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Combinatorial synthesis and biological evaluation of peptide-binding GPCR-targeted library

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ABSTRACT

As an effective strategy of the drug discovery for peptide-binding GPCRs based on the natural ligands, beta-turn peptidomimetic compound library with benzodiazepine skeleton was constructed using solid and solution phase parallel synthesis with four different scaffolds containing Phe, Lys, Ser and Glu, respectively. The usefulness of 162 library compounds was evaluated by the cell based screening at melanocortin 4 receptor in CHO-k1 cells, to find hit compounds showing agonistic effect at the receptor. The screening of library afforded three hit compounds including the most effective analog, (*S*)-3-benzyl-7-(4-fluorobenzyloxy)-4-(4-methoxyphenethyl)-4,5-dihydro-1*H*-benzo[*e*][1,4]diazepin-2(3*H*)-one, **13aiE**, of which EC_{50} was determined as 13 μM.

1. Introduction

G-protein coupled receptors (GPCRs) have been in the center of pharmaceutical research as the most diverse and largest target family showing important physiological relevance with various diseases, participating in a lot of cell signal transduction processes. Peptide-binding GPCRs belong to a small group GPCR family with approximately 120 members which are activated by the binding of intermediate size peptides consisting of typically 30–40 amino acid residues [1]. Most of the peptide-binding GPCRs are related with pathological events in several diseases and most of pharmaceutical companies have put a lot of efforts to modulate these GPCRs for the management of corresponding diseases. Since the lack of three-dimensional structures of GPCRs, an alternative strategy toward drug discovery for peptide-binding GPCRs could be peptidomimetic approaches based on the turn secondary structure of peptide ligands. The turn conformation of natural peptide ligands has been supported by structure–activity relationships of native ligands [2], modified peptide fragments [3], cyclic peptide analogues [4], and turn peptidomimetics [5].

The melanocortin 4 receptor (MC4R), a member of melanocortin receptor family [6] consisting of 332 amino acids, which is activated upon binding of melanocortin peptide and mediates signal transduction via G-protein coupled signaling system. In human, five receptor subtypes which share high homology in primary amino acid sequences have been identified (MC1R–MC5R), but their physiological roles are different each other such as skin pigmentation, anti-inflammatory effects, regulation of blood pressure, immune modulation and food intake [7–11]. Human MC4R is found to be expressed only in the brain and involved in the Leptin pathway; it contributes to the controlling of human feeding behavior and energy homeostasis [12, 13]. Over the last decade, the intensive efforts targeting MC4R have led to the design of selective, peptidic, non-peptidic molecule for the development of anti-obesity drugs [14–22]. Main strategy to identify small-molecule MC4R agonists is the peptidomimetic approach based on piperazine, cyclohexane, and pyrrolidine core, which represent the important pharmacophores from the His-Phe-Arg-Trp message sequence [23, 24].

In our recent study, we successfully developed two kinds of β-turn-like template (Fig. 1) [25–27], adapting the outstanding result of Garland and Dean [28] which are cluster analysis and recombination of the observed patterns yielded a consensus positioning of the Cα atoms among the various β-turn types. The biological activity of the somatostatin and melanocortin mimic analogues with 7'-alkoxy-2',3-dihydro-1'H-spiro[imidazolidine-4,4'-quinoline]-2,5-dione scaffold **1** were demonstrated, respectively [25,27]. Also, we have developed a solid phase combinatorial library synthesis of peptide-binding GPCR targeted library using 1,4-benzodiazepin-2-one based β-turn mimic scaffold with diverse natural amino acids, and building blocks [26]. Several diverse analogs of 1,4-benzodiazepines have been reported to show activities toward diverse drug targets such as cholecystokinin receptor (CCK), benzodiazepine receptor, neurokinin-1 receptor, κ-secretase, and farnesyl transferase [29]. Herein, we report the construction of expanded peptide-binding GPCR targeted library

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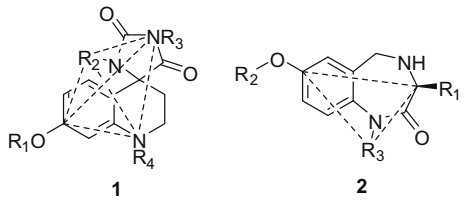


Fig. 1. The β-turn mimic scaffold based on the Garland–Dean triangles.

of 1,4-benzodiazepin-2-one skeleton for the discovery of new agonists by the identification of hit compounds from a screening of the combinatorial library targeting the human MC4R.

2. Materials and methods

Thin-layer chromatography (TLC) was conducted on precoated silica gel plates (Merck silica gel 60; F_{254}, 0.25 mm) and visualized with either short-wave UV light. Starting materials, reagents, and solvents were obtained from Aldrich Chemical Co. (Milwaukee, WI) and used as received unless otherwise noted. PL-FDMP resin was purchased from Polymer Laboratories. Proton nuclear magnetic resonance spectra were collected on a JEOL JNM-LA 300 WB instruments and were recorded in parts per million (ppm) δ values, relative to tetramethylsilane as the internal standard, and spectra were taken in $CDCl_3$. Mass spectroscopy was carried out on VG BIOTECH platform. Parallel solid-phase synthesis was performed on a MiniBlock from Mettler-Toledo Bohdan, Inc. (Vernon Hills, IL). The SPE tube, SAX was purchased from Alltech Associates (Lot No. 2312; Deerfield, IL). Parallel purification was performed on Quad3, Parallel FLASH Purification System, Biotage, Inc. (Charlottesvile, VA).

2.1. Representative synthetic procedure

2.1.1. Preparation of 2,2-dimethyl-propionic acid 3-formyl-4-nitro-phenyl ester (*4*)

Et_3N (7.562 ml, 54.26 mmol) was added to a solution of **3** (9.069 g, 54.26 mmol) in DCM (150 ml), followed by trimethyl acetic chloride (7.34 ml, 59.66 mmol). The reaction was stirred at room temperature for 30 min. The reaction mixture was quenched with saturated aqueous NH_4Cl and extracted with chloroform. The organic phase was separated and dried over Na_2SO_4, then evaporated under reduced pressure. The residue was purified by flash silica gel column chromatography ($CHCl_3$:MeOH = 100:1) to afford **4** (99.5% yield).

^{1}H NMR (300 MHz $CDCl_3$): δ (ppm) 10.44 (s, 1H), 8.10 (d, J = 12 Hz, 1H), 7.25 (s, 1H), 7.07 (d, J = 12 Hz, 1H), 1.336 (s, 9H). MS (ESI) m/z: 252.1 ([M+H]$^+$).

2.1.2. 6-tert-Butoxycarbonylamino-2-[5-(2,2-dimethyl-propionyloxy)-2-nitro-benzylamino]-hexanoic acid methyl ester (*5b*)

L-lysine(Boc) methyl ester hydrochloride salt (13.2 g, 44.57 mmol) was added to a solution of **4** (7 g, 27.86 mmol) in DCE/DMF (70 ml/30 ml). After stirring for 30 min, $NaBH(OAc)_3$ (5.51 g, 26 mmol) was added in the reaction solution and then stirred for 1hr and half at room temperature. The residue obtained was extracted with chloroform and washed well with saturated aqueous $NaHCO_3$. The product was purified by silica gel column chromatography by eluting it with hexane/ethyl acetate (5/1) to afford **5b** (62.8% yield).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.00 (d, J = 9 Hz, 1H), 7.38 (d, J = 2.4 Hz, 1H), 7.11 (dd, J = 9 Hz, J = 2.4 Hz, 1H), 4.61 (bs, NH), 4.03 (ABq, J = 15 Hz, 117.9 Hz, 2H) 3.71 (s, 3H), 3.23 (m, 1H), 3.08 (m, 2H), 1.76–1.37 (m, 6H), 1.42 (s, 9H), 1.38 (s, 9H). MS (ESI) m/z:496.12 ([M+H]$^+$).

2.1.3. 2-[2-Amino-5-(2,2-dimethyl-propionyloxy)-benzylamino]-6-tert-butoxycarbonylamino-hexanoic acid methyl ester (*6b*)

Under supplying hydrogen gas, **5a** (8.47 g, 17.09 mmol) was dissolved in methanol (80 ml) and treated with solid 10% Pd/C (1.5 g). After stirring for 3 h at room temperature, the reaction mixture was filtered through a Celite bed and washed with methanol. The methanol was evaporated, and the product was purified by silica gel column chromatography and eluted with hexane/ethyl acetate (3:1) to afford **6b** (99.3% yield).

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 6.78 (dd, J = 2.7 Hz, 8.5 Hz, 1H), 6.7 (d, J = 2.7 Hz, 1H), 6.62 (d, J = 8.4 Hz, 1H), 4.5 (s, 2H, NH2), 3.64 (ABq, J = 10 Hz, 52.9 Hz, 2H), 3.69 (s, 3H), 3.24 (t, J = 6.6 Hz, 1H), 3.05 (t, J = 5.7 Hz, 2H), 2.04 (s, 3H), 1.79–1.61 (m, 2H), 1.49 (s, 9H), 1.30 (s, 9H), 1.49–1.22 (m, 4H). MS (ESI) m/z: 466.19 ([M+H]$^+$).

2.1.4. 2,2-Dimethyl-propionic acid 3-(4-tert-butoxy carbonylamino-butyl)-2-oxo-2,3,4,5-tetrahydro-1H-benzo[e][1,4]diazepin-7-yl ester (*7b*)

Compound **6a** (6.11 g, 13.12 mmol) was dissolved in toluene (40 ml), and 2 M $AlMe_3$ in toluene (26 ml) was added drop wise for 5 min at 0 °C and stirred for 10 min. The reaction temperature was increased slowly by room temperature and stirred 1½ h. The reaction was quenched with 45 ml of MeOH at 0 °C and stirred more at room temperature. The mixture was partitioned between saturated aqueous $NaHCO_3$ and EtOAc. Before separating the organic layer, the mixture was filtered first. Then the biphasic filtrate was separated and organic phase dried over, filtered, and concentrated. The product was purified by silica gel column chromatography ($CHCl_3$:MeOH = 25:1) to afford **7b** (65.6% yield).

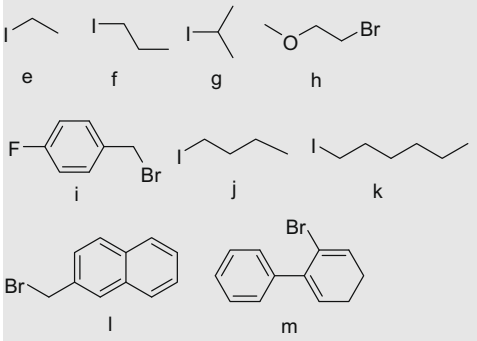
^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.72 (s, NH), 7.01–6.98 (m, 3H), 3.98 (ABq, J = 13.5 Hz, 68.4 Hz, 2H), 3.39 (t, J = 6.6 Hz, 1H), 3.09 (ABq, J = 13.5 Hz, 68.4 Hz, 2H), 1.91–1.76 (m, 2H), 1.87–1.75 (m, 2H), 1.59–1.54 (m, 2H), 1.42 (s, 9H), 1.35 (s, 9H). MS (ESI) m/z: 434.17 ([M+H]$^+$).

2.2. General methods for solid-phase library synthesis

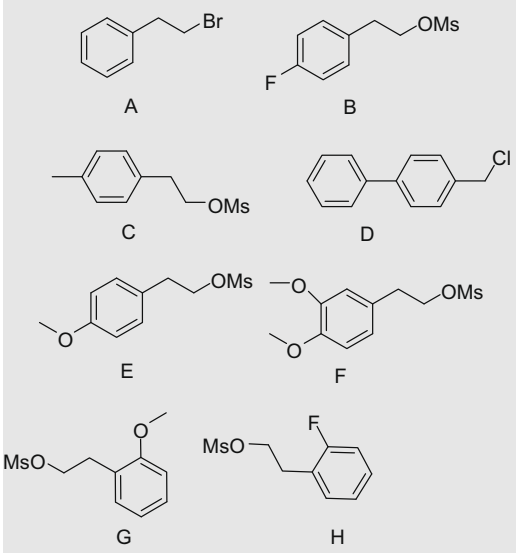
Compounds **13a, 13b'**, and **13c'** can be prepared as previously described for 1,3,4,5-tetrahydrobenzo[*e*][1,4]diazepin-2-one [24]. First, to the **7a, 7b'**, and **7c'** (12.9 mmol) in 1,2-dichloroethane (100 ml) solution was added PL-FDMP resin (1.5 mmol/g, 12.15 mmol) and $NaBH(OAc)_3$ (18.22 mmol) and the reaction mixture was gently stirred for 12 h at room temperature. After the scaffolds disappeared on the TLC, the mixture was filtered and the resin was washed with DMF (3 × 30 ml), DCM (3 × 30 ml), and MeOH (3 × 20 ml). The residue was dried in vacuo to a constant weight (yield: 80.2%). The pivaloyl group of **9a**, **9b**, and **9c** was hydrolyzed to afford free phenolic OH group in 3% KOH solution of dioxane and H_2O (50 ml:50 ml) for 24 h at room temperature. The color of resin changed to yellow-green. The resin was treated as same washing and drying procedure aforementioned. The syntheses were carried out on the solid-phase in two set of 4 × 6 reaction tubes in MiniBlock. Resin-bound benzodiazeipne-2-one **10a**, **10b**, and **10c** (0.168 mmol) distributed in each reaction vessel suspended in 1:1 DMSO/NMP (4 ml) and followed by adding alkyl halide (0.84 mmol) and DBU (0.84 mmol). Reaction mixture was shaken for 24 h at room temperature. After washing the resin with DMF and DCM three times, the reaction was repeated again, and the resin was washed with THF additionally for next N-alkylation reaction. To the resin-loaded O-alkylated benzodiazepine-2-one **11a**, **11b**, and **11c** (0.168 mmol) suspended in THF (3 ml) was added 1 M lithium-tert-butoxide in THF, and the reaction tubes were shaken for 1 h. After removing THF solution, building blocks A–H (Table 1) in DMSO (3 ml) was added to each reaction tube and shaken overnight. The reaction mixture was removed by filtration, and the resins were washed with DMF (4 ml), DCM (4 ml), and MeOH (4 ml) by three

Table 1
Building blocks for the library synthesis.

Building blocks for O-alkylation e-m ($\mathbf{R_2}$)



Building blocks for N-alkylation A-H ($\mathbf{R_3}$)



times. This alkylation was iterated one more time. All the reaction resins were treated with 50% TFA/DCM (3 ml × 2 times) for 2 h, and the solution was collected by filtration and washed with DCM by two times. The solution was evaporated in parallel manner using Genevac DD-4 system, the concentrated products were dissolved in chloroform and passed through the SAX resin to afford the free amine form. Parallel evaporation of the eluents and purification by a Quad3 parallel purification system with an appropriate mixture of Chloroform/MeOH afforded final product **13a**, **13b'**, and **13c'.**

2.3. Solution-phase library synthesis of (S)-7-R_2-10-R_3-10,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]dia zepine-3,11(2H,5H)-diones

Step (i) of Scheme 3. Compound **8** (10.14 mmol) was dissolved in 3% KOH MeOH/Water (8:2) solution and stirred at room temperature for 40 min. Excess KOH was neutralized with 1 N aq. HCl solution, and the resulting reaction mixtures were evaporated to dryness. The residues were washed with pure water, extracted with $CHCl_3$, dried with anhydrous Na_2SO_4, and concentrated to dryness to afford the free phenolic OH **10d** in ~92% yield.

Step (ii) of Scheme 3. The solution of **10d** (0.34 mmol) in DMF (2 ml) was put in wells of 8-channel synthesizer and treated with DBU (0.68 mmol). The mixture was treated with building blocks e–i (Table 1) (0.68 mmol) and shaken for 4 h. When TLC showed that starting material had disappeared, the reaction was quenched with aq. NH_4Cl solution and extracted with $CHCl_3$. All solvent and remaining volatile residues were evaporated in parallel under reduced pressure using a Genevac DD-4 system. The concentrated crude products were dissolved in THF for the next alkylation.

Step (iii) of Scheme 3. To a solution of **11d** (0.09 mmol) in 1 ml of THF was added 1 M lithium-tert-butoxide in THF (0.27 mmol), shaken for 1 h. The reaction mixture were treated with a solution of blocks A–D (Table 1) (0.27 mmol) in 1 ml of DMSO and stirred at room temperature for 9 h. The reaction was quenched with aq. NH_4Cl and extracted with $CHCl_3$. Evaporation of the solvent and purification via silica column chromatography afforded **13d** in ~30–50% yield.

2.4. Representative spectral data: specific examples

2.4.1. (S)-3-benzyl-1-(2-fluorophenethyl)-7-(2-meth oxyethoxy)-4,5-dihydro-1H-benzo[e][1,4]diazepin-2(3H)-one (*13ahH*)

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.26–6.68 (m, 12H), 4.32–4.27 (m, 1H), 4.09–4.06 (m, 2H), 3.85–3.74 (m, 4H), 3.63–3.57 (m, 1H), 3.49–3.44 (m, 1H), 3.45 (s, 3H), 3.20–3.14 (m, 1H), 3.12–2.96 (m, 1H), 2.90–2.82 (m, 2H). MS (ESI) *m*/*z*: 449.20 ([M+H]$^+$).

2.4.2. (S)-3-(4-aminobutyl)-7-ethoxy-1-(4-fluorophen ethyl)-4,5-dihydro-1H-benzo[e][1,4]diazepin-2(3H)-one (*13beB*)

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.14–6.90 (m, 4H), 6.93–6.87 (m, 2H), 6.83 (d, J = 9H, 2H), 4.48–4.38 (m, 1H), 4.04 (q, *J* = 6.9 Hz, 2H), 3.72–3.64 (m, 2H), 3.67 (ABq, *J* = 11.7 Hz, 47.6 Hz, 2H), 3.17 (dd, *J* = 5.4 Hz, 7.8 Hz, 1H), 2.99–2.91 (m, 1H), 2.77–2.71 (m, 1H), 2.65 (t, *J* = 6.9 Hz, 2H), 1.87–1.82 (m, 1H), 1.52–1.45 (m, 1H), 1.43 (t, *J* = 6.9 Hz, 3H), 1.45–1.40 (m, 2H), 1.29–1.21 (m, 2H). MS (ESI) *m*/*z*: 400.13 ([M+H]$^+$).

2.4.3. (S)-1-(biphenyl-4-ylmethyl)-3-(hydroxylmeth yl)-7-propoxy-4,5-dihydro-1H-benzo[e][1,4]diazepin-2(3H)-one (*13cfD*)

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.58–7.49 (m, 5H), 7.46–7.40 (m, 2H), 7.37–7.28 (m, 2H), 7.21 (d, *J* = 9 Hz, 1H), 6.91 (dd, *J* = 2.7 Hz, 9 Hz, 1H), 6.84 (d, *J* = 3H, 2H), 5.28 (d, *J* = 14.7 Hz, 1H), 4.90 (d, *J* = 14.7 Hz, 1H), 4.08 (dd, *J* = 3 Hz, 12 Hz, 1H), 3.715 (ABq, *J* = 11.7 Hz, 34.5 Hz, 2H), 3.71–3.67 (m, 1H), 3.33 (t, J = 3.3 Hz, 1H), 1.82 (tq, *J* = 6.6 Hz, 7.2 Hz, 2H), 0.94 (t, *J* = 7.2 Hz, 3H). MS (ESI) *m*/*z*: 417.01 ([M+H]$^+$).

2.4.4. (S)-7-(ethoxymethyl)-10-(4-methylphenethyl)-10,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-3,11(2H,5H)-dione (*13dhC*)

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.25–7.16 (m, 5H), 7.09 (d, *J* = 8.1 Hz, 1H), 6.94 (dd, *J* = 3 Hz, 8.1 Hz, 1H), 6.80 (d, *J* = 3 Hz, 1H), 4.75–4.58 (m, 2H), 4.06 (ABq, *J* = 13.8 Hz, 288 Hz, 2H), 4.04 (q, *J* = 6.9 Hz, 2H), 3.96 (t, *J* = 7.5 Hz, 2H), 3.74–3.67 (m, 1H), 2.88–2.79 (m, 2H), 2.71–2.61 (m, 1H), 2.59–2.47 (m, 1H), 2.44–2.35 (m, 1H), 2.30 (s, 3H), 2.0–1.89 (m, 1H), 1.41 (t, *J* = 6.9 Hz, 3H). MS (ESI) *m*/*z*: 393.12 ([M+H]$^+$).

2.4.5. (S)-7-ethoxy-10-phenethyl-10,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-3,11(2H,5H)-dione (*13deA*)

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.25–7.16 (m, 5H), 7.09 (d, *J* = 8.1 Hz, 1H), 6.94 (dd, *J* = 3 Hz, 8.1 Hz, 1H), 6.80 (d, J = 3 Hz, 1H), 4.20 (m, 2H), 4.06 (ABq, *J* = 13.8 Hz, 288 Hz, 2H), 4.04 (q, *J* = 6.9 Hz, 2H), 3.96 (t, *J* = 7.5 Hz, 1H), 2.85 (m, 2H), 2.21 (m, 2H), 1.41 (t, *J* = 6.9 Hz, 3H). MS (ESI) *m*/*z*: 365.21 ([M+H]$^+$).

2.4.6. (S)-10-phenethyl-7-propoxy-10,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-3,11(2H,5H)-dione (*13dfA*)

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.31–7.08 (m, 6H), 6.94 (dd, *J* = 3 Hz, 4.3 Hz, 1H), 6.81 (d, *J* = 3 Hz, 1H), 4.35 (m, 2H), 4.07 (ABq,

J = 13.5 Hz, 301 Hz, 2H), 3.99–3.90 (m, 3H), 2.88 (m, 2H), 2.41 (m, 2H), 2.32 (m, 2H), 1.82 (m, 2H), 1.04 (t, J = 7.5 Hz, 3H). MS (ESI) m/z: 379.12 ($[M+H]^+$).

*2.4.7. (S)-10-(4-fluorophenethyl)-7-propoxy-10,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-3,11(2H,5H)-dione (**13dfB**)*

^{1}H NMR (300 MHz, CDCl3) δ (ppm) 7.31–7.08 (m, 5H), 6.94 (dd, J = 3 Hz, 4.3 Hz, 1H), 6.81 (d, J = 3 Hz, 1H), 4.35 (m, 2H), 4.07 (ABq, J = 13.5 Hz, 288 Hz, 2H), 3.99–3.90 (m, 3H), 2.88 (m, 2H), 2.41 (m, 2H), 2.32 (m, 2H), 1.82 (m, 2H), 1.04 (t, J = 7.5 Hz, 3H). MS (ESI) m/z: 397.06 ($[M+H]^+$).

*2.4.8. (S)-10-(4-methylphenethyl)-7-propoxy-10,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-3,11(2H,5H)-dione (**13dfC**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.31–7.08 (m, 5H), 6.94 (dd, J = 3 Hz, 4.3 Hz, 1H), 6.81 (d, J = 3 Hz, 1H), 4.35 (m, 2H), 4.07 (ABq, J = 13.5 Hz, 289 Hz, 2H), 3.99–3.90 (m, 3H), 2.88 (m, 2H), 2.41 (m, 2H), 2.32 (m, 2H), 2.30 (s, 3H), 1.82 (m, 2H), 1.04 (t, J = 7.5 Hz, 3H). MS (ESI) m/z: 393.14 ($[M+H]^+$).

*2.4.9. (S)-7-(4-fluorobenzyloxy)-10-(4-fluorophen ethyl)-10,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-3,11(2H,5H)-dione (**13diB**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.41–7.36 (m,2H), 7.25–6.86 (m, 8H), 5.01 (s,2H), 4.35 (m, 2H), 4.07 (ABq, J = 13.5 Hz, 289 Hz, 2H), 3.99–3.90 (m, 3H), 2.88 (m, 2H), 2.41 (m, 2H), 2.32 (m, 2H), 1.82 (m, 2H), 1.04 (t, J = 7.5 Hz, 3H). MS (ESI) m/z: 463.11 ($[M+H]^+$).

*2.4.10. (S)-7-isopropoxy-10-phenethyl-10,11a-dihydro-1H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-3,11(2H,5H)-dione (**13dgA**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.27–7.10 (m, 6H), 6.94 (dd, J = 3 Hz, J = 4.3 Hz, 1H), 6.81 (d, J = 3 Hz, 1H), 4.48 (m, 1H), 4.20 (m, 2H), 4.18 (ABq, J = 13.5 Hz, 315 Hz, 2H), 3.99 (t, J = 6 Hz, 1H), 2.88 (m, 2H), 2.44 (m, 2H), 2.31 (m, 2H), 1.38 (s, J = 2.1 Hz,3H), 1.36 (s, J = 2.1 Hz, 3H). MS (ESI) m/z: 379.09 ($[M+H]^+$).

*2.4.11. (S)-3-(4-aminobutyl)-7-ethoxy-1-phenethyl-4,5-dihydro-1H-benzo[e][1,4]diazepin-2(3H)-one (**13beA**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.48–7.14 (m, 5H), 7.03 (d, J = 4.5 Hz, 1H), 6.88 (dd, J = 1.5 Hz, 4.5 Hz, 1H), 6.81 (d, J = 1.5H, 1H), 4.05 (q, J = 6.9 Hz, 2H), 3.72 (m, 2H), 3.67 (ABq, J = 11.7 Hz, 41.7 Hz, 2H), 3.17 (t, J = 5.7 Hz, 1H), 2.77 (m, 2H), 2.65 (t, J = 6.9 Hz, 2H), 1.85 (m, 2H), 1.55–1.21 (m, 4H), 1.43 (t, J = 6.6 Hz, 3H), 1.31 (m, 2H). MS (MALDI), m/z: 380.2 ($[M+H]^-$).

*2.4.12. (S)-3-(4-aminobutyl)-1-(4-fluorophenethyl)-7-isopropoxy-4,5-dihydro-1H-benzo[e][1,4]diazepin-2(3H)-one (**13bgB**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.20–6.81 (m, 7H), 4.48 (m, 1H), 3.72 (m, 2H), 3.67 (ABq, J = 11.7 Hz, 41.6 Hz, 2H), 3.17 (t, J = 5.7 Hz, 1H), 2.77 (m, 2H), 2.65 (t, J = 6.9 Hz, 2H), 1.85 (m, 2H), 1.38 (s, J = 2.1 Hz,3H), 1.36 (s, J = 2.1 Hz, 3H). MS (ESI) m/z: 414.22 ($[M+H]^+$).

*2.4.13. (S)-3-(4-aminobutyl)-7-(hexyloxy)-1-phenethyl-4,5-dihydro-1H-benzo[e][1,4]diazepin-2(3H)-one (**13bkA**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.36–7.14 (m, 5H), 7.03 (d, J = 8.4 Hz, 1H), 6.88 (dd, J = 3 Hz, 8.4 Hz, 1H), 6.80 (m, J = 3H, 1H), 3.96 (t, J = 6.3 Hz, 2H), 3.72 (m, 2H),3.69 (ABq, J = 11.4 Hz, 43.5 Hz, 2H), 3.17 (t, J = 5.7 Hz, 1H), 2.77 (m, 2H), 2.65 (t, J = 6.9 Hz, 2H), 1.9 4–1.75 (m, 4H), 1.49–1.14 (m, 10H), 0.91 (t, J = 6.6 Hz, 3H). MS (ESI) m/z: 438.12 ($[M+H]^+$).

*2.4.14. (S)-3-(4-aminobutyl)-1-(biphenyl-4-ylmethyl)-7-isopropoxy-4,5-dihydro-1H-benzo[e][1,4]diazepin-2(3H)-one (**13bgD**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 7.53–7.23 (m, 9H), 7.07 (d, J = 8.4 Hz, 1H), 6.81 (dd, J = 2.7 Hz, 8.4 Hz, 1H), 6.69 (d, J = 2.7 Hz, 1H), 4.97 (ABq, J = 14.7 Hz, 119 Hz, 2H), 3.94 (m, 1H), 3.52 (ABq, J = 11.7 Hz, 48.3 Hz, 2H), 3.17 (t, J = 5.7 Hz, 1H), 2.84 (t, J = 6.9 Hz, 2H), 1.85 (m, 2H), 1.64 (m, 2H), 1.38 (s, J = 2.1 Hz, 3H), 1.36 (s, J = 2.1 Hz, 3H) 1.18 (m, 2H). MS (ESI) m/z: 458.16 ($[M+H]^+$).

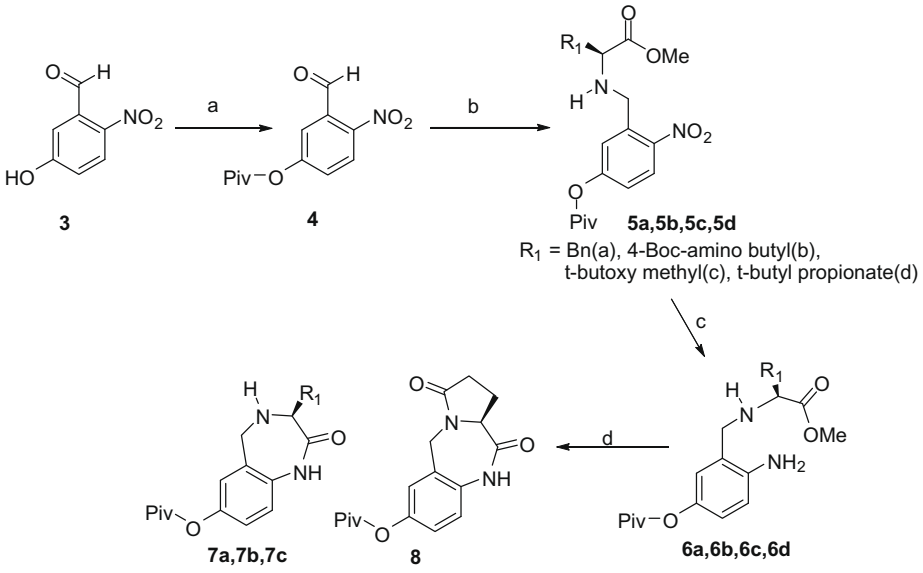
2.5. Functional assay of the melanocortin 4 receptors

The MC4R agonist assay is a whole cell transcriptional assay that detects the production of the β-lactamase enzyme induced by agonist stimulation of the MC4 GPCR. The assay consists of an engineered clonal Chinese Hamster Ovary (CHO-K1) cell line that has been transfected with plasmids that allow for the expression of the receptor and an adenylate cyclase responsive reporter element (CRE) coupled to the coding sequence of the β-lactamase enzyme. The transfected cells were plated at 5000 cells per well in a 96-well plate (black, clear bottom) in 50 μl per well of DMEM supplemented with 10% fetal bovine serum. These cells were allowed to adhere to the plate. The next day the media was removed from the cells and replaced with serum-free DMEM media. After 12 h of serum starvation, media was aspirated, and then 25 μl of DMEM was added. The library compounds were distributed to the each well at 10 μM final concentration in DMEM media. Responses were compared to the effect of α-MSH and only DMEM as positive and negative control, respectively. After plates were incubated for 4 h at 37 °C in a 5% CO_2, 7 μl of CCF2 dye stock prepared per the manufacturer's protocol was then added to each well. Plates were then incubated for 1 h at room temperature, and the fluorescence was determined on a BMG polarstar reader with bottom reading optics.

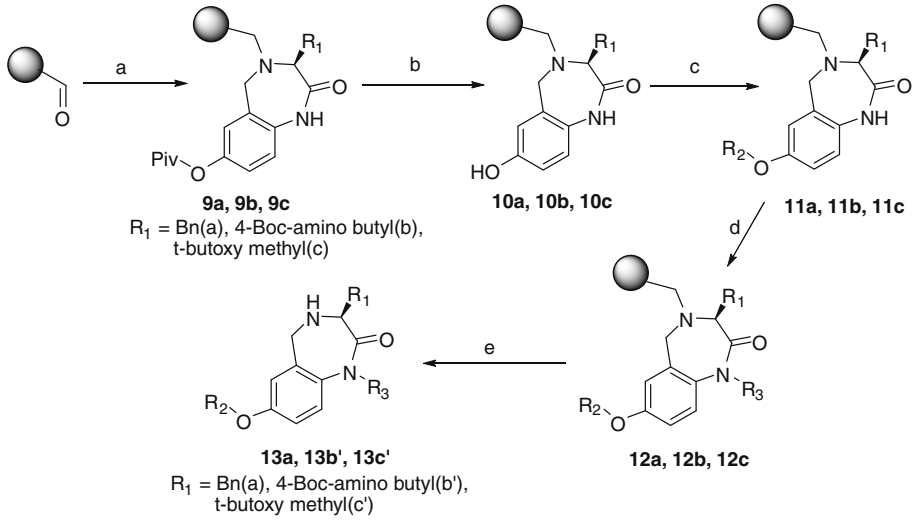
3. Results and discussion

The synthesis of benzodiazepine library was summarized in Schemes 1 and 2. Hydroxyl group of 5-hydroxy-2-nitro-benzaldehyde (3) was first protected with trimethylacetyl chloride in CH_2Cl_2. Four amino acid esters, phenylalanine methyl ester **a**, lysine methyl ester **b**, serine methyl ester **c**, and glutamate methyl ester **d**, were used to form secondary amine unit of the benzodiazepine skeleton with reductive alkylation reactions using $NaBH(OAc)_3$. For the cyclization, the nitro group of compound **5a**–**d** was reduced to amine in a condition of catalytic hydrogenation and subsequent intramolecular cyclization with $AlMe_3$ in toluene gave the skeleton of tetrahydro-1,4-benzodiazepin-2-one **7a**–**c** and **8** as the key scaffold for solid and solution phase library synthesis (Scheme 1).

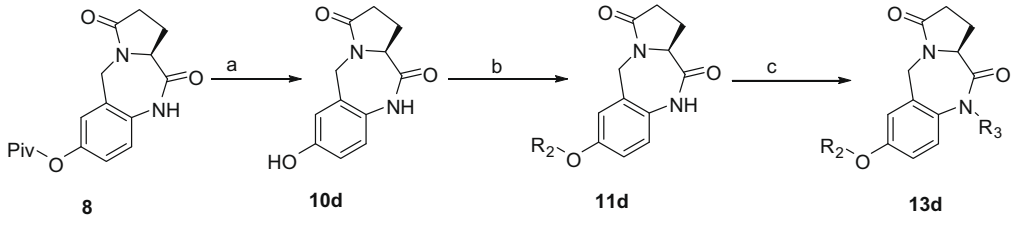
For the efficient and fast library synthesis, Phe **7a**, Lys **7b'**, and Ser-scaffold **7c'** were loaded on the 4-formyl-3,5-dimethoxyphenoxy (PL-FDMP) resin by reductive alkylation with high yield (>80%). To incorporate R_2 building blocks at the phenolic oxygen, the pivaloyl group of **9a**–**c** was first hydrolyzed in 3% KOH in dioxane/H_2O (1:1) and resulting **10a**–**c** were treated with 9 alkyl halides (Table 1) in the presence of DBU as a base in the mixture of DMSO/NMP (1:1), respectively (Scheme 2). Each resin-bound O-substituted benzodiazepine-2-one **9a**–**c** were treated with 1 M lithium-tert-butoxide in THF, and after wash, subsequently reacted with 8 alkyl halide as the R_3 building blocks (Table 1) in DMSO. To cleave the final product out of each resin, 50% TFA/ CH_2Cl_2 (3 ml) was treated for 2 h, and the filtrate was collected, evaporated, passed through SAX resin to remove TFA in a parallel fashion. In the case of glutamate scaffold **8**, the t-butyl protective group was cleaved by $AlMe_3$ during cyclization process, forming pyrrolidinone structure. Thus, parallel solution phase synthesis was applied using eight channel parallel synthesizers for the introduction of limited number of R_2 and R_3 groups to afford 24 compounds (Scheme 3). After each step of parallel



Scheme 1. Preparation of four tetrahydro-1,4-benzodiazepine-2-one scaffolds. (a) trimethylacetyl chloride, TEA, DCM, 30 min RT. (b) Amino acid ester, $NaBH(OAc)_3$, DCE/DMF, 2 h, RT. (c) Pd/C, H_2, MeOH, 3 h, RT. (d) $AlMe_3$, toluene, 1 h, 0–25 °C.



Scheme 2. Solid phase synthetic route of tetrahydro-1,4-benzodiazepine-2-one library. (a) PL-FDMP resin, **7a** (**7b** or **7c**), $NaBH(OAc)_3$, DCE, 12 h, RT. (b) 5% KOH dioxane/H_2O (1:1) 24 h, RT. (c) R_2–X, DBU, NMP/DMSO, 48 h, RT. (d) i, LiOtBu in THF, 1 h, RT. ii, R_3–X in DMSO 24 h, RT. (e) TFA, DCM.

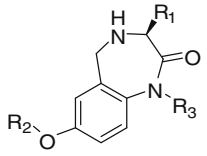


Scheme 3. Solution phase synthetic route of tetrahydro-1,4-benzodiazepine-2-one library. (a) 5% KOH dioxane/H_2O, 40 min (1:1) (b) R_2–X, DBU, NMP/DMSO, 4 h, RT (c) i, LiOtBu in THF; ii, R_3–X in DMSO, 10 h, RT.

procedure including extraction, washing and evaporation, all products were purified by Quad3™ parallel purification system with chloroform/methanol elution condition to give 162 final library compounds. The purified products were characterized randomly by ^1H NMR and ESI mass.

All the compounds described here were evaluated for cellular activity using a whole cell transcriptional assay that detects the production of the beta lactamase enzyme induced by agonist stimulation of the MC4 G-protein coupled receptor using a publishing procedure [25]. In the assay, every compound was tested in three

Table 2
Biological evaluation of the benzodiazepine derivatives.



13

Compounds	R_1	R_2	R_3	% Activity @ 10 μM	EC_{50} (μM)
13beC	4-Amino-butyl	Ethyl	4-Methyl-phenethyl	27	n.d.[a]
13bfD	4-Amino-butyl	Propyl	4-Phenyl-benzyl	23	n.d.
13ahH	Bn	2-Methoxy-ethyl	2-Fluoro-phenethyl	27	n.d.
13alC	Bn	2-Methyl-naphtalene	4-Methyl-phenethyl	53	20.0
13afC	Bn	propyl	4-Methyl-phenethyl	75	17.0
13amG	Bn	3-Phenyl-benzyl	2-Methoxy-benzyl	26	n.d.
13aiE	Bn	4-Fluoro-benzyl	4-Methoxy-phenethyl	88	13.0

[a] n.d., not determined.

times. Only the compounds showing over 50% activation at 10 μM concentration were determined in EC_{50}. The results are listed in Table 2. Some of these compounds showed micromolar activity including compounds **13alC** (20.0 μM), **13afC** (17.0 μM), and **13aiE** (13.0 μM), for MC4R. The benzyl group at R_1 position showed better agonistic activity than others groups. Relatively small groups are more tolerated at the R_2 position, as shown by the comparison between compounds **13afC** and **13ahC** (2-methyl-naphtalene vs. propyl) and **13alC** and **13aiE** (2-methyl-naphtalene vs. 4-fluoro-benzyl). A 4-methoxy-phenethyl group at R_3 position displayed improved agonistic activity than 4-methyl-phenethyl group. The results of screening and conformational analysis supported that the rationale of designing β-turn mimic non-peptide small molecules using tetrahydro-1,4-benzodiazepin-2-one scaffold could be valid.

4. Conclusions

A 162-member compound library was synthesized from tetrahydro-1,4-benzodizepin-2-one by an efficient solid and solution phase synthetic route with three major points of diversity. We present verification of our designing rationale, β-turn mimic approach, with a screening of peptide-binding GPCR targeted library of tetrahydro-1,4-benzodizepin-2-one derivatives at MC4R. Based on the results in this study, progress on the increase of agonist potency by the introduction of key amino acid moiety of α-melanocyte stimulating hormone (α-MSH) and selectivity against subtype of MCR family is presently underway.

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