CHAPTER 2

MATERIALS AND METHODS

2.1) Construction of CHO-K1 cell line over-expressing endothelin receptor A and ligand binding assay

The lipofectin-mediated transfection method described by Tseng *et al.*[116] 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 expressed plasmid harboring ET_A using Lipofectin reagent for 6-8 h in serum-free medium. Cells were then returned to 5% FBS, cultured 36 h, then replated at reduced density in 150-mm plates in the presence of 0.75 mg/ml (active) G418. G-418 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 the day before the binding assay. For cell binding assays, [^{125}I] ET-1 (10 pM) 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 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. Nonspecific binding was determined in the presence of 100 nM ET-1.

2.2) Preparation of a cell-immobilized capillary column

A fused-silica capillary column (60 cm effective length × 200 μm inner diameter, ~1.88 mL whole volume) was activated by washing successively with MeOH

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(ca. 20 μL), 1 N HCl (ca. 20 μL), deionized water (ca. 20 μL), 1 N NaOH (ca. 20 μL) and deionized water (ca. 20 μL). The column was stored in the presence of 1 mM PBS buffer (containing 31.7 mg of NaH_2PO_4 and 206 mg of Na_2HPO_4 at pH 7.3 per liter). The transfected CHO cells harbouring ET_A (~ 2.5×10^5 cells/mL) recovered from the culture media were fixed by treatment with formaldehyde (3.7% in water, 5 mL) for 30 min to furnish the desired cross linkage. The fixed cells were stored at 4 °C in PBS (1 mM, pH 7.3). For loading of the fixed cells onto the capillary column, the column was washed with ethanol (95%, ca. 40 μL), purged with air in order to dry it, charged with poly-L-lysine (15,000-30,000 molecular weight, 0.5 mg/mL in water) for 5 min, and incubated for 30 min [117,118]. The column was then charged again with poly-L-lysine for 5 min and incubated for 2 h. The column was then dried by airflow for 2 h, afterwards; the fixed cells in the PBS buffer were purged into the poly-L-lysine-coated column. After 5 min of incubation time, another batch of fixed cells was purged into the column for 30 min of incubation. After which, 1% FBS (fetal bovine serum) in PBS (ca. 20 μL) was purged to cap the exposed area of poly-L-lysine. The column immobilized with the transfected CHO cells was finally washed with PBS (ca. 40 μL), and stored at room temperature (~ 25 to 27°C). No apparent degradation was observed after 7 days.

The capillary electrophoresis experiments were performed on a P/ACE system (Beckman Instruments, USA) at a constant voltage of 10 kV. The sample (~ 1.5 μL of ~ 10^{-6} to 10^{-7} M solution in 1 mM PBS, pH 7.0) was introduced into the capillary column by pressure injection at 0.5 psi/3 sec. The background electrolyte was PBS (1 mM, pH 7.0). Electrophoresis was monitored by an absorbance detector held at 214 nm. The low concentration of PBS (1 mM) ensured no interference with 214-nm absorbance. The temperature of the capillary column was maintained at 25°C.

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2.3) Samples and reagents

2.3.1) Cyclical peptide synthesis

Unless otherwise stated, all reagents and solvents were obtained commercially as reagent grade and used without further purification. All peptides were synthesized by the solid-phase method using a 433A peptide synthesizer (ABI). Endothelin-1 (0.25 mmol) was synthesized on HMP (*p*-hydroxymethyl phenoxymethyl polystyrene) resin (with a loading of 1.0 mmol/g, AnaSpec Inc., USA), whereas BQ123 and JKC302 were synthesized on 2-chlorotrityl chloride resin (with a loading of 1.35 mmol Cl/g, AnaSpec Inc., USA). The synthesis was performed using a stepwise FastMoc protocol using the manufacturer's prepacked cartridges (1.0 mmol each).

Elongated ET-1 peptide was cleaved from the resin by using at 0°C, 1 h mixture containing phenol (0.75 g, Acros), 1,2-ethandithiol (0.25 mL, Merck), thioanisole (0.5 mL, Sigma), water (0.5 mL) and trifluoroacetic acid (10 mL, Sigma). Disulfide bond cermates was perform by long air-oxidation and the progress of the oxidation was beelone by HPLC. The peptide was lyophilized, rinsed with cold ether (200 mL), and filtered. The filtrate was dissolved in a diluted acetic acid solution (5% in water, 50 μg/mL) to the solution was added, and methanol (10 mL) was added drop by drop until white solids dissolved. After the residual resin was filtered off, the solution was adjusted to pH 8.33 with ammonium hydroxide. The peptide was further purified by HPLC using a C_{18} column (10-μm particle size, 25 cm × 1 cm) with continuous gradients of buffers A and B (from 35% B/A to 39% in 10 min) at a flow rate of 2.5 mL/min, in which buffer A contained 0.1% TFA in 2% aqueous acetonitrile and buffer B contained 0.1% TFA in 90% aqueous acetonitrile. The UV monitor was set at an absorbance of 214 nm. The structure of peptide was identified by electrospray ionization mass spectrometry on a Finnigan LCQ™ mass spectrometer

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(ThermoFinnigan, USA) using thioglycerol or 3-nitrobenzyl alcohol as the matrix. D-Val-Leu-D-Trp(Boc)-D-Ser(^{t}Bu)-Pro and D-Trp(Boc)- D-Asp(^{t}Bu)-Pro-D-Val-Leu, were cleavaged from resin by using a mixture containing acetic acid (1 mL), trifluoroethanol (2 mL) and dichloromethane (7 mL) to give the linear precursors of BQ123 and JKC302. Cyclization of the linear peptide (0.1 mmol) was carried out by using *N*,*N*'-dicyclohexylcarbodiimide (DCC, 0.117 mmol), 1-hydroxybenzotriazole (HOBt, 0.114 mmol) and diisopropylethylamine (DIEA, 0.55 mmol). BQ123 and JKC302 were purified by HPLC, using gradients of buffers A and B (37-42% B/A for BQ123 and 36-39% B/A for JKC 302).

2.3.2) The active herbal components

The active herbal components, Magnolol, Geniposide and Honokiol, were purchased from Wako Pure Chemical Industries (Japan).

2.3.3) Nonpeptide (I): Carbazolothiophene-2-Carboxylic Acid Derivatives

We established method of three-component coupling reactions of thiophene-2-carboxylate[101] to synthesize carbazolothiophene-2- carboxylate derivatives 7-22, and examined their antagonism against the binding of ET-1 with ET_A receptor. The three-component coupling reaction of methyl thiophene-2-carboxylate (1) with N-methylindole-2-carbaldehyde (2) and 4-methoxyacetophenone (3) occurred smoothly to afford a 77% yield of 4 (Fig. 2.1).[123] This one-pot operation presumably proceeded by an initial coupling of ester 1 with aldehyde 2 to give a dienolate intermediate A, which was then trapped by ketone 3. Although diol 4 existed as a mixture of diastereomers, the subsequent oxidation and dehydration would yield a single product. Conversion of 4 to 5 was achieved by a three-step sequence: an

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oxidation with DDQ to give the corresponding ketone, an acid-catalyzed dehydration to eliminate a water molecule, and an oxidative aromatization by using $Pd(OAc)_2$ to afford the thiophene product 5. The subsequent intramolecular Friedel-Crafts alkylation thus furnished the tetracyclic skeleton, giving carbazolothiophene-2-carboxylate 6 as a pivotal compound for the synthesis of other derivatives 7-10. Saponification of 6 afforded acid 7, whereas demethylation of 6 with BBr_3 gave phenol 8. Alkylation of phenol 8 with methyl 2-bromoacetate or 4-bromobutanenitrile, followed by hydrolysis in alkaline conditions, gave diacids 9 and 10 in high yields.

A series of carbazolothiophenes 11-12 were similarly prepared, initially by the SmI_2-promoted three-component coupling reactions with appropriate partner substrates. For example, the SmI_2-promoted coupling product of thiophene ester 1, indole aldehyde 2 and 3-methoxyacetophenone was further elaborated, according to the procedure similar to that shown in Scheme 1, to give compounds 11-13 bearing MeO, CH_2CO_2H or $(CH_2)_3CO_2H$ substituents on the *meta* positions of the phenyl rings as the "*meta*-analogues" of 7, 9 and 10. Compounds 14-19 with the (substituted)benzyl groups on the nitrogen atoms were also prepared in high yields by elaboration of the coupling products obtained from ester 1, 1-benzylindole-2-carbaldehydes and appropriate ketones. Compounds 20-22 are analogues of 17-19 having the substituents switched over. It was noted that the benzyl protons in compounds 14-22 occurred at low fields (*ca.* δ 6.1) presumably due to the deshielding effect of the adjacent carbonyl group.

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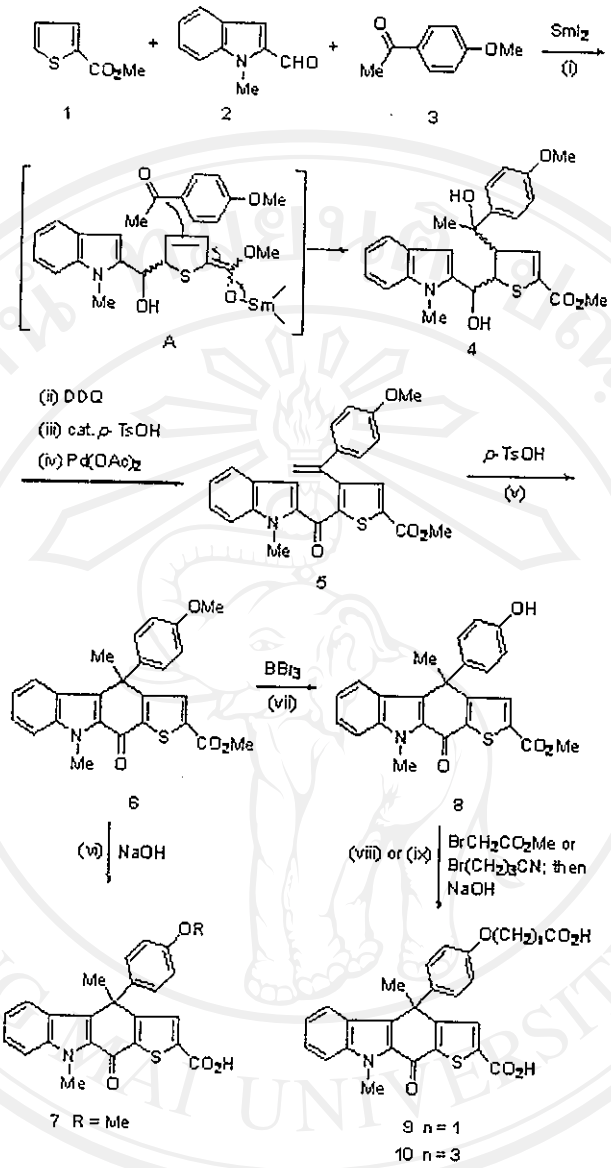

Figure 2.1 Liquid-phase synthesis of carbazolothiophene-2-carboxylic acid derivatives: (i) 1 (1 mmol), 2 (1 mmol), SmI_2 (3.6 mmol), THF, HMPA, 0°C to r.t., 1.5 h; then 3 (1.2 mmol), 0°C to r.t., 10 h; 77% yield of 4. (ii) DDQ, PhH, r.t., 4 h; 88%. (iii) cat. *P*-TsOH, PhH, reflux, 10 h; 95%. (iv) $Pd(OAc)_2$ (5 equiv), K_2CO_3, CH_3CN, r.t., 12 h; 93%. (v) *p*-TsOH, CH_2Cl_2, r.t., 2 h; 98%. (vi) aq. NaOH (0.5 %), THF, 0°C to r.t., 2.5 h; 99%. (vii) BBr_3, CH_2Cl_2,-78°C; 96%. (viii) $BrCH_2CO_2Me$, K_2CO_3, CH_3CN, 80°C, 15 h; aq. NaOH (0.5 %), THF, 0°C to r.t., 2.5 h; 95% yield of 9. (ix) $Br(CH_2)_3CN$, K_2CO_3, CH_3CN, 80°C, 48 h; aq. NaOH (40%), MeOH, reflux, 1 h; 84% yield of 10.

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2.3.4) Nonpeptide (II): 1,4-Benzodiazepine-2,5-diones Derivatives

2.3.4.1) Liquid-phase synthesis

The dilute solution of borontribromide, 1 M borontribromide in CH_2Cl_2, was considered, and it was applied to 24 and 25 at the step of demethylation. The reacting result gave exclusively the corresponding demethylated products, 28 and 29, while the methyl ester moiety in both products was retained. Compound 28 and 29 were *O*-alkylated to yield the relative side-chained esters 30 and 31 as the only products, and the same reacting condition were also applied to the *N*-1 benzylations, giving the desired products 24 and 25 cleanly. The above improvement increased the total yield to around 20 % for target compounds 32 and 33, and the detailed synthetic pathway is shown in Fig. 2.2.

Owing to the easy of dealkylation of the methyl and isopropyl ester, the alternate approach to target 32 or 33 was emphasized that the side chain, attached to the aromatic ring, was modified before the benzylation. The sketch of this approach was started from two series of synthesis. One was the construction of the ring of 1,4-benzodiazepine, and the other was the modification of the side chain. The benzyl moiety with the modified side chain was then introduced to the ring of 1,4-benzodiazepine, and the further hydrolysis to the target.

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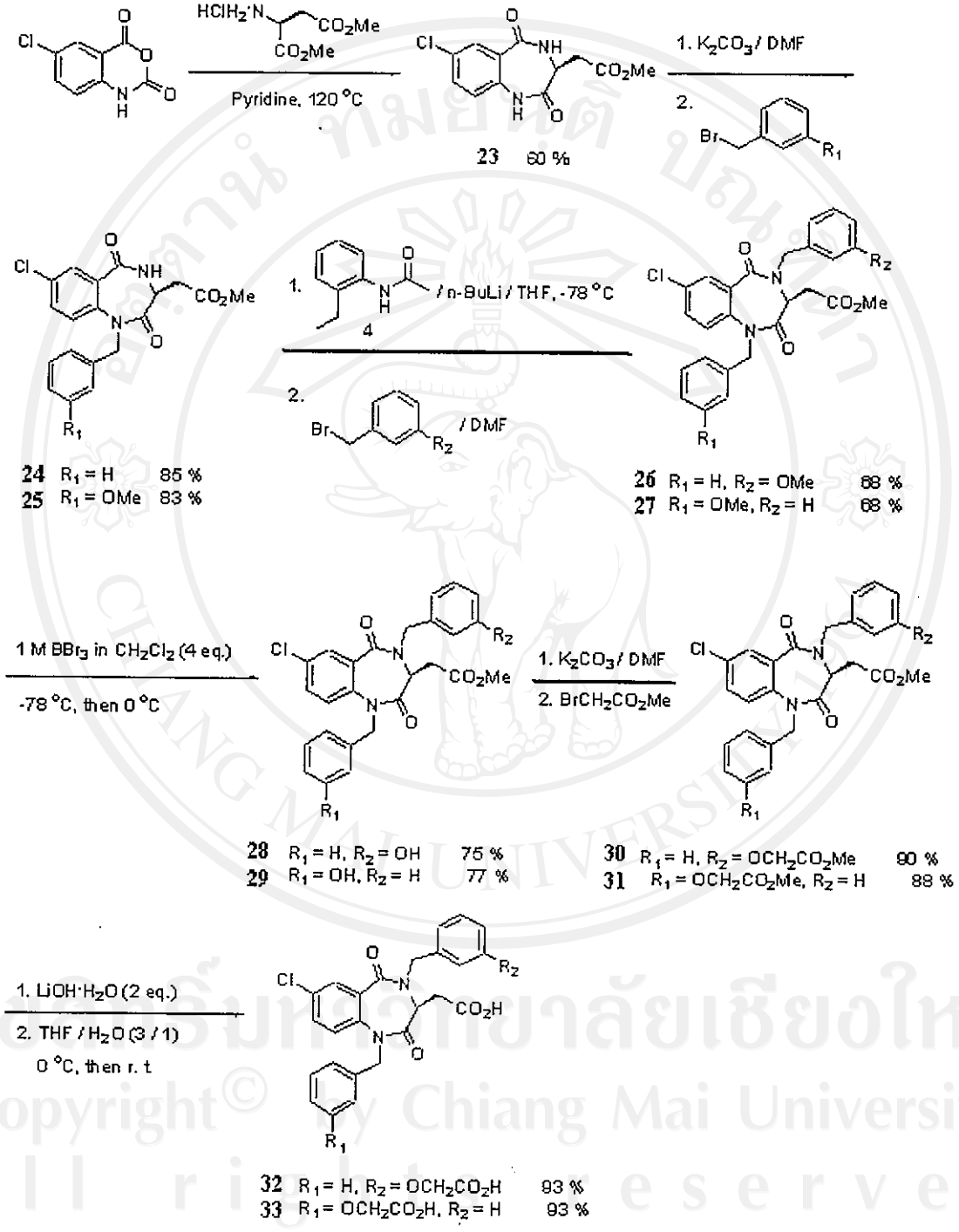

Figure 2.2 Liquid-phase synthesis of 1,4-benzodiazepine-2,5-dione dimethyl ester derivatives.

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2.3.4.2) Polyethylene resin-bound liquid-phase synthesis

Ellman and co-workers have utilized strategy A to construct a library of 1,4-benzodiazepine-2,5-diones from three components of anthranilic acids, R-amino esters, and alkylating agents.5a,b Merrifield resin is derivatized by alkylation with the sodium salt of 4-hydroxy-2,6-dimethoxy-benzaldehyde, and the resin-bound aldehyde (A3) is linked to R-amino esters via reductive amination. Amidation of these resin-bound amino esters with anthranilic acids, followed by cyclization and alkylation, leads to the polymer-bound 1,4-benzodiazepine-2,5-dione derivatives (A1). Treatment of A1 with $CF_3CO_2H/Me_2S/H_2O$ releases the solid support to give 1,4-benzodiazepine-2,5-diones without substitution at the N4-position. This solid-phase synthesis thus successfully pro-vides a library of 2508 members. This study does not mention the further alkylation at N4-position, which may cause the general problem of regioselectivity in the N-o*r*O-alkylations in an amide moiety.7 In the strategy devised by Goff and co-workers, 5c R-amino esters (B4) are first reacted with the resin-bound bromo-acetate reagent B3 and then subjected to amidation with 2-azidobenzoyl chlorides (B2). The azido group is reduced by Ph3P (Staudinger reaction), and the intermediate is heated to 130°C to effect an aza-Wittig reaction. Hydrolysis of the iminoether intermediate and cleavage of the Rink resin are achieved concomitantly by treatment with an acid (CF_3CO_2H/H_2O) to afford the desired 1,4-benzodiazepine-2,5-diones.

As the target 1,4-benzodiazepine-2,5-dione requires an N4-aryl substituent with a carboxylic group to mimic the Asp-18 residue of ET-1, we deliberately used the PEG-bound benzaldehydes E3 to couple with R-amino esters E5. By this means, the required carboxylic group can be revealed at the final step along with removal of the PEG support. We demonstrate herein this strategy by a liquid-phase synthesis of a

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small library of 1,4-benzodiazepine-2,5-diones (10a-p) using PEG5000 monomethyl ether as the support (Fig. 2.3).

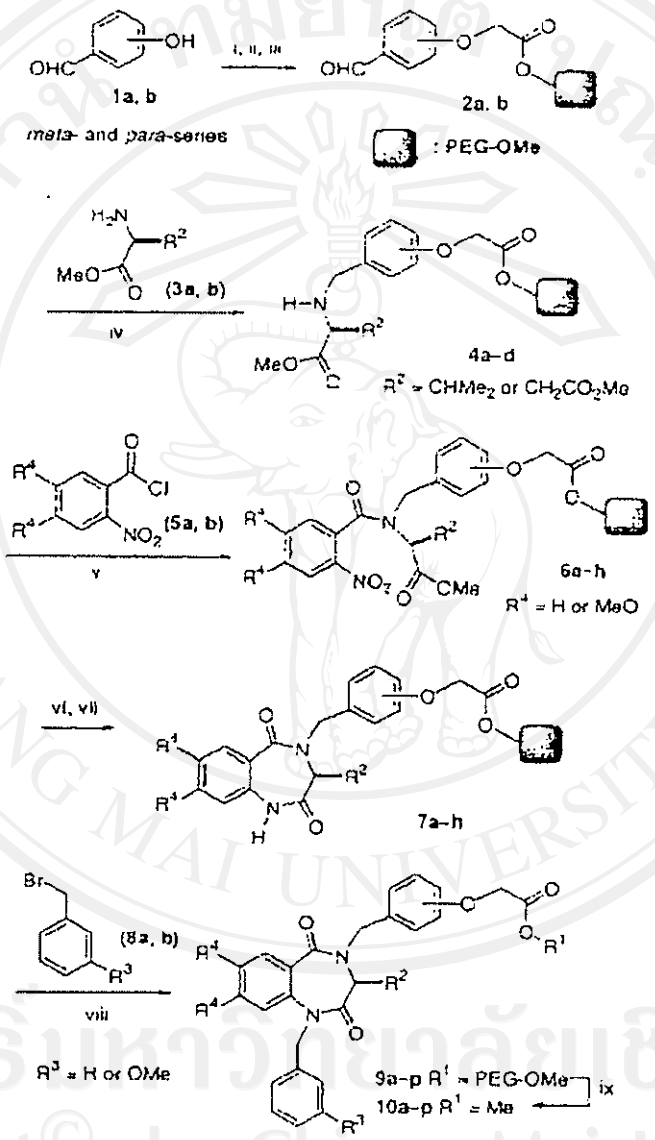

Figure 2.3 Polyethylene resin-bound liquid-phase synthesis. The Reagents and conditions: (i) Methyl bromoacetate, K_2CO_3, CH_3CN, reflux 4 h, 98%. (ii) 1 N NaOH(aq), THF, room temperature, 2 h, 90%. (iii) Monomethoxy-PEG-OH5000, DCC, DMAP, CH_2Cl_2, room temperature, 23 h. (iv) L-Aspartic acid dimethyl ester hydrochloride or L-valine methyl ester hydrochloride, $NaBH(OAc)_3$, NaOAc, CH_2Cl_2,

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room temperature, 5 h. (v) 2-Nitrobenzoyl chloride or 4,5-dimethoxy-2-nitrobenzoyl chloride, Bu_4NI, K_2CO_3,CH_2Cl_2, room temperature, 5 h. (vi) Zn, HOAc, room temperature, 2 h. (vii) CF_3COOH, CF_3COONa, CH_3CN, room temperature, 13 h. (viii) Benzyl bromide or 3-methoxybenzyl bromide, Cs_2CO_3, DMF, room temperature, 2 h. (ix) Na_2CO_3, MeOH, room temperature, 5 min.

Melting points are uncorrected. 1 H NMR spectra were recorded at 300 or 400 MHz; 13 C NMR spectra were recorded at 75 or 100 MHz. $CDCl_3$ (™÷) 7.24 and ™X) 77.0 (central line of triplet)) was used as an internal standard in 1 H and 13 C NMR spectra, unless otherwise stated. Mass spectra were recorded at an ionizing voltage of 70 or 20 eV. 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 ì) 254 nm using the eluents of EtOAc/hexane (3:2 or 4:1) at a flow rate of 1 mL/min. Merck silica gel 60F sheets were used for analytical 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. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl under N_2. Mono-methoxy- PEG-OH was dried by azeotropical removal of water with refluxing acetonitrile.

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2.3.4.3) Methyl (3-Formylphenoxy)acetate and Methyl (4-Formylphenoxy)acetate

A mixture of 3-hydroxybenzalde-hyde (or 4-hydroxybenzaldehyde, 10.0 g, 81.9 mmol) and K_2CO_3 (34.0 g, 245.7 mmol) in CH_3CN was stirred under reflux. After 1 h, methyl bromoacetate was added, and the mixture was allowed to stir under reflux for another 3 h. After the reaction was finished, CH_3CN was removed and the residue

was extracted with EtOAc (40 mL). The organic layer was dried over MgSO4 and evaporated to give methyl (3-formylphenoxy) acetate [or methyl (4-formyl-phenoxy) acetate] (15.6 g, 80.3 mmol, 98%) as a yellow oil without purification.

2.3.4.4) (3-Formylphenoxy) acetic acid and (4-Formylphenoxy)-acetic acid

To a solution of (3-formylphenoxy) acetate [or methyl (4-formylphenoxy) acetate] (14.9 g, 76.8 mmol) in THF (75 mL) was added 1 N NaOH(aq) (45 mL) at 0 °C for 30 min. The reaction mixture was allowed to stir at room temperature for 1.5 h. After the reaction was completed, 1 N NH_4Cl (aq) (65 mL) was added, and the mixture was extracted with EtOAc (40 mL). The organic layer was dried over Na_2SO_4 and purification by recrystallization from EtOAc to give (3-formylphenoxy) acetic acid [or (4-formyl-phenoxy) acetic acid] (12.4 g, 69.1 mmol) as colorless solids.

2.3.4.5) Methoxy-PEG (3-Formylphenoxy) acetate (2a) and Meth-oxy- PEG (4-Formylphenoxy) acetate (2b)

A solution of methoxy-PEG-OH5000 (15.0 g, 3.0 mmol) was treated with DCC (1.9 g, 9.0 mmol), DMAP (367 mg, 3.0 mmol) and sodium triacetoxy-borohydride (918 mg, 4.2 mmol) and NaOAc (230 mg, 2.8 mmol) at 0°C. The suspension was allowed to warm to room temperature and stirred for 5 h. The progress of reaction was monitored by TLC analysis (EtOAc/hexane, 3:2) of aliquots, which were treated with NaOMe/MeOH for 10 s before analysis. After the reaction completed, the mixture was added with brine (30 mL) and extracted with CH_2Cl_2 (40 mL × 3). The organic layer was dried over $MgSO_4$ and concentrated to about 7 mL. The residue was triturated with Et_2O (50 mL) to give precipitates. The mixture was cooled in an ice bath; the precipitates were filtered and washed successively with

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Et_2O/2-propanol (1: 1, 30 mL) to give 4a-d. Elemental analysis (six measure-ments) showed a nitrogen content of 0.24-0.27%, equivalent to a loading of 0.182 mmol/g on average (91-99%).

2.3.4.6) Linkage of N-(PEG-Bound) Amino Acid Methyl Esters 4a-d with 2-Nitrobenzoyl Chloride or 4,5-Dimethyl-2-nitrobenzoyl Chloride, Giving Amides 6a-h.

To a solution of N-(PEG-bound) amino acid methyl ester 4a (or 4c,d) (3.0 g, 0.6 mmol) in CH_2Cl_2 (20 mL) was added K_2CO_3 (312 mg, 2.4 mmol) and Bu_4NI (111 mg, 0.3 mmol). 2-Nitro-benzoyl chloride (5a, 0.17 mL, 1.2 mmol) [or 4,5-dimethyl-2-nitrobenzoyl chloride (5b, 295 mg, 1.2 mmol)] was then added dropwise into the mixture. The mixture was stirred at room temperature, and the progress of reaction was moni-tored by TLC analysis (EtOAc/hexane, 3:2) of aliquots, which were treated with NaOMe/MeOH for 10 s before analysis. After 5 h, the mixture was filtered through Celite, and the filtrate was concentrated to about 5 mL. The residue was cooled in an ice bath and triturated with Et_2O (30 mL) to give precipitates, which were filtered and washed suc-cessively with Et_2O/2-propanol (1:1, 25 mL). Yellow powders of PEG-bound amide 6a (or 6b-h) were obtained by drying under reduced pressure.

2.3.4.7) PEG-Bound Benzodiazepines 7a-h

PEG-bound amide 6a (or 6b-d) (3.0 g, 0.6 mmol) was dissolved in HOAc (20 mL) and treated with zinc powder (392 mg, 6 mmol) at room temperature for 2 h. The suspension was filtered through Celite, and the filtrate was evaporated under reduced pressure to give a thick mass of PEG-bound anilines without further purification. To a solution of the PEG-bound anilines (3.0 g, 0.6 mmol) in CH_3CN (60 mL) was added

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trifluoroacetic acid (1.5 mL) and its sodium salt (600 mg) at room temperature. The mixture was stirred for 13 h, after which CH_3CN was removed under reduced pressure. After addition of brine (25 mL), the mixture was extracted with CH_2Cl_2 (25 mL × 3). The organic layer was dried over $MgSO_4$ and concentrated to about 5 mL. The residue was cooled in an ice bath and triturated with Et_2O (30 mL) to give precipitates. The precipitates were filtered and washed successively with Et_2O/ 2-propanol (1:1, 30 mL) to give light yellow solids of the PEG-bound benzodiazepines 7a (or 7b-d). For the more reactive substrates 6e-h, reduction of the nitro group and the subsequent cyclization were conducted in a one pot procedure (zinc powder, HOAc, room temperature, 2 h) to give the PEG-bound benzodiazepines 7e-h. Alkylation of PEG-Bound Benzodiazepines 7a-h, Giv-ing Benzodiazepines 9a-p. To a mixture of PEG-bound benzodiazepines 7a-h (1.5 g, 0.3 mmol) and Cs_2CO_3 (1.5 g, 4.5 mmol) in DMF (15 mL) was added benzyl bromide (8a, 0.7 mL, 6.0 mmol) [or 3-methoxybenzyl bromide (8b, 0.9 mL, 6.0 mmol)] at room temperature. The progress of reaction was monitored by TLC analysis (EtOAc/hexane, 3:2) of aliquots, which were treated with NaOMe/MeOH for 10s before analysis. The reaction was completed in 2 h, and DMF was then removed under reduced pressure. After addition of brine (10 mL), the mixture was extracted with CH_2Cl_2 (10 mL × 3). The organic layer was dried over $MgSO_4$ and concentrated to about 5 mL. The residue was cooled in an ice bath and triturated with Et_2O (15 mL) to give precipitates, which were filtered and washed succes-sively with Et_2O/2-propanol (1:1, 20 mL). Light yellow powders of the PEG-bound benzodiazepines 9a-p were obtained by drying under reduced pressure. 1,4-Benzodiazepine-2,5-dione Dimethyl Ester Derivatives 10a-p. The PEG-bound benzodiazepine (9a-p, 1.5 g, 0.3 mmol) was dissolved in MeOH (5 mL) and treated with Na_2CO_3 (31.7 mg, 0.3 mmol) at room temperature. The progress of reaction was

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monitored by TLC analysis (EtOAc/hexane, 3:2) of aliquots, which were treated with NaOMe/MeOH for 10s before analysis. After the reaction was completed (stirring for 5 min), the mixture was cooled in an ice bath and triturated with Et_2O (5 mL) to give precipitates of MeO-PEG resin. The mixture was filtered through Celite, and the filtrate was concentrated under reduced pressure to give a crude product of 1,4-benzodiazepine-2,5-diones (10a-p). The purity of these crude products was determined by HPLC analyses. Purification of the crude product was carried out by preparative TLC by elution with gradients of EtOAc/ hexane (2:3 to 3:2).

2.3.5) Measurement of intracellular calcium concentration($[Ca^{2+}]_i$ assay)

The interaction of ET-1 with endothelin receptor is known to trigger an increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as the consequence of multistep biological events initiated by G-protein. To evaluate the potency of an endothelin receptor antagonist, one can measure its inhibitory ability against the ET-1 induced $[Ca^{2+}]_i$ change. According to the reported experimental protocol, Chinese hamster ovary (CHO-K1) cells were transfected with the rat ET_A-expression plasmid DNA using lipofectin reagent (Life Technologies Inc., USA). The ET_A overexpression CHO-K1 cells were prior incubated with calcium chelating agent fura-2 applied as its penta(acetoxymethyl) ester, and then treated with ET-1 in 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. This increment of functional assay was taken as the standard value (100%) to assess the inhibitory potency of compounds the ET-1 binding with receptor. On addition of the test sample (10^{-6} M) along with ET-1 (10^{-7} M), only increment of $[Ca^{2+}]_i$ was observed (a mean value of three measurements). By comparisons with the known ET_A antagonists, 10^{-6} M of SB209670 completely

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inhibited the ET-1 induced $[Ca^{2+}]_i$, whereas BQ123 showed ~ 60% inhibition under our assay conditions.

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