Practical synthesis of potential endothelin receptor antagonists of 1,4-benzodiazepine-2,5-dione derivatives bearing substituents at the C_3-, N_1- and N_4-positions†

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The expedient synthesis of various 1,4-benzodiazepine-2,5-dione compounds, particularly those having substituents at the C_3-, N_1- and N_4-positions is achieved. The important features in these synthetic strategies include: (i) using the coupling reaction of isatoic anhydride with α-amino ester for direct construction of the core structure of 1,4-benzodiazepine-2,5-dione; (ii) using potassium carbonate as the base of choice for selective alkylation at the N_1-site, while using lithiated 2-ethylacetanilide as the required base to furnish the N_4-alkylation; and (iii) using 2-nitrobenzoyl chloride as a synthetic equivalent of anthranilic acid to facilitate the polyethylene resin-bound liquid-phase combinatorial synthesis. The prepared 1,4-benzodiazepine-2,5-dione compounds are evaluated for endothelin receptor antagonism by a functional assay that measures the inhibitory activity against the change of intramolecular calcium ion concentration induced by endothelin-1. The preliminary results indicate that 1,4-benzodiazepine-2,5-diones bearing two flanked aryl substituents at the N_1- and N_4-sites show better inhibitory activity than the corresponding unalkylated and *N*-monoalkylated compounds. A promising candidate, 1-benzyl-7-chloro-3-isopropyl-4-(3-methoxybenzyl)-1,4-benzodiazepine-2,5-dione (**17b**), exhibits an IC_{50} value in low nM range.

Introduction

Since the first discovery in the mid 1950s,[1] many benzodiazepine derivatives have been developed to show potent pharmacological activities,[2] such as in anxiolytics, anticonvulsants, anti-HIV agents, and the drugs active to the central nervous system. The benzodiazepine scaffold is considered to be one of the most important structures in drug discovery due to its high functional group diversity.[3] For example, 1,4-benzodiazepine-2,5-dione and its analogues exhibit remarkable potency in various biological targets, including antithrombotics, antibiotics and antitumor activities.[4] Furthermore, 1,4-benzodiazepine peptidomimetics are employed as enzyme inhibitors[5] and as the ligands to bind with various G-protein coupled receptors.[6]

Endothelins are a group of isopeptides locally produced in various cell types under different physiological stimuli. Human endothelin-1 (ET-1) is a 21-amino-acid peptide that exhibits a potent vasoconstrictor activity, conceivably through its selective interaction with specific receptor ET_A.[7] In recent studies, a series of compounds having planar core structures flanked by two aryl side-arms have been explored as effective endothelin receptor antagonists.[8] The structures of some representative antagonists (Fig. 1) include an indan-type compound SB209670,[9] an indole-type compound PD159433,[10] a pyrazole-type compound HMR865,[11] and a carbazolothiophene-type compound JMF222.[12]

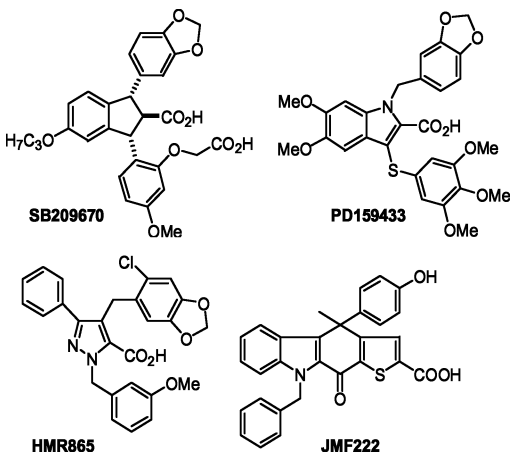

Fig. 1 Examples of non-peptide endothelin receptor antagonists.

In comparison with the low-energy conformations of ET-1 derived by ^{1}H NMR analysis, the calculated 3-dimensional structure of SB209670 suggests that the two phenyl substituents may mimic the Tyr-13 and Phe-14 residues in ET-1 (Fig. 2).[9a] The two carboxyl groups in SB209670 may also mimic the Asp-18 residue and the *C*-terminus of ET-1, which ligate a Zn^{2+} ion on binding with the endothelin receptor. This molecular modeling experiment thus provides useful information for the selection of specific side chains on a flat core structure for the subsequent exploration of other endothelin receptor antagonists.

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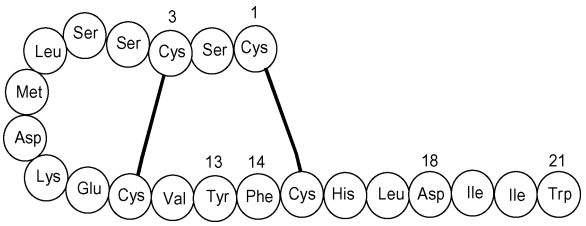


Fig. 2 Structure of human endothelin-1.

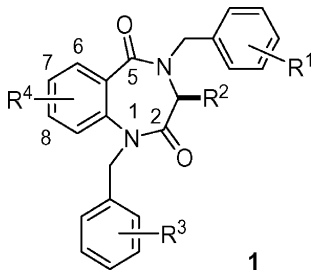


Fig. 3 1,4-Benzodiazepine-2,5-dione derivatives **1** with a nearly planar core ring structure flanked by two aryl side-arms are designed to evaluate their activities in endothelin receptor antagonism.

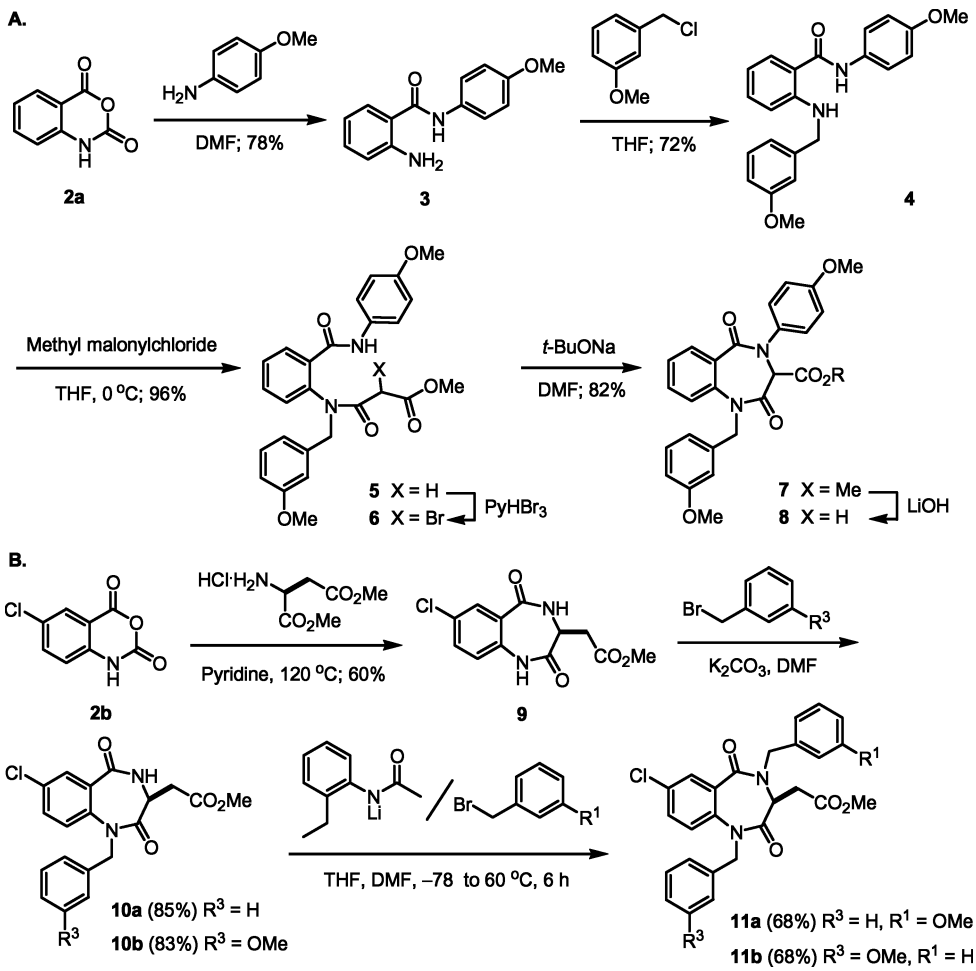
We speculated that 1,4-benzodiazepine-2,5-dione derivatives **1** bearing appropriate substituents (Fig. 3) might also function as endothelin receptor antagonists, because the benzodiazepine structure consists of a nearly planar core platform similar to the indan ring in SB209670. For a better binding affinity toward endothelin receptors, various aryl substituents might be implanted on the N_1- and N_4-positions, and carboxyl groups might be introduced at the desired sites on the benzodiazepine scaffold.

To construct the main framework of 1,4-benzodiazepine-2,5-dione, two general methods have been applied.[13,14] One method utilizes the condensation reaction of an α-amino acid with 2-aminobenzoic acid (anthranilic acid), in which the amino group can be used in the protected or latent forms, *e.g.*, NHFmoc, azido and nitro groups.[13] The other method utilizes the Ugi four-component reaction of anthranilic acid with aldehyde, amine and isocyanate.[14] Although various 1,4-benzodiazepine-2,5-dione derivatives can be prepared by the above-mentioned methods,[13,14] difficulties may be encountered in placing specific side chains and carboxyl groups at the N_1-, N_4- and C_3-positions. We report herein the synthetic approaches to circumvent this problem.

Results and discussion

Synthesis of 1,4-benzodiazepine-2,5-dione compounds

In our first approach (Scheme 1, A), isatoic anhydride is used as the activated derivative of anthranilic acid.[15,16] The reaction



Scheme 1 Approaches using isatoic anhydrides for the synthesis of 1,4-benzodiazepine-2,5-dione derivatives that bear carboxyl group and aryl substituents at the C_3-, N_1-, and N_4-positions.

of isatoic anhydride (**2a**) with *p*-methoxyaniline gave amide **3**, which was subsequently alkylated with 3-methoxybenzyl chloride to afford **4** in reasonable yield. Compound **4** was subjected to amidation with methyl malonyl chloride, giving **5**, which was treated with pyridinium tribromide to introduce a bromine atom at the methylene carbon. The product **6**, without isolation, was treated with two equivalents of sodium *tert*-butoxide to effect an intramolecular alkylation reaction, forming 1,4-benzodiazepine-2,5-dione **7** in 82% yield (from **5**). Though ester **7** and the corresponding acid **8** are obtained with the desired C_3-, N_1- and N_4-substituents on the core structure of 1,4-benzodiazepine-2,5-dione, this approach lacks flexibility in tuning the length of the carboxyl substituent.

In another approach (Scheme 1, B), isatoic anhydride was reacted with an amino acid to construct the skeleton of 1,4-benzodiazepine-2,5-dione in a direct manner. The C_3-substituent can be varied by using different α-amino acids. For example, 5-chloroisatoic anhydride (**2b**) was heated with the hydrochloride salt of L-aspartic acid dimethyl ester in pyridine to afford the desired 1,4-benzodiazepine-2,5-dione **9** in 60% yield. Selective alkylation at the N_1-position was realized by using K_2CO_3 as the required base. Thus, the reaction of **9** with benzyl bromide (1 equiv.) was promoted by using K_2CO_3 (1 equiv.) in DMF solution to give exclusively the N_1-alkylation product **10a** in 85% yield. A similar reaction of **9** with *m*-methoxybenzyl bromide (K_2CO_3, DMF) also occurred selectively at the N_1-site, giving the monoalkylation product **10b** in 83% yield. The structure of the N_1-alkylation products were characterized by ^{1}H NMR analyses. For example, compound **9** in DMSO-d_6 solution showed the N_1–H as a singlet at δ 10.59 and the N_4–H as a doublet (J = 5.6 Hz) at δ 8.71. The monoalkylation product **10a** in $CDCl_3$ solution exhibited the N_4–H signal as a doublet (J = 5.6 Hz) at δ 8.18, but no signal for N_1–H. The regioselectivity in the alkylation might be attributable to a selective removal of the more acidic N_1–H, in comparison with N_4–H, by the weak base K_2CO_3.[17]

A novel base was generated by lithiation of 2-ethylacetanilide with BuLi in THF (−78 °C, 1 h), similar to that reported by Ellman.[13a,b,18] This base was successfully utilized to effect the alkylations of **10a,b** at the N_4-positions, giving **11a,b**, without complication of *O*-alkylations.[17,18] Therefore, this method demonstrates a feasible approach to various 1,4-benzodiazepine-2,5-dione compounds bearing C_3-, N_1- and N_4-substituents.

The combinatorial or parallel synthetic approaches are especially useful in constructing a molecular library for the study of structure–activity relationship.[19] A combinatorial approach to the synthesis of 1,4-benzodiazepine-2,5-diones was designed to incorporate four units of 2-nitrobenzoic acid, benzaldehyde, benzyl halide and α-amino acid (Fig. 4). To test this approach, 2-nitrobenzoyl chloride was used as an equivalent of anthranilic acid in the solution-phase synthesis of 1,4-benzodiazepine-2,5-diones (Scheme 2, A).

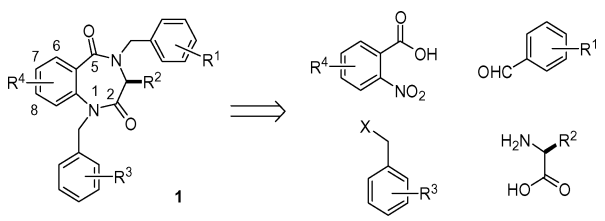


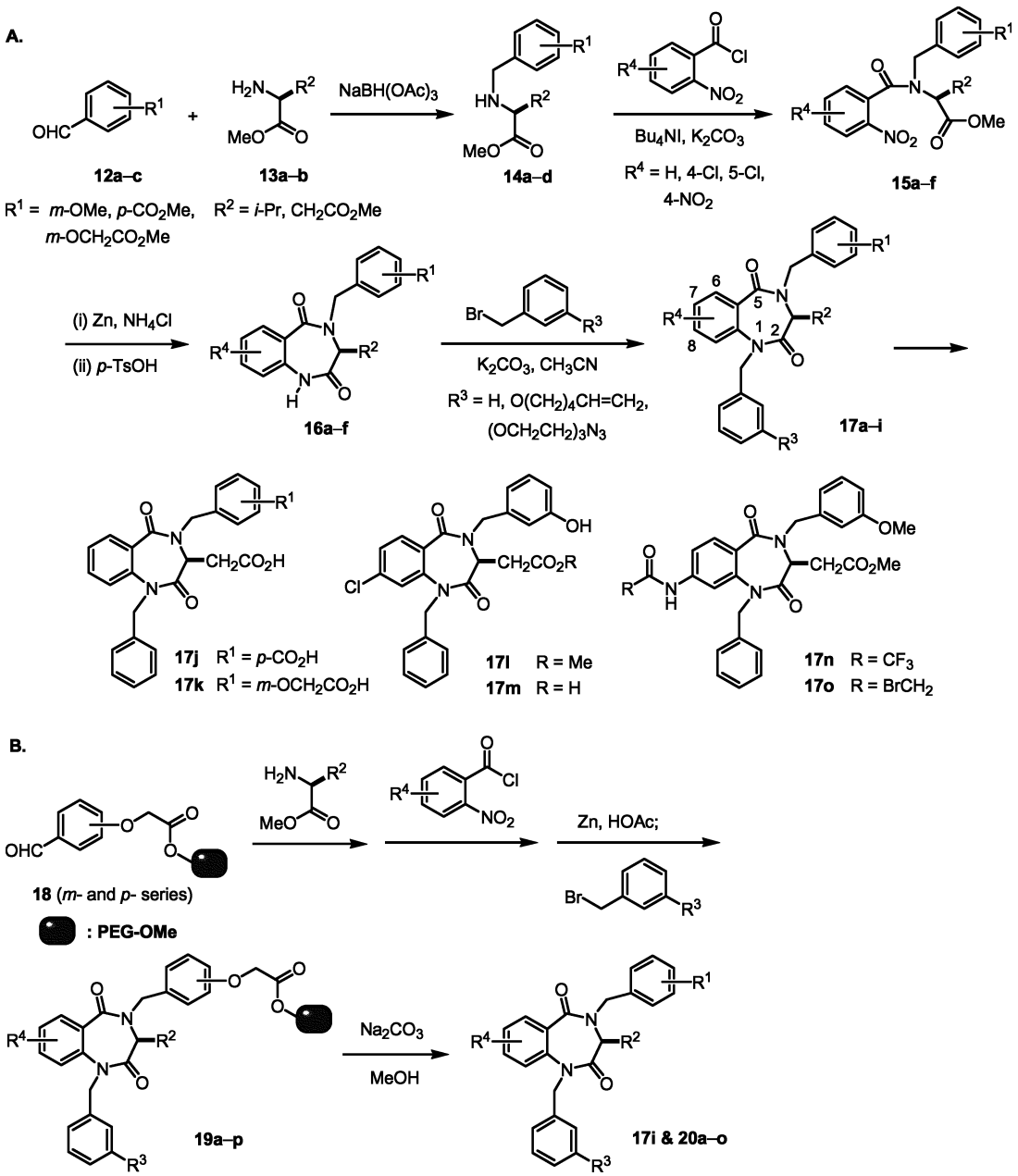
Fig. 4 A combinatorial approach to the synthesis of 1,4-benzodiazepine-2,5-diones.

Thus, the (substituted)benzaldehydes **12a–c** were coupled with α-amino acid derivatives, *e.g.*, the esters of L-valine and L-aspartic acid, *via* reductive amination reactions using $NaBH(OAc)_3$ as the reducing agent. The resulting amines **14a–d** were then reacted with 2-nitrobenzoyl chloride (or its analogs) to afford amides **15a–f**. The nitro groups in **15a–f** were smoothly reduced by zinc powder, and the resulting anilines, without isolation, cyclized on treatment with *p*-toluenesulfonic acid to give 1,4-benzodiazepine-2,5-diones **16a–f** in 75–90% yields. In this process, formation of the core structure of 1,4-benzodiazepine-2,5-dione was facilitated because the tertiary amide moiety existed preferably in the C–N *cis* conformation to render the cyclization reaction.[20] Alkylation of **16a–f** with benzyl bromides was conducted in a dipolar solvent (CH_3CN) using K_2CO_3 as the base to give the desired products **17a–i**. Elaboration of **17a–i** was feasible. For example, diesters **17a** (R^1 = *p*-CO_2Me, R^2 = CH_2CO_2Me, R^3 = R^4 = H) and **17i** (R^1 = *m*-OCH_2CO_2Me, R^2 = CH_2CO_2Me, R^3 = R^4 = H) were hydrolyzed by LiOH to give the corresponding diacids **17j** and **17k**, respectively. When **17c** (R^1 = *m*-OMe, R^2 = CH_2CO_2Me, R^3 = H, R^4 = 8-Cl) was treated with BBr_3, a phenol **17l** (47%) was obtained by demethylation of the anisole moiety, along with an acid **17m** (30%) derived from a simultaneous removal of the methyl group on the ester moiety. Aniline **17d** (R^1 = *m*-OMe, R^2 = CH_2CO_2Me, R^3 = H, R^4 = 8-NH_2) was subjected to amidation with trifluoroacetic anhydride and bromoacetyl bromide to give **17n** and **17o**, respectively.

Upon the successful solution-phase synthesis of 1,4-benzodiazepine-2,5-diones, we also explored a liquid-phase synthesis using PEG_{5000} monomethyl ether as the support to construct a library of 1,4-benzodiazepine-2,5-diones (**17i** and **20a–o**).[21] As the target 1,4-benzodiazepine-2,5-dione requires an N_4-aryl substituent, we deliberately used the PEG-bound benzaldehydes **18** to couple with the esters of α-amino acids (Scheme 2, B). By a similar reaction sequence to that shown in the solution-phase synthesis, the desired 1,4-benzodiazepine-2,5-diones **19a–p** bound to a PEG support were prepared. Removal of the PEG support with concurrent formation of the methyl ester was realized by stirring with Na_2CO_3 in MeOH at room temperature for a short time (<5 min). Thus, sixteen 1,4-benzodiazepine-2,5-dione dimethyl ester derivatives were obtained in 80–99% crude yields by a liquid-phase combinatorial synthesis. The HPLC analyses indicated that the purity was around 53–77% (corresponding to 90–96% average yield in each synthetic step). The final products **17i** and **20a–o** were simply purified by silica gel chromatography, and fully characterized by physical and spectroscopic methods.

Preliminary functional bioassay

The interaction between endothelin receptor and its ligand, *e.g.*, ET-1, is coupled with the activation of a G-protein. This interaction triggers a series of biological events to induce an increase of intracellular calcium concentration, $[Ca^{2+}]_i$.[22] The measurement of inhibitory potency against the ET-1 induced $[Ca^{2+}]_i$ change thus provides a functional assay for preliminary evaluation of endothelin receptor antagonists. According to the



Scheme 2 Approach using 2-nitrobenzoyl chloride as the equivalent of anthranilic acid for the synthesis of 1,4-benzodiazepine-2,5-dione derivatives: (A) solution-phase synthesis, and (B) liquid-phase synthesis.

reported experimental protocol,[23] Chinese hamster ovary (CHO–K1) cells were transfected with the rat ET_A-expression plasmid DNA using a lipofectin reagent.[24] The ET_A overexpression CHO–K1 cells were prior incubated with calcium chelating agent fura-2 applied as its penta(acetoxymethyl) ester,[24] and then treated with ET-1 at 10^{-7} M. The $[Ca^{2+}]_i$ increase was monitored at 510 nm fluorescence emission by a ratiometric method using dual excitations at 340 and 380 nm wavelengths.[25] This increment of $[Ca^{2+}]_i$ was taken as the standard value (100%) to assess the inhibitory potency of the test compounds against the ET-1 binding with receptor (Fig. 5).

The 1,4-benzodiazepine-2,5-dione compounds **11a**, **17b** and **17n** (Fig. 6) showed high antagonistic activity against ET-1 according to the functional assays. In comparison, these endothelin receptor antagonists are more potent than a cyclopentapeptide inhibitor BQ123 [cyclo(D-Trp-D-Asp-Pro-D-Val-Leu)],[26] which showed only ~60% inhibition at 10^{-6} M in our functional assay. Compound **17b** showed an IC_{50} value of slightly lower than 10 nM; however, SB209670 is an even more effective antagonist showing ~85% inhibition at 10 nM under similar assay conditions. Unalkylated and *N*-monoalkylated 1,4-benzodiazepine-2,5-diones, *e.g.* **9** and **10b**, are less active than the corresponding N_1,N_4-dialkylated compound **11a**. This trend of inhibitory activity appeared to be in agreement with the previous prediction on the need of two flanked aryl substituents for the endothelin receptor antagonism.[9a]

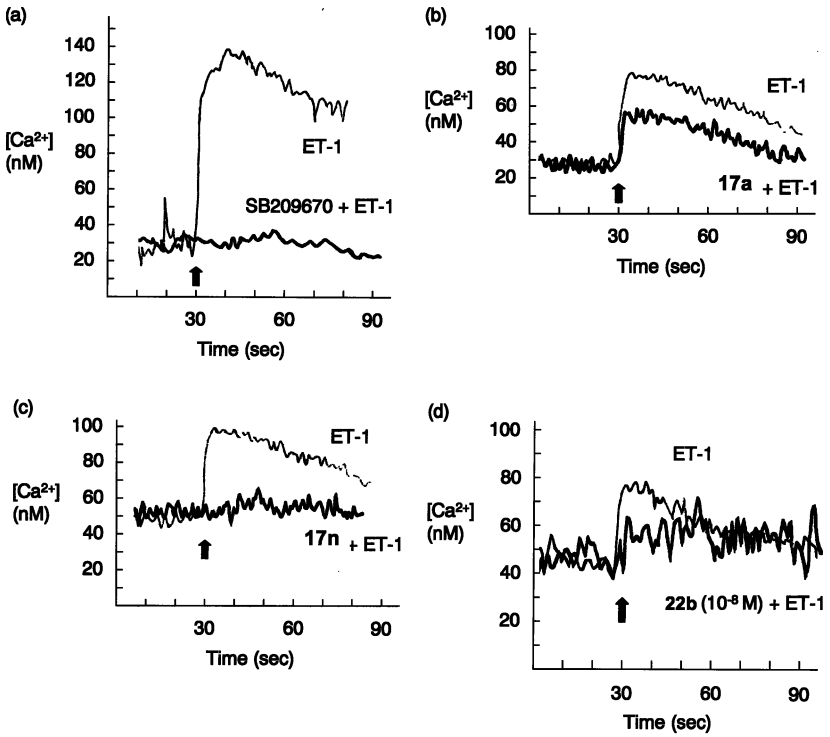


Fig. 5 Fluorescence measurements of intracellular calcium concentration ($[Ca^{2+}]_i$) by addition of test samples (as shown by the arrow) to the ET_A-overexpressing CHO cells in the presence of fura-2. The induced $[Ca^{2+}]_i$ change is taken as a measure of antagonist potency against ET-1, shown in thin lines. (a) Treatment with a mixture of SB209670 (10^{-6} M, 100% inhibition) and ET-1 (10^{-7} M), (b) treatment with a mixture of **17a** (10^{-6} M, 55% inhibition) and ET-1 (10^{-7} M), (c) treatment with a mixture of **17n** (10^{-6} M, 100% inhibition) and ET-1 (10^{-7} M), (d) treatment with a mixture of **17b** (10^{-8} M, 58% inhibition) and ET-1 (10^{-7} M).

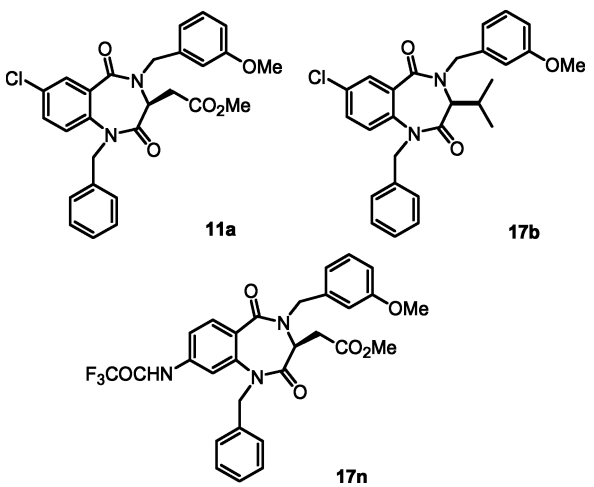


Fig. 6 Potent endothelin receptor antagonists of 1,4-benzodiazepine-2,5-diones that show complete inhibition at 10^{-6} M against the $[Ca^{2+}]_i$ change induced by ET-1 at 10^{-7} M.

Conclusion

We have demonstrated straightforward approaches, including a liquid-phase combinatorial method, for the expedient synthesis of various novel 1,4-benzodiazepine-2,5-dione compounds, particularly those having substituents at the C_3-, N_1- and N_4-positions. The coupling reaction of isatoic anhydrides with amino acids leads to the direct formation of the core structure of 1,4-benzodiazepine-2,5-dione. N_1–H is selectively removed by using a mild base, K_2CO_3, without the interference of N_4–H, and exclusive N_1-alkylation is thus achieved. On the other hand, N_4-alkylation requires a special base of lithiated 2-ethylacetanilide. Using 2-nitrobenzoyl chloride as an equivalent of anthranilic acid, either in the solution- or liquid-phase approach, is advantageous for the construction of 1,4-benzodiazepine-2,5-diones. The preliminary functional assay of our prepared 1,4-benzodiazepine-2,5-dione compounds has revealed some potentially useful endothelin receptor antagonists. However, the accurate measurement of inhibition constants (K_i values) by competitive assay[23] with the radioactive ^{125}I-labeled endothelin is pending on collaboration with other laboratories.

Experimental

General

Melting points are uncorrected. ^{1}H NMR spectra were recorded at 400 MHz; ^{13}C NMR spectra were recorded at 100 MHz. Chemical shifts (δ) are given in parts per million (ppm) relative to residual solvent [δ 7.24 (s) for $CHCl_3$ and δ 2.49 (m) for DMSO-d_6]. The splitting patterns are reported as s (singlet), d (doublet), t (triplet) and multiplet (m). Chemical shifts of ^{13}C NMR spectra are reported relative to $CDCl_3$ (δ 77.0 for the central line of triplet) and DMSO-d_6 [δ 39.5 (m)]. Mass spectra were recorded at an ionizing voltage of 70 or 20 eV. Merck silica gel 60F sheets were used for analytical thin-layer chromatography (TLC). Merck silica gel 60F

glass plates (20 cm × 20 cm with 2 mm thickness) were used for preparative TLC. Column chromatography was performed on silica gel (70–230 mesh) using gradients of EtOAc/hexane as eluents. HPLC (Hewlett Packard 1100) analysis was performed on a vp250/10 Nucleosil 100–7 column (25 cm × 1 cm I.D.) with UV detection at $\lambda = 254$ nm using the eluents EtOAc/hexane (3 : 2 or 4 : 1) at a flow rate of 1 mL min^{-1}.

Reactions requiring dry conditions were carried out under an inert atmosphere using standard techniques. All the reagents and solvents were reagent grade and were used without further purification unless otherwise specified. THF was distilled from sodium benzophenone under N_2. Polyethylene glycol monomethyl ether was dried by azeotropic removal of water with refluxing acetonitrile.

Representative procedure for the formation of 1,4-benzodiazepine-2,5-dione by condensation of isatoic anhydride with α-amino ester

A mixture of 5-chloroisatoic anhydride (0.5 g, 2.5 mmol) and L-aspartic acid dimethyl ester hydrochloride (0.5 g, 2.5 mmol) in pyridine (5 mL) was heated to 120 °C for 18 h. After cooling to room temperature, the mixture was acidified to pH = 1 by adding 6 N HCl (30 mL), and the acidified aqueous phase was extracted with EtOAc (20 mL × 3). The combined organic phase was washed with brine (50 mL), dried over $MgSO_4$, and concentrated to 20 mL of mixture. The precipitated white solids were collected, and washed with water and EtOAc to provide benzodiazepine **9** (0.42 g, 60%).

(+)-(*S*)-7-Chloro-3-(methoxycarbonyl)methyl-1,4-benzodiazepine-2,5-dione (9). Solid, mp = 197–203 °C; $R_f = 0.35$ (hexane/EtOAc, 2 : 3); $[\alpha]^{25}_D = +258.5$ ($c = 1.0$, DMSO); ν_{max} (KBr, cm^{-1}) 3086, 1736, 1685; δ_H (DMSO-d_6, 400 MHz) 2.71 (1 H, dd, J = 16.8, 6.4 Hz), 2.86 (1 H, dd, $J = 16.8$, 8.4 Hz), 3.57 (3 H, s), 4.06 (1 H, ddd, $J = 8.4$, 6.4, 5.6 Hz), 7.14 (1 H, d, $J = 8.8$ Hz), 7.59 (1 H, dd, $J = 8.8$, 2.6 Hz), 7.69 (1 H, d, $J = 2.6$ Hz), 8.71 (1 H, d, $J = 5.6$ Hz), 10.59 (1 H, s); δ_C (DMSO-d_6, 100 MHz) 170.4 (2×), 166.4, 135.5, 132.3, 129.7, 128.3, 127.7, 123.1, 51.6, 48.5, 32.4; MS (70 eV) m/z 284 (18%, $M^+ + 2$), 282 (55, M^+), 181 (100); HRMS $C_{12}H_{11}N_2O_4{}^{35}Cl$ requires 282.0407, found m/z 282.0416. Anal. $C_{12}H_{11}N_2O_4Cl$ requires: C, 50.99; H, 3.92; N, 9.91. Found: C, 50.83; H, 3.77; N, 9.48%.

Representative procedure I for the selective alkylation at the *N*-1 position

Under an atmosphere of argon, a mixture of **9** (1 g, 3.54 mmol) and K_2CO_3 (0.4 g, 3.56 mmol) in DMF (4 mL) was stirred at room temperature (25 °C) for 1 h. Benzyl bromide (0.42 mL, 3.54 mmol) was added, and the mixture was stirred for another 4 h. The reaction was quenched by pouring the mixture into ice water (50 mL), and the mixture was extracted with EtOAc (40 mL × 3). The combined organic phase was washed with brine (50 mL), dried ($MgSO_4$), filtered, and concentrated to afford a pale-yellow oil. The crude product was chromatographed on a silica gel column by elution with hexane/EtOAc (6 : 4) to afford **10a** (1.12 g, 85%).

(+)-(*S*)-1-Benzyl-7-chloro-3-(methoxycarbonyl)methyl-1,4-benzodiazepine-2,5-dione (10a). Oil; $R_f = 0.40$ (hexane/EtOAc, 1 : 1); $[\alpha]^{26}_D = +41.1$ ($c = 0.75$, $CDCl_3$); ν_{max} (film, cm^{-1}) 3231, 2945, 1715, 1670; δ_H ($CDCl_3$, 400 MHz) 2.80 (1 H, dd, $J = 16.8$, 5.2 Hz), 3.09 (1 H, dd, $J = 16.8$, 8.8 Hz), 3.70 (3 H, s), 4.30 (1 H, ddd, $J = 8.8$, 5.6, 5.2 Hz), 4.97 (1 H, d, $J = 16$ Hz), 5.14 (1 H, d, J = 16 Hz), 7.10–7.38 (7 H, m), 7.78 (1 H, d, $J = 2.4$ Hz), 8.18 (1 H, d, $J = 5.6$ Hz); δ_C ($CDCl_3$, 100 MHz) 170.7, 169.1, 167.8, 138.5, 136.1, 132.6, 131.9, 130.0, 129.8, 128.8 (2×), 127.5, 126.6 (2×), 123.7. 52.2, 52.1, 49.5, 33.1; MS (70 eV) m/z 372 (46%, M^+), 180 (100), 91 (76); HRMS $C_{19}H_{17}N_2O_4Cl$ requires 372.0877, found m/z 372.0904. Anal. $C_{19}H_{17}N_2O_4Cl$ requires: C, 61.21; H, 4.60; N, 7.51. Found: C, 60.90; H, 4.83; N, 7.14%.

Representative procedure II for alkylation at *N*-4 position

To a solution of *N*-(2-ethylphenyl)acetamide (0.16 g, 0.98 mmol) in THF (2 mL) was added butyllithium (95 μL, 1 mmol, 1.6 M solution in hexane) dropwise under argon at −78 °C (dry ice/acetone bath). The mixture was stirred at −78 °C for 1 h to give a yellow solution. Compound **10a** (0.36 g, 0.98 mmol) in THF (1 mL) and DMF (1 mL) was added dropwise. After stirring for 1 h, a solution of 3-methoxybenzyl bromide (200 μL, 0.99 mmol) and lithium iodide (0.44 g, 0.99 mmol) in DMF (1 mL) was added in one portion. The reaction mixture was heated to 60 °C over a period of 6 h. The mixture was cooled, and partitioned between CH_2Cl_2 (30 mL) and 1 N HCl (30 mL). The organic phase was separated, washed with water (30 mL) and brine (30 mL), dried ($MgSO_4$), filtered, concentrated, and chromatographed on a silica gel column by elution with hexane/EtOAc (7 : 3) to afford compound **11a** (0.33 g, 68%).

(+)-(*S*)-1-Benzyl-7-chloro-4-(3-methoxybenzyl)-3-(methoxycarbonyl)methyl-1,4-benzodiazepine-2,5-dione (11a). Oil; $R_f = 0.35$ (hexane/EtOAc, 3 : 2); $[\alpha]^{26}_D = +25.8$ ($c = 0.75$, $CDCl_3$); ν_{max} (film, cm^{-1}) 2952, 1740, 1690; δ_H ($CDCl_3$, 400 MHz) 2.68 (1 H, dd, $J = 16.4$, 4.0 Hz), 3.59 (1 H, dd, $J = 16.4$, 3.6 Hz), 3.79 (3 H, s), 3.83 (3 H, s), 4.47 (1 H, d, J = 16 Hz), 4.80 (1 H, dd, $J = 4.0$, 3.6 Hz), 4.91 (1 H, d, $J = 16.0$ Hz), 5.13 (1 H, d, $J = 16.0$ Hz), 5.20 (1 H, d, $J = 16.0$ Hz), 6.77–7.40 (11 H, m), 7.91 (1 H, d, $J = 2.8$ Hz); δ_C ($CDCl_3$, 100 MHz) 170.4, 168.3, 167.5, 160.0, 142.5, 138.8, 138.5, 136.1, 132.4, 131.7, 130.6, 129.8, 127.5, 126.5, 122.8, 119.3, 119.0, 113.2, 112.8, 112.7, 112.1, 55.2, 53.0, 52.1, 51.5, 47.2, 31.8.; MS (70 eV) m/z 492 (22%, M^+), 357 (50), 180 (25), 121 (62), 91 (100); HRMS $C_{27}H_{25}N_2O_5Cl$ requires 492.1452, found m/z 492.1458. Anal. $C_{27}H_{25}N_2O_5Cl$: C, 65.79; H, 5.11; N, 5.68. Found: C, 65.40; H, 5.20; N, 5.19%.

Representative procedure III for reductive amination

A mixture of L-valine methyl ester hydrochloride (2.03 g, 12 mmol) and *m*-anisaldehyde (1.25 mL, 10 mmol) in CH_2Cl_2 (100 mL) was treated with sodium triacetoxyborohydride (3.18 g, 15 mmol) and NaOAc (1.23 g, 15 mmol) at 0 °C. The suspension was warmed to room temperature and stirred for 5 h. After the reaction was finished, brine (50 mL) was added, and the mixture was extracted with CH_2Cl_2 (30 mL × 2). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel eluting with EtOAc/hexane (4 : 6) to give *N*-(3-methoxybenzyl) valine methyl ester **14b** (2.46 g, 98%).

(−)-(*S*)-*N*-(3-Methoxybenzyl) valine methyl ester (14b). Oil; $R_f = 0.63$ (EtOAc/hexane, 3 : 2); $[\alpha]^{23}_D = -65.4$ (CH_2Cl_2, c = 0.75); ν_{max} (film, cm^{-1}) 3344, 1735, 1604; δ_H($CDCl_3$, 400 MHz)

7.20 (1 H, d, J = 8.2 Hz), 6.90–6.87 (2 H, m), 6.77 (1 H, d, J = 8.2 Hz), 3.80 (1 H, d, J = 13.2 Hz), 3.78 (3 H, s), 3.71 (3 H, s), 3.54(1 H, d, J = 13.2 Hz), 3.00 (1 H, d, J = 6.0 Hz), 1.93–1.86 (1 H, m), 0.95–0.91 (6 H, m); δ_C($CDCl_3$, 100 MHz) 175.5, 159.5, 141.6, 129.1, 120.4, 113.4, 112.5, 66.5, 55.2, 52.5, 51.4, 31.8, 19.5, 18.8; HRMS $C_{14}H_{22}NO_3$ (M +H^+) requires 252.1600, found m/z 252.1598.

Representative procedure IV for amidation of 2-nitrobenzoyl chloride

To a mixture of amine **14b** (2.26 g, 9 mmol) K_2CO_3 (3.73 g, 27 mmol) and Bu_4NI (1.66 g, 4.5 mmol) in CH_2Cl_2 (90 mL) was added 4-chloro-2-nitrobenzoyl chloride (2.38 g, 10.8 mmol) dropwise. After the mixture was stirred at room temperature for 4 h, water (70 mL) was added, and the mixture was extracted with CH_2Cl_2 (30 mL × 2). The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel eluting with EtOAc/hexane (3 : 7 to 1 : 1) to give *N*-(5-chloro-2-nitrobenzoyl)-*N*-(3-methoxybenzyl) valine methyl ester **15b** (3.68 g, 94%).

(−)-(*S*)-*N*-(5-Chloro-2-nitrobenzoyl)-*N*-(3-methoxybenzyl) valine methyl ester (15b). Gel; R_f = 0.63 (EtOAc/hexane, 3 : 2); $[\alpha]^{23}_D$ = −19.5 (CH_2Cl_2, c = 0.60); ν_{max} (film, cm^{-1}) 2970, 1745, 1654; δ_H ($CDCl_3$, 400 MHz) 8.25–8.04 (1 H, m), 7.56–7.32 (2 H, m), 7.24–6.58 (4 H, m), 5.00–4.87 (1 H, m), 4.44–4.36 (1 H, m), 3.82–3.43 (7 H, m), 2.57–2.52 (0.5 H, m), 2.34–2.27 (0.5 H, m), 1.10–1.06 (3 H, m), 0.77–0.68 (3 H, m); δ_C ($CDCl_3$, 100 MHz) 169.7–166.7 (2×), 159.0–133.2 (5×), 129.7–112.9 (7×), 77.3–63.7, 55.4–52.0 (2×), 47.0–46.5, 28.8–28.3 (CH), 21.1–19.6 (2×); HRMS $C_{21}H_{24}ClN_2O_6$ (M + H^+) requires 435.1323, found m/z 435.1325.

Representative procedure V for reduction of nitro compounds and *in situ* cyclization to benzodiazepines

A solution of the nitro compound **15b** (1.74 g, 4 mmol) in MeOH (40 mL) was treated with zinc powder (2.61 g, 40 mmol) and NH_4Cl (1.07 g, 20 mmol). The suspension was stirred at room temperature for 10 min, and filtered through a pad of Celite. The filtrate was concentrated to about 40 mL, and *p*-TsOH monohydrate (76 mg, 0.4 mmol) was added. The mixture was heated under reflux for 6 h, cooled, and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel eluting with EtOAc/hexane (1 : 1) to give 7-chloro-3-isopropyl-4-(3-methoxybenzyl)-1,4-benzodiazepine-2,5-dione **16b** (1.12 g, 75%).

(−)-(*S*)-7-Chloro-3-isopropyl-4-(3-methoxybenzyl)-1,4-benzodiazepine-2,5-dione (16b). Solid, mp = 75–77 °C; R_f = 0.43 (EtOAc/hexane, 1 : 1); $[\alpha]^{23}_D$ = −199.6 (CH_2Cl_2, c = 0.47); ν_{max} (KBr, cm^{-1}) 3238, 2970, 1690, 1636; δ_H ($CDCl_3$, 400 MHz) 9.37 (1 H, br), 7.97 (1 H, s), 7.34 (1 H, d, J = 8.8 Hz), 7.10 (1 H, t, J = 8.0 Hz), 6.88–6.82 (3 H, m), 6.65 (1 H, d, J = 8.4 Hz), 5.29 (1 H, d, J = 14.4 Hz), 4.30 (1 H, d, J = 14.4 Hz), 3.65 (3 H, s), 3.53 (1 H, d, J = 14.0 Hz), 1.72–1.65 (1 H, m), 0.82 (3 H, d, J = 6.8 Hz), 0.72 (3 H, d, J = 6.4 Hz); δ_C ($CDCl_3$, 100 MHz) 171.7, 164.6, 159.6, 137.3, 133.2, 132.4, 131.0, 130.1, 129.5, 127.8, 121.3, 120.7, 114.1, 113.3, 71.2, 55.4, 55.1, 27.9, 19.7, 19.5; HRMS $C_{20}H_{22}ClN_2O_3$ (M + H^+) requires 373.1319, found 373.1317.

(−)-(*S*)-1-Benzyl-7-chloro-3-isopropyl-4-(3-methoxybenzyl)-1,4-benzodiazepine-2,5-dione (17b). Alkylation of **16b** (1.30 g, 3.5 mmol) with benzyl bromide (0.42 mL, 3.5 mmol), according to the representative procedure II, gave **17b** (90%). Solid, mp = 46–48 °C; R_f = 0.60 (EtOAc/hexane, 1 : 1); $[\alpha]^{23}_D$ = −229.9 (CH_2Cl_2, c = 0.35); ν_{max} (KBr, cm^{-1}) 2970, 1673, 1647, 1600, 1472, 1266, 1154, 1052; δ_H ($CDCl_3$, 400 MHz) 7.81 (1H, d, J = 2.8 Hz), 7.32 (1H, d, J = 8.8 Hz), 7.24–7.06 (3H, m), 7.04 (2H, d, J = 6.0 Hz), 6.99 (2H, d, J = 7.6 Hz), 6.93–6.85 (2H, m), 6.82 (1H, d, J = 7.2 Hz), 5.08–4.93 (3H, m), 4.52 (1H, d, J = 14.0 Hz), 3.81 (1H, d, J = 11.6 Hz), 3.76 (3H, s), 1.45–1.37 (1H, m), 0.81 (3H, d, J = 6.8 Hz), 0.61 (3H, d, J = 6.4 Hz); δ_C ($CDCl_3$, 100 MHz) 169.2, 164.8, 159.6, 137.4, 137.2, 136.4, 132.0, 131.3, 131.2, 130.3, 129.5, 128.7 (2×), 127.4, 126.9 (2×), 122.7, 121.3, 114.3, 113.8, 72.6, 55.3, 51.9, 28.3, 19.9, 19.7; HRMS $C_{27}H_{28}ClN_2O_3$ (M +H^+) requires 463.1788, found 463.1788. Anal. $C_{27}H_{27}ClN_2O_3$ requires: C, 70.05; H, 5.88; N, 6.05; O, 10.37. Found: C, 70.40; H, 5.82; N, 6.19%.

(+)-(*S*)-1-Benzyl-4-(3-methoxybenzyl)-3-(methoxycarbonyl)-methyl-8-(trifluoroacetyl-amino)-1,4-benzodiazepine-2,5-dione (17n). The reductive amination of *m*-anisaldehyde (0.69 mL, 5.53 mmol) with dimethyl L-aspartate hydrochloride (1.35 g, 6.63 mmol), according to the representative procedure III, gave *N*-(3-methoxybenzyl) aspartic acid dimethyl ester **14c** (1.45 g, 93%). Amidation of **14c** (3.60 g, 12.8 mmol) with 2,4-dinitrobenzoyl chloride (3.29 g, 15.4 mmol), according to the representative procedure IV, gave *N*-(2,4-dinitrobenzoyl)-*N*-(3-methoxybenzyl) aspartic acid dimethyl ester **15d** (5.53 g, 91%). Reduction and *in situ* cyclization of **15d** (1.00 g, 2.10 mmol), according to the representative procedure V, gave 8-amino-4-(3-methoxybenzyl)-3-(methoxycarbonyl)methyl-1,4-benzodiazepine-2,5-dione **16d** (0.81 g, 78%). Alkylation of **16d** (2.00 g, 5.23 mmol) with benzyl bromide (0.76 mL, 6.26 mmol), according to the representative procedure I, gave the *N*-1 alkylation product, 8-amino-1-benzyl-4-(3-methoxybenzyl)-3-(methoxycarbonyl)methyl-1,4-benzodiazepine-2,5-dione **17d** (1.24 g, 50%), accompanied by the 8-benzylamino derivative **17e** (0.68 g, 23%) and the 8-dibenzylamino derivative **17f** (0.34 g, 10%).

Compound **17d** (142 mg, 0.3 mmol) was dissolved in trifluoroacetic anhydride (3 mL) and stirred at 0 °C for 1 h. Excess of trifluoroacetic anhydride was removed under reduced pressure, and the residue was extracted with CH_2Cl_2 (5 mL × 2). The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford pure **17n** (143 mg, 0.25 mmol) in 83% yield.

Compound **17n**: Solid, mp = 101–103 °C; R_f = 0.48 (EtOAc/hexane, 3 : 2); $[\alpha]^{23}_D$ = +9.5 (CH_2Cl_2, c = 0.17); ν_{max} (KBr, cm^{-1}) 3443, 3294, 2966, 1735, 1686, 1640, 1438, 1282, 1157, 1050; δ_H ($CDCl_3$, 400 MHz) 8.64 (1H, br), 7.78 (1H, d, J = 8.4 Hz), 7.71 (1H, s), 7.28–7.19 (5H, m), 7.09 (2H, d, J = 7.2 Hz), 6.86–6.79 (3H, m), 5.12 (1H, d, J = 16.0 Hz), 5.07 (1H, d, J = 16.0 Hz), 5.02 (1H, d, J = 16.0 Hz), 4.78 (1H, dd, J = 4.4, 10.4 Hz), 4.38 (1H, d, J = 16.0 Hz), 3.79 (3H, s), 3.57 (3H, s), 3.30 (1H, dd, J = 10.4, 16.6 Hz), 2.70 (1H, dd, J = 4.4, 16.6 Hz); δ_C ($CDCl_3$, 100 MHz) 170.6, 168.4, 168.1, 159.9, 155.1 (q, J = 38.0 Hz), 140.6, 139.1, 138.8, 136.0, 131.9, 129.8, 128.7 (2×), 127.6, 126.9 (2×), 126.7, 119.4, 117.7, 116.8, 113.1, 113.0, 112.7, 55.2, 53.1, 52.2, 51.1, 47.2, 31.9; HRMS $C_{29}H_{27}F_3N_3O_6$ (M + H^+) requires 570.1852, found

570.1852. Anal. $C_{29}H_{26}F_3N_3O_6$ requires: C, 61.16; H, 4.60; N, 7.38. Found: C, 61.18; H, 4.88; N, 7.04%.

Materials and methods for the $[Ca^{2+}]_i$ assay

Endothelin-1 (ET-1) and the cyclic peptide antagonists BQ123 [cyclo(D-Trp-D-Asp-L-Pro-D-Val-L-Leu)][26] were synthesized by using an ABI 433A peptide synthesizer (ABI, USA). Non-peptide endothelin receptor antagonist SB209670 was synthesized according to the reported procedure.[9] The ^{125}I-labeled ET-1 ((3-[^{125}I]iodotyrosyl)endothelin-1, 81.4 TBq mmol^{-1}) was purchased from NEN Life Science Products (USA). The fluorescent reagent, fura-2 penta(acetoxymethyl) ester, was purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA).

Construction of CHO–K1 cell line over-expressing endothelin receptor A and ligand binding assay[22c]

The lipofectin-mediated transfection method described by Tseng and co-workers[24] was used to construct stable CHO cell lines over-expressing ET_A. Cells were grown to 30–40% confluence in 60 mm dishes and transfected with 1 μg of pcDNA-3 expressing plasmid harboring ET_A using lipofectin reagent for 6–8 h in a serum-free medium. Cells were then returned to 5% FBS, cultured for 36 h, then replated at reduced density in 150 mm plates in the presence of 0.75 mg mL^{-1} (active) G418. The G418 resistant colonies were selected and screened for ET_A by binding of (3-[^{125}I]iodotyrosyl)endothelin-1 ([^{125}I]ET-1). Binding was conducted to cells plated in 24-well dishes at 2–3 × 10^5 cells mL^{-1} the day before the binding assay. For cell binding assays [^{125}I]ET-1 (10^{-12} M) was added to HR buffer (5 mM NaCl, 4.7 mM KCl, 1 mM Na_2PO_4, 1.28 mM $CaCl_2$, 10 mM HEPES, pH 7.4, with 0.5% bovine serum albumin, and 0.1 mg mL^{-1} soybean trypsin inhibitor). Cells were incubated to equilibrium (2 h at 37 °C) then washed twice with ice-cold phosphate-buffered saline. The cells were then solubilized with 1 mL of 0.1 N NaOH and radioactivity quantified in a γ-counter. Non-specific binding was determined in the presence of 100 nM ET-1.

Identification of transfected CHO cell[22c,23]

The cultured CHO cells were subjected to transfection with the ET_A-expression plasmid DNA using a lipofectin reagent. The efficacy of ET_A expression was shown by the competitive binding assay with a synthetic sample of ET-1 and the radiolabeled [^{125}I]-ET-1. The binding affinity of the transfected cell line by the agonist ET-1 was established to have a dissociation constant of K_d = 1.52 nM. The receptor density (B_{max}) of 6.3 × 10^5 sites per cell was estimated from a Scatchard plot.[27] This result indicated that ET_A receptors were successfully over-expressed in the CHO cells.

Functional assay

According to the reported experimental protocol,[23] the transfected CHO cells harboring ET_A (~10^6 cells mL^{-1}) in HR buffer was incubated with fura-2 penta(acetoxymethyl) ester at 0 °C for 30 s. After washing away the extracellular residual fura-2, a mixture of ET-1 (10^{-7} M) and test compound of known concentration (*e.g.*, 10^{-6} M) in HR buffer was added. At 100 s, digitonin was added to destroy the cell membrane, and caused all the ET-1 induced Ca^{2+} ions to bind with fura-2. The $[Ca^{2+}]_i$ was indirectly deduced by the intensity of 510 nm fluorescence with excitation at 340 nm.[25] At 120 s, EGTA [ethylene glycol bis(2-aminoethyl ether) tetraacetic acid] was added to remove Ca^{2+} ions, and the concentration of free fura-2 was measured by the intensity of the 510 nm fluorescence with excitation at 380 nm. The intracellular calcium concentration, $[Ca^{2+}]_i$, was calculated according the following equation: $[Ca^{2+}] = K_d \times (sf/sb) \times [(R - R_{min})/(R_{max} - R)]$ in which, K_d is the dissociation constant of fura-2 to Ca^{2+}; R, R_{min} and R_{max} represent, respectively, the ratio of fluorescence intensity at 340 nm to 380 nm, that for the concentration of Ca^{2+} close to zero, and that for the concentration of Ca^{2+} close to saturation; sf and sb represent, respectively, the ratio of fluorescence intensity at 380 nm for the concentration of Ca^{2+} close to zero, and that for the concentration of Ca^{2+} close to saturation.

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