hz, 2H, 4-AcNH phenyl C₃,₅-H), 7.64 (dd, J = 7.6 Hz, J = 2.0 Hz, 1H, 2-Cl phenyl C₅-H), 7.78 (d, J = 8.4 Hz, 1H, 2-Cl phenyl C₆-H). ¹³C NMR (CD₃OD, 100 MHz) 22.2 (C₆), 24.1 (AcNH), 28.0 (C₅), 37.7 (C₇), 59.5 (C₉), 109.4 (C₈a), 125.9 (2C, 4-AcNH phenyl C₃,₅), 128.5 (2-Cl phenyl C₅), 128.9 (2C, 4-AcNH phenyl C₂,₆), 129.8 (2-Cl phenyl C₆), 130.8 (2-Cl phenyl C₃), 131.3 (2-Cl phenyl C₄), 132.8 (2-Cl phenyl C₂), 133.0 (4-AcNH phenyl C₁), 134.3 (4-AcNH phenyl C₄), 139.9 (2-Cl phenyl C₁), 151.0 (C₈a), 154.9 (CN₂N₄), 160.4 (CN₁N₃), 168.6 (CH₃CO), 197.2 (CO).

4.1.32. 2-(2-Chlorophenyl)-9-(4-ethylphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (9f)
Yield: 45.7 %, yellowish powder. LR-ESI: 403.3 [M-H]⁻. HR-ESI m/z calcd for C₂₃H₂₀ClN₄O [M-H]⁻ 403.1326, found 403.1324. ¹H NMR (CD₃OD, 400 MHz) 1.17 (t, J = 7.6 Hz, 3H, CH₂CH₃), 2.04 (m, 2H, C₆-H), 2.39 (m, 2H, C₅-H), 2.58 (q, J = 7.6 Hz, 2H, CH₂CH₃), 2.76 (m, 2H, C₇-H), 3.95 (s, 1H, C₉-H), 7.12 (d, J = 8.0 Hz, 2H, 4-Et phenyl C₃,₅-H), 7.21 (d, J = 8.0 Hz, 2H, 4-Et phenyl C₂,₆-H), 7.32 (dt, J = 7.6 Hz, J = 1.6 Hz, 1H, 2-Cl phenyl C₄-H), 7.37 (dt, J = 8.0 Hz, J = 2.0 Hz, 1H, 2-Cl phenyl C₅-H), 7.46 (dd, J = 8.0 Hz, J = 1.6 Hz, 1H, 2-Cl phenyl C₃-H), 7.63 (dd, J = 7.6 Hz, J = 2.0 Hz, 1H, 2-Cl phenyl C₆-H). ¹³C NMR (CD₃OD, 125 MHz) 16.2 (CH₂CH₃), 22.2 (C₆), 28.0 (C₅), 29.6 (CH₂CH₃), 37.7 (C₇), 59.6 (C₉), 109.6 (C₈a), 128.0 (2-Cl phenyl C₅), 128.4 (2C, 4-Et phenyl C₃,₅), 129.1 (2C, 4-Et phenyl C₂,₆), 131.4 (2-Cl phenyl C₂), 131.5 (2-Cl phenyl C₆), 131.8 (2-Cl phenyl C₃), 132.6 (2-Cl phenyl C₄), 134.2 (4-Et
phenyl C\(_1\)), 139.9 (2-Cl phenyl C\(_1\)), 145.7 (4-Et phenyl C\(_4\)), 148.9 (C\(_{5a}\)), 154.8 (CN\(_2\)N\(_4\)), 160.3 (CN\(_1\)N\(_3\)), 197.2 (CO).

4.1.33. 2-(2-Chlorophenyl)-9-(4-(methylthio)phenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b] quinazolin-8(4H)-one (9g)


\(^1\)H NMR (CD\(_3\)OD, 400 MHz) 2.06 (m, 2H, C\(_6\)-H), 2.41 (m, 2H, C\(_5\)-H), 2.43 (s, 3H, CH\(_3\)S), 2.76 (m, 2H, C\(_7\)-H), 6.39 (s, 1H, C\(_9\)-H), 7.19 (d, \(J = 8.8\) Hz, 2H, 4-CH\(_3\)S phenyl C\(_{2,6}\)-H), 7.24 (d, \(J = 8.4\) Hz, 2H, 4-CH\(_3\)S phenyl C\(_{3,5}\)-H), 7.33 (dt, \(J = 7.6\) Hz, \(J = 1.6\) Hz, 1H, 2-Cl phenyl C\(_4\)-H), 7.38 (dt, \(J = 8.0\) Hz, \(J = 2.0\) Hz, 1H, 2-Cl phenyl C\(_5\)-H), 7.46 (dd, \(J = 8.0\) Hz, \(J = 1.6\) Hz, 1H, 2-Cl phenyl C\(_3\)-H), 7.64 (dd, \(J = 7.6\) Hz, \(J = 2.0\) Hz, 1H, 2-Cl phenyl C\(_6\)-H). 

\(^{13}\)C NMR (CD\(_3\)OD, 125 MHz) 15.6 (CH\(_3\)S), 22.2 (C\(_6\)), 28.0 (C\(_5\)), 37.7 (C\(_7\)), 59.5 (C\(_9\)), 109.3 (C\(_{8a}\)), 127.5 (2C, 4-CH\(_3\)S phenyl C\(_{3,5}\)), 128.0 (2-Cl phenyl C\(_3\)), 128.9 (2C, 4-CH\(_3\)S phenyl C\(_2,6\)), 131.5 (2-Cl phenyl C\(_3\)), 131.6 (2-Cl phenyl C\(_6\)), 131.8 (2-Cl phenyl C\(_5\)), 132.6 (2-Cl phenyl C\(_4\)), 134.3 (4-CH\(_3\)S phenyl C\(_1\)), 139.3 (2-Cl phenyl C\(_1\)), 140.3 (4-CH\(_3\)S phenyl C\(_4\)), 148.9 (C\(_{5a}\)), 155.0 (CN\(_2\)N\(_4\)), 160.4 (CN\(_1\)N\(_3\)), 197.1 (CO).

4.1.34. 2-(2-Chlorophenyl)-9-(4-(pyrrolidin-1-yl)phenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b] quinazolin-8(4H)-one (9h)


\(^1\)H NMR (CD\(_3\)OD, 400 MHz) 1.98 (m, 4H, pyrrolidinyl C\(_3,4\)-H), 2.03 (m, 1H, C\(_6\)-H), 2.20 (m, 1H, C\(_6\)-H), 2.40 (m, 2H, C\(_5\)-H), 2.77 (m, 2H, C\(_7\)-H), 3.22 (m, 4H, pyrrolidinyl C\(_2,5\)-H), 6.31 (s, 1H, C\(_9\)-H), 6.48 (d, \(J = 8.4\) Hz, 2H, 4-pyrrolidinyl phenyl C\(_{3,5}\)-H), 7.12 (d, \(J = 8.8\) Hz, 2H, 4-pyrrolidinyl phenyl C\(_{2,6}\)-H), 7.33 (dt, \(J = 7.6\) Hz, \(J = 1.6\) Hz, 1H, 2-Cl phenyl C\(_4\)-H), 7.38 (dt, \(J = 7.2\) Hz, \(J = 1.6\) Hz, 1H, 2-Cl phenyl C\(_5\)-H), 7.47 (dd, \(J = 8.0\) Hz, \(J = 1.6\) Hz, 1H, 2-Cl phenyl C\(_3\)-H), 7.63 (dd, \(J = 7.6\) Hz, \(J = 2.0\) Hz, 1H, 2-Cl phenyl C\(_6\)-H). 

\(^{13}\)C NMR (CD\(_3\)OD, 125 MHz) 22.3 (C\(_6\)), 26.5 (2C, pyrrolidinyl C\(_3,4\)), 28.0 (C\(_3\)), 37.7 (C\(_7\)), 58.5 (2C, pyrrolidinyl C\(_2,5\)), 59.5 (C\(_9\)), 110.1 (C\(_{8a}\)), 112.7 (2C, 4-pyrrolidinyl phenyl C\(_3,5\)), 128.0 (2-Cl phenyl C\(_3\)), 128.1 (2-Cl phenyl C\(_6\)), 129.1 (2C, 4-pyrrolidinyl phenyl C\(_2,6\)), 129.3 (4-pyrrolidinyl phenyl C\(_1\)), 131.5 (2-Cl phenyl C\(_2\)), 131.9 (2-Cl phenyl C\(_3\)), 132.7 (2-Cl phenyl C\(_4\)), 140.0 (2-Cl phenyl C\(_1\)), 148.8 (C\(_{5a}\)), 149.6 (4-pyrrolidinyl phenyl C\(_4\)), 154.9 (CN\(_2\)N\(_4\)), 160.1 (CN\(_1\)N\(_3\)), 197.4 (CO).

4.1.35. 2-(2-Chlorophenyl)-9-(4-mercaptophenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo [5,1-b] quinazolin-8(4H)-one (9i)
LR-ESI: 409.1 [M+H]+. HR-ESI m/z calcd for C_{21}H_{18}ClN_{4}OS [M+H]+ 409.0890, found 409.0895. 1H NMR (CD$_{2}$OD, 400 MHz) 2.09 (m, 2H, C$_{6}$-H), 2.40 (m, 2H, C$_{5}$-H), 2.76 (m, 2H, C$_{7}$-H), 6.34 (s, 1H, C$_{9}$-H), 7.04 (d, $J = 8.8$ Hz, 2H, 4-SH phenyl C$_{2,6}$-H), 7.24 (d, $J = 8.4$ Hz, 2H, 4-OH phenyl C$_{3,5}$-H), 7.33 (dt, $J = 1.2$ Hz, $J = 7.6$ Hz, 1H, 2-Cl phenyl C$_{5}$-H), 7.38 (dt, $J = 1.6$ Hz, $J = 7.6$ Hz, 1H, 2-Cl phenyl C$_{4}$-H), 7.47 (d, $J = 8.0$ Hz, 1H, 2-Cl phenyl C$_{3}$-H), 7.63 (dd, $J = 2.0$ Hz, $J = 7.6$ Hz, 1H, 2-Cl phenyl C$_{6}$-H).

4.1.36. 2-(2-Chlorophenyl)-9-(4-hydroxyphenyl)-6,6-dimethyl-5,6,7,9-tetrahydro-[1,2,4]triazolo [5,1-b]quinazolin-8(4H)-one (11a)

Yield: 57.2%, yellowish powder, m.p. 228-229°C. LR-ESI: 419.2 [M+H]+. HR-ESI m/z calcd for C$_{23}$H$_{20}$ClN$_{4}$O$_{2}$ [M+H]+ 419.1275, found 419.1278. 1H NMR (CD$_{3}$OD, 400 MHz) 1.06 (s, 3H, C$_{6}$-CH$_{3}$), 1.14 (s, 3H, C$_{6}$-CH$_{3}$), 2.21 (d, $J = 16.4$ Hz, 1H, C$_{5}$-H), 2.34 (d, $J = 16.4$ Hz, 1H, C$_{5}$-H), 2.59 (d, $J = 17.2$ Hz, 1H, C$_{7}$-H), 2.65 (d, $J = 16.8$ Hz, 1H, C$_{7}$-H), 6.31 (s, 1H, C$_{9}$-H), 6.71 (d, $J = 8.0$ Hz, 2H, 4-OH phenyl C$_{3,5}$-H), 7.13 (d, $J = 8.0$ Hz, 2H, 4-OH phenyl C$_{2,6}$-H), 7.34 (t, $J = 7.6$ Hz, 1H, 2-Cl phenyl C$_{4}$-H), 7.39 (t, $J = 7.6$ Hz, 1H, 2-Cl phenyl C$_{5}$-H), 7.47 (d, $J = 8.0$ Hz, 1H, 2-Cl phenyl C$_{3}$-H), 7.63 (d, $J = 7.2$ Hz, 1H, 2-Cl phenyl C$_{6}$-H). 13C NMR (CD$_{3}$OD, 125 MHz) 27.6 (C$_{6}$-CH$_{3}$), 29.3 (C$_{3}$-CH$_{3}$), 33.7 (C$_{2}$), 41.3 (C$_{5}$), 51.3 (C$_{3}$), 59.6 (C$_{9}$), 108.9 (C$_{8a}$), 116.3 (2C, 4-OH phenyl C$_{3,5}$), 128.0 (2-Cl phenyl C$_{5}$), 129.6 (2C, 4-OH phenyl C$_{2,6}$), 131.5 (2-Cl phenyl C$_{6}$), 131.8 (2-Cl phenyl C$_{3}$), 132.7 (2-Cl phenyl C$_{4}$), 132.9 (C$_{8a}$), 133.6 (4-OH phenyl C$_{1}$), 134.3 (2-Cl phenyl C$_{2}$), 148.9 (2-Cl phenyl C$_{1}$), 152.6 (CN$_{2}$N$_{4}$), 158.6 (4-OH phenyl C$_{4}$), 160.3 (CN$_{2}$N$_{3}$), 196.9 (CO).

4.1.37. 2-(2-Chlorophenyl)-9-(4-hydroxyphenyl)-5,5-dimethyl-5,6,7,9-tetrahydro-[1,2,4]triazolo [5,1-b]quinazolin-8(4H)-one (11b)

Yield: 67.2%, white powder, m.p. 227-229°C. LR-ESI: 419.1 [M+H]+. HR-ESI m/z calcd for C$_{23}$H$_{20}$ClN$_{4}$O$_{2}$ [M+H]+ 419.1275, found 419.1279. 1H NMR (CD$_{3}$OD, 400 MHz) 1.02 (s, 3H, C$_{6}$-CH$_{3}$), 1.11 (s, 3H, C$_{5}$-CH$_{3}$), 1.91 (m, 2H, C$_{6}$-H), 2.74 (m, 2H, C$_{7}$-H), 6.29 (s, 1H, C$_{9}$-H), 6.70 (d, $J = 8.4$ Hz, 2H, 4-OH phenyl C$_{3,5}$-H), 7.12 (d, $J = 8.4$ Hz, 2H, 4-OH phenyl C$_{2,6}$-H), 7.32 (t, $J = 7.6$ Hz, 1H, 2-Cl phenyl C$_{3}$-H), 7.37 (t, $J = 8.0$ Hz, 1H, 2-Cl phenyl C$_{5}$-H), 7.46 (d, $J = 8.0$ Hz, 1H, 2-Cl phenyl C$_{3}$-H), 7.62 (d, $J = 7.2$ Hz, 1H, 2-Cl phenyl C$_{6}$-H). 13C NMR (CD$_{3}$OD, 125 MHz) 24.8 (C$_{6}$-CH$_{3}$), 27.6 (C$_{3}$-CH$_{3}$), 35.7 (2C, C$_{6}$-), 41.3 (C$_{5}$), 59.6 (C$_{9}$), 108.2 (C$_{8a}$), 116.3 (2C, 4-OH phenyl C$_{3,5}$), 128.0 (2-Cl phenyl C$_{5}$), 129.6 (2C, 4-OH phenyl C$_{2,6}$), 131.5 (2-Cl phenyl C$_{6}$), 131.8 (2-Cl phenyl C$_{3}$), 132.6 (2-Cl phenyl C$_{4}$), 133.6 (4-OH phenyl C$_{1}$), 134.3 (2-Cl phenyl C$_{2}$), 148.7 (2-Cl phenyl C$_{1}$), 152.6 (CN$_{2}$N$_{4}$), 158.5 (4-OH phenyl C$_{4}$), 160.2
4.1.38. 2-(2-Chlorophenyl)-9-(4-hydroxyphenyl)-7,9-dihydro-4H-pyrano[3,4-d][1,2,4]triazolo[1,5-a]pyrimidin-8(5H)-one (11c)

Yield: 58.8%, yellowish powder, m.p. 208-209°C. LR-ESI: 393.1 [M-H]. HR-ESI m/z calcld for C_{20}H_{14}ClN_{4}O_{3} [M-H] 393.0754, found 393.0757. ¹H NMR (CD_{3}OD, 400 MHz) 4.13 (s, 2H, C_{5}-H), 4.61 (d, J = 16.0 Hz, 1H, C_{7}-H), 4.69 (d, J = 16.4 Hz, 1H, C_{7}-H), 6.40 (s, 1H, C_{9}-H), 6.73 (d, J = 7.6 Hz, 2H, 4-OH phenyl C_{3,5}-H), 7.16 (d, J = 7.6 Hz, 2H, 4-OH phenyl C_{2,6}-H), 7.33 (t, J = 7.2 Hz, 1H, 2-Cl phenyl C_{4}-H), 7.38 (t, J = 7.6 Hz, 1H, 2-Cl phenyl C_{5}-H), 7.46 (d, J = 8.0 Hz, 1H, 2-Cl phenyl C_{3}-H), 7.65 (d, J = 7.2 Hz, 1H, 2-Cl phenyl C_{6}-H). ¹³C NMR (CD_{3}OD, 125 MHz) 58.8 (C_{9}), 65.0 (C_{7}), 72.6 (C_{3}), 107.1 (C_{8a}), 116.4 (2C, 4-OH phenyl C_{3,5}), 128.0 (2-Cl phenyl C_{5}), 129.6 (2C, 4-OH phenyl C_{2,6}), 131.4 (4-OH phenyl C_{1}), 131.5 (2-Cl phenyl C_{6}), 131.8 (2-Cl phenyl C_{3}), 132.6 (2-Cl phenyl C_{4}), 132.8 (2-Cl phenyl C_{2}), 134.2 (2-Cl phenyl C_{1}), 148.3 (CN_{2}N_{4}), 152.4 (C_{5a}), 158.8 (4-OH phenyl C_{4}), 160.3 (CN_{1}N_{3}), 192.6 (CO).

4.1.39. 2-(2-Chlorophenyl)-9-(4-hydroxyphenyl)-5,9-dihydro-4H-thiopyrano[3,4-d][1,2,4]triazolo[1,5-a]pyrimidin-8(7H)-one (11d)

Yield: 58.6%, yellowish powder, m.p. 211-213°C. LR-ESI: 409.1 [M-H]. HR-ESI m/z calcld for C_{20}H_{14}ClN_{4}O_{2}S [M-H] 409.0526, found 409.0531. ¹H NMR (CD_{3}OD, 400 MHz) 3.18 (dd, J = 2.0 Hz, J = 16.0 Hz, 1H, C_{5}-H), 3.56 (dd, J = 2.0 Hz, J = 16.5 Hz, 1H, C_{5}-H), 3.62 (dd, J = 1.5 Hz, J = 17.0 Hz, 1H, C_{7}-H), 3.95 (d, J = 17.0 Hz, 1H, C_{7}-H), 6.39 (s, 1H, C_{9}-H), 6.72 (d, J = 8.5 Hz, 2H, 4-OH phenyl C_{3,5}-H), 7.18 (d, J = 8.5 Hz, 2H, 4-OH phenyl C_{2,6}-H), 7.34 (dt, J = 1.5 Hz, J = 7.5 Hz, 1H, 2-Cl phenyl C_{4}-H), 7.39 (dt, J = 1.5 Hz, J = 7.5 Hz, 1H, 2-Cl phenyl C_{5}-H), 7.47 (dd, J = 1.5 Hz, J = 8.0 Hz, 1H, 2-Cl phenyl C_{3}-H), 7.64 (dd, J = 1.5 Hz, J = 7.5 Hz, 1H, 2-Cl phenyl C_{6}-H). ¹³C NMR (CD_{3}OD, 125 MHz) 27.6 (C_{3}), 35.4 (C_{7}), 59.3 (C_{9}), 108.8 (C_{8a}), 116.3 (2C, 4-OH phenyl C_{3,5}), 128.0 (2-Cl phenyl C_{5}), 129.6 (2C, 4-OH phenyl C_{2,6}), 131.4 (4-OH phenyl C_{1}), 131.5 (2-Cl phenyl C_{6}), 131.8 (2-Cl phenyl C_{3}), 132.7 (2-Cl phenyl C_{4}), 133.2 (2-Cl phenyl C_{2}), 148.3 (CN_{2}N_{4}), 152.4 (C_{5a}), 158.8 (4-OH phenyl C_{4}), 160.3 (CN_{1}N_{3}), 192.6 (CO).

4.1.40. 9-(4-Hydroxyphenyl)-5,6,7,9-tetrahydro-[1,2,4]triazolo[5,1-b]quinazolin-8(4H)-one (13a)

Yield: 69.5%, white powder, m.p. 230-231°C. LR-ESI: 281.2 (M-1). HR-ESI m/z calcld for C_{15}H_{13}N_{4}O_{2} [M-H] 281.1039, found 281.1042. ¹H NMR (DMSO-d_{6}, 400 MHz) 1.94 (m, 2H, C_{6}-H), 2.25 (m, 2H, C_{5}-H), 2.65 (m, 2H, C_{7}-H), 6.11 (s, 1H, C_{6}-H), 6.64
(d, J = 7.6 Hz, 2H, 4-OH phenyl C₃-5-H), 6.99 (d, J = 7.6 Hz, 2H, 4-OH phenyl C₆-H), 7.65 (s, 1H, C₂-H), 9.36 (s, OH), 11.0 (s, NH). ¹³C NMR (DMSO-d₆, 125 MHz) 20.7 (C₆), 26.4 (C₅), 36.4 (C₇), 57.1 (C₉), 106.8 (C₈a), 114.9 (2C, 4-OH phenyl C₃,5), 128.1 (2C, 4-OH phenyl C₂,6), 132.1 (4-OH phenyl C₁), 146.7 (C₅a), 149.8 (C₂), 152.3 (CN₃), 156.9 (4-OH phenyl C₄), 193.2 (CO).

4.1.41. 9-(4-Hydroxyphenyl)-5,6,7,9-tetrahydrotetrazolo[5,1-b]quinazolin-8(4H)-one (13b)
Yield: 75.8%, yellowish powder. LR-ESI: 282.1 [M-H]. HR-ESI m/z calcd for C₁₄H₁₂N₂O₂ [M-H]: 282.0991, found 282.0993. ¹H NMR (CD₃OD, 400 MHz) 2.09 (m, 2H, C₆-H), 2.40 (m, 2H, C₅-H), 2.78 (m, 2H, C₇-H), 6.58 (s, 1H, C₉-H), 6.72 (d, J = 8.0 Hz, 2H, 4-OH phenyl C₃,5-H), 7.13 (d, J = 8.0 Hz, 2H, 4-OH phenyl C₂,6-H). ¹³C NMR (CD₃OD, 125 MHz) 22.2 (C₆), 28.3 (C₅), 37.7 (C₇), 58.9 (C₉), 109.2 (C₈a), 116.5 (2C, 4-OH phenyl C₃,5), 129.7 (2C, 4-OH phenyl C₂,6), 132.7 (4-OH phenyl C₁), 150.3 (C₅a), 155.5 (4-OH phenyl C₄), 159.0 (tetrazole), 196.9 (CO).

4.1.42. 2-(2-Chlorophenyl)-9-(4-hydroxyphenyl)-5,6,7,9-tetrahydropyrazolo[5,1-b]quinazolin-8(4H)-one (13c)
Yield: 75.0%, yellowish powder, m.p. 256-258°C. LR-ESI: 390.1 [M-H]. HR-ESI m/z calcd for C₂₂H₁₇ClN₃O₂ [M-H]: 390.1009, found 390.1012. ¹H NMR (CD₃OD, 400 MHz) 2.02 (m, 2H, C₆-H), 2.35 (m, 2H, C₅-H), 2.70 (m, 2H, C₇-H), 6.21 (s, 1H, C₉-H), 6.32 (s, 1H, C₃-H), 6.66 (d, J = 7.6 Hz, 2H, 4-OH phenyl C₃,5-H), 7.07 (d, J = 7.6 Hz, 2H, 4-OH phenyl C₂,6-H), 7.28 (m, 2H, 2-Cl phenyl C₄,5-H), 7.43 (m, 1H, 2-Cl phenyl C₃-H), 7.56 (m, 1H, 2-Cl phenyl C₆-H). ¹³C NMR (CD₃OD, 125 MHz) 22.3 (C₆), 28.1 (C₅), 37.6 (C₇), 58.7 (C₉), 91.3 (C₃), 108.8 (C₈a), 116.0 (2C, 4-OH phenyl C₃,5), 128.1 (2-Cl phenyl C₆), 129.2 (2C, 4-OH phenyl C₂,6), 130.6 (2-Cl phenyl C₄), 131.3 (2-Cl phenyl C₃), 131.8 (CN₂), 132.0 (2-Cl phenyl C₅), 133.6 (2-Cl phenyl C₁), 135.1 (4-OH phenyl C₁), 139.2 (2-Cl phenyl C₂), 150.9 (C₅a), 153.9 (C₂), 158.0 (4-OH phenyl C₄), 196.9 (CO).

4.1.43. 2-(2-Bromophenyl)-9-(4-hydroxyphenyl)-7,9-dihydro-4H-thiopyrano[3,4-d][1,2,4]triazolo[1,5-a]pyrimidin-8(5H)-one (14a)
Yield: 49.5%, yellowish powder, m.p. 295-296°C. LR-ESI: 455.0 [M+H]. HR-ESI m/z calcd for C₂₀H₁₆BrN₄O₂S [M+H]: 455.0172, found 455.0162. ¹H NMR (CD₃OD, 400 MHz) 3.16 (d, J = 16.0 Hz, 1H, C₅-H), 3.54 (d, J = 17.2 Hz, 2H, C₅,7-H), 3.89 (d, J = 17.2 Hz, 1H, C₇-H), 6.38 (s, 1H, C₉-H), 6.71 (d, J = 8.8 Hz, 2H, 4-OH phenyl C₃,5-H), 7.18 (d, J = 8.8 Hz, 2H, 4-OH phenyl C₂,6-H), 7.30 (t, J = 8.0 Hz, 1H, 2-Br phenyl C₄-H),...
H), 7.38 (t, J = 7.6 Hz, 1H, 2-Br phenyl C₅-H), 7.57 (dd, J = 1.6 Hz, J = 7.6 Hz, 1H, 2-Br phenyl C₃-H), 7.66 (d, J = 8.4 Hz, 1H, 2-Br phenyl C₆-H). ¹³C NMR (CD₃OD, 125 MHz) 27.5 (C₅), 35.4 (C₇), 59.3 (C₉), 108.9 (C₈ₚ), 116.3 (2C, 4-OH phenyl C₃,₅), 123.4 (2-Br phenyl C₂), 128.5 (2-Br phenyl C₅), 129.7 (2C, 4-OH phenyl C₂,₆), 132.0 (2-Br phenyl C₆), 132.8 (2-Br phenyl C₄), 133.2 (4-OH phenyl C₁), 133.6 (2-Br phenyl C₃), 148.2 (2-Br phenyl C₁), 151.9 (CN₂N₄), 158.7 (4-OH phenyl C₄), 161.3 (CN₁N₃), 191.5 (CO).

4.1.44. 9-(4-Hydroxyphenyl)-2-(2-iodophenyl)-7,9-dihydro-4H-thiopyran[3,4-d][1,2,4]triazolo[1,5-a]pyrimidin-8(5H)-one (14b)


¹H NMR (CD₃OD, 400 MHz) 3.15 (dd, J = 2.0 Hz, J = 16.0 Hz, 1H, C₅-H), 3.53 (m, 2H, C₅,₇-H), 3.87 (dd, J = 2.0 Hz, J = 17.2 Hz, 1H, C₇-H), 6.36 (s, 1H, C₉-H), 6.71 (d, J = 8.8 Hz, 2H, 4-OH phenyl C₃,₅-H), 7.11 (dt, J = 1.6 Hz, J = 7.6 Hz, 1H, 2-I phenyl C₅-H), 7.18 (d, J = 8.8 Hz, 2H, 4-OH phenyl C₂,₆-H), 7.39 (dt, J = 1.2 Hz, J = 7.6 Hz, 1H, 2-I phenyl C₄-H), 7.48 (dd, J = 1.6 Hz, J = 7.6 Hz, 1H, 2-I phenyl C₆-H), 7.93 (d, J = 8.0 Hz, 1H, 2-I phenyl C₃-H). ¹³C NMR (CD₃OD, 125 MHz) 27.6 (C₅), 35.4 (C₇), 59.3 (C₉), 97.4 (2-I phenyl C₂), 108.8 (C₈ₚ), 116.3 (2C, 4-OH phenyl C₃,₅), 127.4 (2-I phenyl C₅), 129.2 (2-I phenyl C₆), 129.7 (2C, 4-OH phenyl C₂,₆), 132.0 (2-I phenyl C₄), 133.2 (4-OH phenyl C₁), 137.6 (C₅ₐ), 141.3 (2-I phenyl C₃), 148.2 (2-I phenyl C₁), 152.0 (CN₂N₄), 158.6 (4-OH phenyl C₄), 163.0 (CN₁N₃), 191.5 (CO).

4.1.45. 9-(4-Hydroxyphenyl)-2-(2-(trifluoromethyl)phenyl)-7,9-dihydro-4H-thiopyran[3,4-d][1,2,4]triazolo[1,5-a]pyrimidin-8(5H)-one (14c)


¹H NMR (CD₃OD, 400 MHz) 3.18 (dd, J = 16.4 Hz, J = 2.4 Hz, 1H, C₅-H), 3.57 (dd, J = 16.0 Hz, J = 2.0 Hz, 1H, C₇-H), 3.60 (dd, J = 16.8 Hz, J = 2.0 Hz, 1H, C₇-H), 3.93 (d, J = 16.8 Hz, 1H, C₇-H), 6.38 (s, 1H, C₉-H), 6.71 (d, J = 8.8 Hz, 2H, 4-OH phenyl C₃,₅-H), 7.16 (d, J = 8.8 Hz, 2H, 4-OH phenyl C₂,₆-H), 7.64 (m, 3H, 2-CF₃ phenyl C₃,₄,₅-H), 7.78 (d, J = 7.6 Hz, 1H, 2-CF₃ phenyl C₆-H). ¹³C NMR (CD₃OD, 125 MHz) 27.5 (C₅), 35.4 (C₇), 59.3 (C₉), 108.9 (C₈ₚ), 116.3 (2C, 4-OH phenyl C₃,₅), 127.6 (CF₃), 127.7 (2-CF₃ phenyl C₃), 129.6 (2C, 4-OH phenyl C₂,₆), 130.5 (2-CF₃ phenyl C₂), 131.0 (2-CF₃ phenyl C₄), 131.5 (C₈ₚ), 133.0 (2-CF₃ phenyl C₆), 133.1 (2-CF₃ phenyl C₃), 133.2 (4-OH phenyl C₁), 148.4 (2-CF₃ phenyl C₁), 152.1 (CN₂N₄), 158.7 (4-OH phenyl C₄), 160.6 (CN₁N₃), 191.5 (CO).
4.2. Chiral resolution of compound 7a

The enantiomers of compound 7a (1.57 g) were resolved by CHIRALPAK IC (IC00CD-NA012) column (0.46 cm × 15 cm) on Shimadzu LC-20AT HPLC eluting with dichloromethane/ethanol [90/10 (v/v)] with a flow rate of 1.0 mL/min at 35°C under the wavelength of UV 254 nm. Two peaks were separately collected at the \( t_R = 2.090 \) min (isomer 1) and \( t_R = 2.409 \) min (isomer 2) and the enantiomeric excess (e.e.) value of each product is determined by HPLC as >98%. Two isomers were thus obtained as light yellowish powder (isomer 1: 0.71 g; isomer 2: 0.71 g). The absolute configuration of isomer 1 was determined by X-ray diffraction as 9-(R)-7a. Specific optical rotation was also detected for these two isomers. Isomer 1: 9-(R)-7a, \([\alpha]^{D20}_{20} -110^\circ\) (c 0.1 in methanol); Isomer 2: 9-(S)-7a, \([\alpha]^{D20}_{20} +107^\circ\) (c 0.1 in methanol).

4.3. Chiral resolution of compound 14b

The enantiomers of compound 14b (0.0705 g) were resolved by CHIRALPAK IC column (0.46 cm × 15 cm) on Shimadzu LC-2010 HPLC eluting with ethanol with a flow rate of 1.0 mL/min at 25°C under the wavelength of UV 210 nm. Two peaks were separately collected at the \( t_R = 4.568 \) min (isomer 1) and \( t_R = 5.981 \) min (isomer 2) and the enantiomeric excess (e.e.) value of each product is determined by HPLC as >99%. Two isomers were thus obtained as white powder (isomer 1: 0.0309 g; isomer 2: 0.0255 g). Specific optical rotation was detected for these two isomers. Isomer 1: 9-(R)-14b, \([\alpha]^{D20}_{20} -22^\circ\) (c 0.1 in methanol); Isomer 2: 9-(S)-14b, \([\alpha]^{D20}_{20} +22^\circ\) (c 0.1 in methanol).

4.4. X-ray structure determination of 9-(R)-7a (isomer 1)

Diffraction data were collected on a Bruker D8 VENTURE single-crystal diffractometer using a graphite-monochromated MoKα radiation (0.71073Å) at 193 K in the \( \omega-2\theta \) scan mode. In this case, an empirical absorption correction by SADABS was applied to the intensity data. The structure was solved by direct methods and refined by full-matrix least-squares on \( F^2 \) methods using the SHELXTL crystallographic software package. All non-hydrogen atoms were refined anisotropically with hydrogen atoms included in calculated positions (riding model). Crystallographic data for compound 9-(R)-7a is given in Table S2-7. CCDC2004638 contains the supplementary crystallographic data for compound 9-(R)-7a, which can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

4.5. Bioassays

4.5.1. Reagents
Human INSL5 (hINSL5), R3/I5 and relaxin-3 were purchased from Phoenix Pharmaceuticals (Burlingame, USA). LANCE Ultra cAMP and AlphaScreen SureFire p-ERK1/2 assay kits were obtained from PerkinElmer (Waltham, MA, USA). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), dimethyl sulfoxide (DMSO) and bovine serum albumin were supplied by Sigma-Aldrich (St. Louis, USA). The methods for plasmid construction, cell culture and assay validation were described in our previous paper [14].

4.5.2. cAMP accumulation assay
For off-target examination, inhibition of forskolin-induced cAMP accumulation by test compounds was carried out in parental CHO cells. For selective agonist effect valuation, compounds were tested for their ability to inhibit cAMP accumulation in CHO-K1 cells stably over-expressing human RXFP4 or human RXFP3. Cells were seeded at a density of 8 × 10^5 cells/mL and stimulated with different concentrations of individual testing compounds (250, 100, 40, 16, 6.4, 2.56, 1.024 and 0.4096 μM) plus 500 nM forskolin for 40 min at RT in the presence of 500 nM IBMX. Peptides INSL5 and R3I5 were used as positive controls at different concentrations (μM). For cAMP assay on hRXFP1-overexpressing 293T cells, relaxin-3 was used as positive control without forskolin stimulation. Eu-cAMP tracer and ULight-anti-cAMP working solution were then applied followed by incubation for 40 min at RT. Time-resolved fluorescence resonance energy transfer (TR-FRET) signals were read on an EnVision® multimode plate reader (PerkinElmer) with excitation at 320 or 340 nm and emission at 665 nm and 615 nm. Each compound was tested in duplicate and each experiment was performed independently three times. Agonist activity was expressed as % INSL5 in hRXFP4-CHO-K1 cells, % R3/I5 in hRXFP3-CHO-K1 cells or % relaxin-3 in hRXFP1-293T cells. For each ligand-concentration, the value of 665/615 was calculated, and normalized to the corresponding maximum value obtained for INSL5 in hRXFP4-CHO-K1 cells, R3/I5 in hRXFP3-CHO-K1 cells and relaxin-3 in hRXFP1-293T cells. The normalized values were plotted vs. ligand concentration using GraphPad PRISM 8 and are expressed as means ± SEM.

4.5.3. Cytotoxicity assay
Cytotoxicity was assessed in hRXFP4-CHO cells using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cells were seeded into 96-well plates at a density of 30,000 cells/well and incubated overnight, in which different concentrations of compounds were added and incubated for 24 h. CCK-8 solution was then added and incubated for another 1 h. Absorbance values at 450 nm were quantitated on a
SpectraMax M5 plate reader (Molecular Devices). Data were normalized to the vehicle-treated samples (Supplementary Information).

4.5.4. Receptor binding assay

CHO-K1 cells stably expressing human RXFP4 were plated out at the density of 50,000 cells per well per 200 μL in a 96-well ViewPlate with clear bottom and white walls precoated with poly-L-lysine. Competitive binding assay was performed with 5 nM of europium-labeled Eu(A)-R3/I5 in the presence of increasing amounts of test compounds dissolved in DMSO following the protocol described previously [18]. Fluorescence measurement was carried out at an excitation wavelength of 340 nm and an emission wavelength of 614 nm on a Victor Plate Reader (PerkinElmer, Melbourne, Australia). Each concentration point was measured in triplicate, and each experiment was performed independently three times. Data were analyzed using GraphPad PRISM 8 (GraphPad Inc., San Diego, CA) and expressed as means ± SEM.

4.5.5. Statistical analysis

Dose-response data were analyzed with Prism software (GraphPad PRISM 8) using a sigmoidal model with variable slope. Statistical significance was determined using two tailed student’s t-test, and P < 0.05 was considered significant.

4.6. Molecular modelling

Molecular docking for the binding of derivatives to hRXFP4 and hRXFP3 was performed using the LibDock docking protocol in BIOVIA Discovery Studio 2016 (Accelrys).

4.6.1. Preparation of target protein

Homology models of RXFP4 (SWISS-MODEL: Q8TDU9) and RXFP3 (SWISS-MODEL: Q9NSD7), which were modeled on the template of agonist-bound apelin receptor (PDB code: 5VBL), were downloaded from SWISS-MODEL at https://swissmodel.expasy.org/repository/uniprot/. The energy of the system was minimized using CHARMM forcefield.

4.6.2. Preparation of ligands

Ligands were prepared by energy minimization with the top 10 poses to be presented and scored while and keeping the other options in their default values using CHARMM forcefield until RMS gradient of 0.01 was reached.

4.6.3. Molecular docking

Cavity searching was performed to find the hRXFP4 orthosteric binding site constructed by residues L1183.29, T1764.60, R2085.42, F2917.35, Q2055.39, T2666.55,
G269^{6.58}, V265^{6.54}, Q287^{7.31}, K273^{6.62}, Y284^{7.28}, T288^{7.32}, L201^{5.35}, P196^{5.30}, L193, L192, L190 and Y204^{5.38}, and the hRXFP3 orthosteric binding site constructed by residues T346^{6.55}, Y369^{7.33}, L345^{6.54}, L365^{7.29}, C366^{7.30}, S349^{6.58}, Y267^{5.38}, L264^{5.35}, I350^{6.59}, K353^{6.62}, F262^{ECL2}, W263^{5.34}, R250^{ECL2} and F251^{ECL2}. Then the prepared ligand 7a and 14b were docked into the binding site using LibDock protocol, with the top 10 poses presented and scored while keeping other options in their default values. LibDock fitness scores of 114.064 (7a) and 111.977 (14b) for hRXFP4, and 111.545 (7a) and 111.749 (14b) for hRXFP3 were thus obtained.

4.6.4. Sequence alignment
The sequences of hRXFP3 (Q8BGE9), hRXFP4 (Q8TDU9) and apelin receptor (5VBL) were downloaded from SWISS-MODEL (https://swissmodel.expasy.org/repository/uniprot/) and the alignment by CLUSTALW was performed on https://www.genome.jp/tools-bin/clustalw. The figure was plotted with ENDscript/ESPript 3.0 on http://espiript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi [19].

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions
QL, DHY and M-WW designed research. LL, GYL, QTZ, GQG and QL performed research, LL, GYL, QTZ, QL, RADB, DHY and M-WW analyzed data. LL, QL and M-WW wrote the manuscript.

Appendix A. Supplementary Data
Supplementary data for this article can be found online at XXX.
References and notes


Highlights
A series of tricyclic derivatives were synthesized via Biginelli cyclocondensation. The analogues were screened for their biological activities using a HTRF assay that measures the inhibition of forskolin-stimulated cAMP accumulation in human RXFP4-overexpressing CHO cells.
The specificity of the analogues for RXFP4 was also examined in human RXFP3-overexpressing CHO-K1 cells and human RXFP1-overexpressing 293T cells. Compared with 7a, compound 9-(S)-14b behaved as a RXFP4 agonist and exhibited 2.3-fold higher efficacy and better selectivity for RXFP4 vs. RXFP3. A relatively high LibDock fitness score of 111.977 was obtained for the docking of 14b with RXFP4, and several functions were involved including two hydrogen bonds, Pi-Pi stack and Pi-cation individually, and one additional Pi-sulfur function, which may explain the efficacy difference between 14b and 7a.