Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 261-266

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

DESIGN AND SYNTHESIS OF A BIOTINYLATED DOPAMINE TRANSPORTER LIGAND FOR THE PURIFICATION AND LABELING OF DOPAMINERGIC NEURONS

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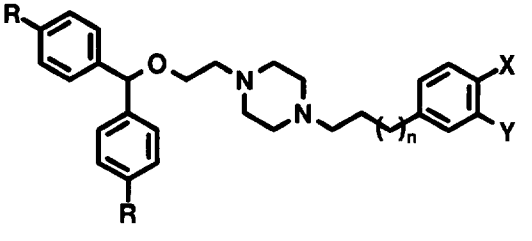
Received 15 September 1997; accepted 22 December 1997

Abstract: The design and synthesis of a new tool for labeling and purification of dopaminergic neurons is described.

Rationale: Dopaminergic neuron cultures, obtained by dissection from fetal rat mesencephalon, can maximally reach purities of 20% under optimized conditions. In order to get consistent results using mesencephalic dopaminergic neuron cultures (MDNs) for testing the survival promoting effects of neuroprotective compounds, new purification and identification procedures were envisaged.

Approach: GBR12935 is a highly selective ligand for the dopamine transporter system (DAT). Biotin was tethered to a GBR12935 derivative via a short linker yielding **CGP 81077**. The purification and identification methods were taking advantage of the high affinity of biotin to streptavidin. Streptavidin coupled fluorescein and streptavidin coated magnetic beads were bound to **CGP 81077**, allowing fluorescent staining of living dopaminergic neurons as well as affinity purification in a strong magnetic field.



R, X, Y = H; n = 1: **GBR12935** [1]
[^{3}H]DA uptake: IC_{50} = 3.7 ± 0.4 nM

R = F; X, Y = H; n = 1: **GBR12909** [1]
[^{3}H]DA uptake: IC_{50} = 4.3 ± 0.3 nM

R = H; X = N_3; Y = ^{125}I; n = 0: **^{125}I-DEEP** [2]

X = $O(CH_2)_3NH$-biotin; R, Y = H; n = 1: **CGP 81077**

Design of the linker: GBR12935 (1-[2-(diphenyl-methoxy)-ethyl]-4-(3-phenylpropyl) piperazine) has been reported to selectively inhibit ^{3}H-dopamine uptake with high potency [1, 3]. Using a close analog of GBR12935 (DEEP), a photoaffinity-labeled ligand had been synthesized by introducing an azido-group and 125-iodine into the terminal phenyl ring [2]. This successful use of the terminal phenyl ring for photoaffinity crosslinking indicated, that a longer side-chain could possibly be introduced in the para-position of the phenyl ring (position X).

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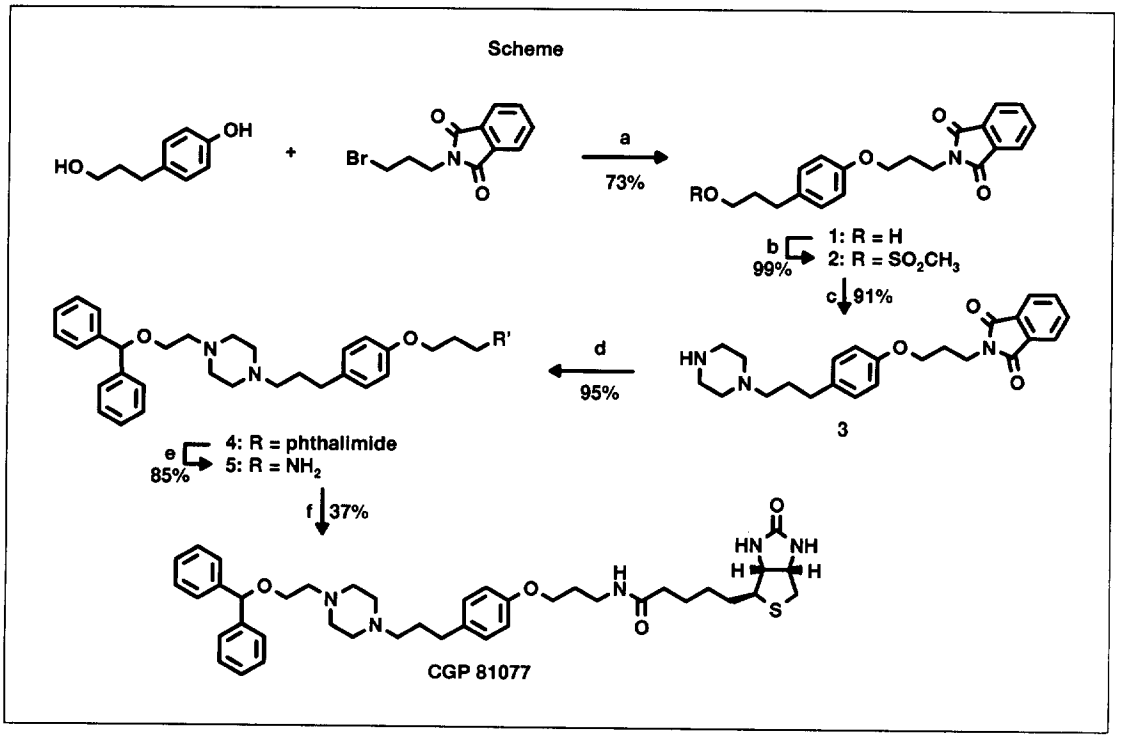
0960-894X/98/$19.00
PII: S0960-894X(98)00003-1

Synthesis of the biotinylated dopamine transport inhibitor CGP 81077 (see scheme): Commercially available 3-(4-hydroxyphenol)-propanol and N-(3-bromopropyl)-phthalimide were coupled under basic conditions giving the alcohol **1**. After introduction of the mesyl leaving group (**2**) the product was condensed with BOC-protected piperazine under vigorous conditions (NMP, 200°C), immediately followed by removal of the protecting group giving the piperazine derivative **3** in excellent yield. Under elevated temperature and base catalysis, 2-chloroethyl diphenylmethyl ether [4] was coupled to the secondary nitrogen of the piperazine ring (**4**). The phthalimide group was removed using N-methyl-hydrazine in EtOH, giving the free amine **5** in good yield. The biotinylated ligand **CGP 81077** was obtained by direct coupling of the free amine to (+)-biotin succinimide ester.



a) K_2CO_3, acetone, rflx, 24h; b) CH_3SO_2Cl, pyridine, 0°, 2h; c) piperazine-N-carboxylic acid tert.-butyl ester, N-methyl-pyrrolidone (NMP), 200°, 2h, then 1H HCl/dioxane; d) chloroethyl diphenylmethyl ether, K_2CO_3, DMF, 150°, 15h; e) N-methyl hydrazine, EtOH, 80°, 7h; f) (+)-biotin-N-succinimide ester, CH_2Cl_2, rt, 18h.

Dopamine uptake experiments: To demonstrate that the introduction of the biotinylated linker did not destroy the biological activity, dopamine uptake experiments were performed. A DAT expressing cell line was preincubated with various concentrations of **CGP 81077** or GBR12909 [1] and then the uptake of ^{3}H-dopamine was measured. GBR12909 is the bis-4-fluoro-phenyl derivative of GBR12935 and is widely used as a highly selective and potent dopamine uptake inhibitor. The results presented in figure 1 demonstrate that the

modified inhibitor **CGP 81077** was still able to bind to the dopamine transporter system (DAT) of a DAT expressing cell line and to inhibit dopamine uptake.

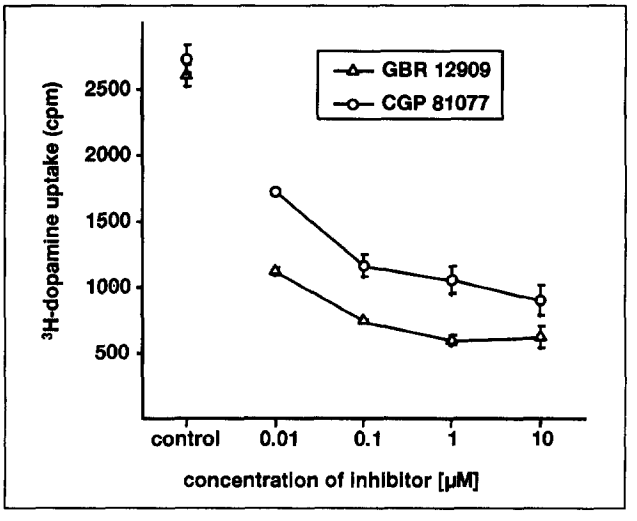


Figure 1: Inhibition of ^{3}H-dopamine uptake after preincubation with GBR12909 (△) or **CGP 81077** (○)

Cell culture, purification of dopaminergic neurons and selectivity: Dopaminergic neuron cultures are routinely prepared from embryonic rat ventral mesencephalon. However, the percentage of dopaminergic tyrosine hydroxylase positive (TH^+) cells present in those cultures is usually only between 0.1 and 2.5%. With an optimized dissection technique [5] cultures containing up to 20% TH^+ cells can be obtained. These dopaminergic neurons are intermingled with glutamatergic, GABA-ergic and serotonergic neurons [6, 7].

In order to study dopaminergic neurons in more detail, DAT expressing cells from the ventral mesencephalon were purified with the new tool **CGP 81077**. Dopaminergic neuron cultures with purities up to >95% were obtained by capturing **CGP 81077**-labeled neurons with streptavidin coated magnetic micro-beads in a strong magnetic field.

As demonstrated in figure 2, virtually all neurons isolated using the new tool **CGP 81077** were TH^+. TH can be used as a marker for dopaminergic cells in these cultures, since it is excluded by the dissection procedure that the tissue sample includes other tyrosine hydroxylase expressing cells like noradrenergic neurons [5]. Although GBR12935 has the highest affinity to the dopamine transporter it also binds with lower affinity to the serotonin and norepinephrine transporter (IC_{50} = 3.7nM, 289nM, 1261nM resp. [1]). The presence of noradrenergic neurons is excluded by the dissection procedure, but the ventral mesencephalon contains a low percentage of serotonergic neurons [7]. However, the selectivity of **CGP 81077** is sufficient to differentiate between dopaminergic and serotonergic neurons under the purification conditions described below. Co-

purification of serotonergic neurons from the ventral mesencephalon seemed not to occur, since no significant number of TH-negative neurons was detectable after the purification.

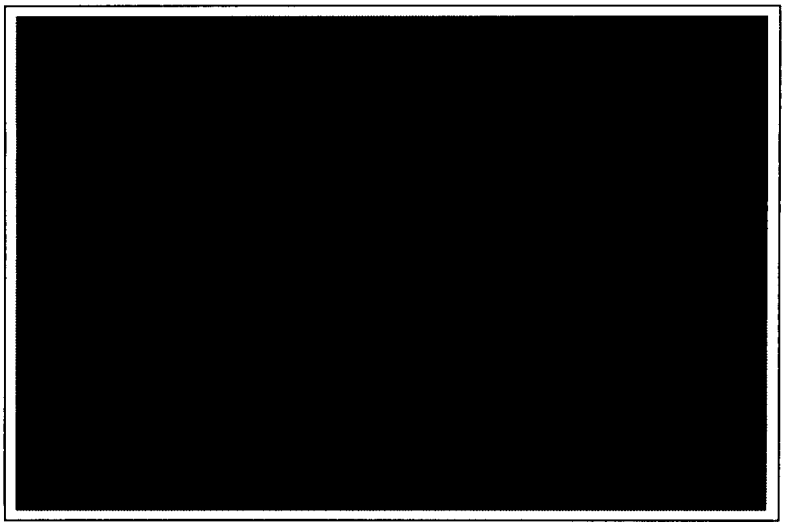


Figure 2: TH^+ cells from embryonic rat ventral mesencephalon after purification with **CGP 81077**

Discussion: The new tool **CGP 81077** allowed the affinity purification of freshly prepared mesencephalic dopaminergic neurons to purities up to >95% by capturing the **CGP 81077** labeled neurons with streptavidin coated magnetic micro-beads in a strong magnetic field. All neurons obtained were TH^+. A high selectivity for dopaminergic neurons is guaranteed due to the applied dissection technique together with the favorable affinity of the ligand to the DAT. In addition the fluorescent staining of living dopaminergic neurons became possible using streptavidin coupled fluorescein. Experimental details will be reported elsewhere. The new tool will also allow further applications, e.g. purification of MDNs from different species or quantification of MDNs in lesioned animals.

Experimental Section:

Cell culture and dopamine uptake: The rat ventral mesencephalon from E15 rat embryos was prepared as described [5] and the cells were dispersed by trituration with a fire-polished pasteur pipette after treatment with papain (150 µg/ml in Hank's buffered salt solution, HBSS) for 5 min. at 37°C. Dopaminergic neurons were labeled for 5 min. with 0.1 µM **CGP 81077**, washed three times with HBSS to remove the unbound inhibitor and incubated with 10 µl MACS Streptavidin micro-beads (Miltenyi Biotech GmBH, Bergisch Gladbach, Germany) for 15 min. on ice. The magnetic beads-labeled cells were purified on a MINI-MACS column (Miltenyi Biotech GmBH; Bergisch Gladbach, Germany), eluted from the column by releasing the magnetic field and plated on poly-lysine coated 8-well chamber slides (Biocoat, Becton Dickinson, Bedford,

MA, USA). The dopaminergic cells in the cultures were stained with an anti-TH antibody (Boehringer Mannheim, Germany) as recommended by the manufacturer. Dopamine uptake was measured as described [8] except that HBSS was used for preparing the uptake buffer.

Preparation of 2-{3-[4-(3-hydroxy-propyl)-phenoxy]-propyl}-isoindole-1,3-dione (1): 3-(4-Hydroxyphenol)-propanol (1.52 g, 10 mmol), N-(3-bromopropyl)-phthalimide (3.20 g, 12mmol) and dry potassium carbonate (6.91 g) were refluxed in 25 ml acetone for 24 h. After filtration and removal of solvent, the residue was chromatographed (SiO_2, hexane-AcOEt (1:1)), yielding **1** (2.48 g, 7.3 mmol, 73%) as white crystals. TLC (SiO_2, hexane-AcOEt (1:1)): R_f = 0.13. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.88 (m, 2H), 2.16 (quint., 2H), 2.62 (t, 2H), 3.65 (t, 2H), 3.90 (t, 2H), 4.00 (t, 2H), 6.74 (m, 2H), 7.05 (m, 2H), 7.71 (m, 2H), 7.85 (m, 2H). ES-MS: 340 (M+1).

Preparation of methanesulfonic acid 3-{4-[3-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-propoxy]-phenyl}-propylester (2): To a 0°C pyridine solution (100 ml) of **1** (12.60 g, 37.13 mmol) methanesulfonylchloride (4.32 g, 55.69 mmol) was slowly added, then stirred for 16 h. All volatiles were evaporated, 500 ml H_2O was added to the residue, acidified with 2N HCl_{aq}, extracted with AcOEt, the organic layer washed with H_2O, dried over Na_2SO_4 and the solvent evaporated, giving **2** (15.30 g, 36.65 mmol, 99%) as crude white crystals. TLC (SiO_2, AcOEt): R_f = 0.70. ^{1}H-NMR ($CDCl_3$, 200 MHz): 2.03 (m, 2H), 2.16 (quint., 2H), 2.68 (t, 2H), 3.00 (s, 3H), 3.92 (t, 2H), 4.01 (t, 2H), 4.20 (t, 2H), 6.73 (m, 2H), 7.05 (m, 2H), 7.71 (m, 2H), 7.85 (m, 2H). ES-MS: 418 (M+1).

Preparation of 2-{3-[4-(3-piperazine-1-yl-propyl)-phenoxy]-propyl}-isoindole-1,3-dione (3): A mixture of **2** (12.83 g, 30.76 mmol) and piperazine-N-carboxylic acid tert.-butylester (11.44 g, 61.52 mmol) in 1.5 ml N-methyl-pyrrolidone (NMP) was heated at 200°C for 2 h, then cooled, filtered, dioxane and 7H HCl in dioxane added (finally ca. 1N HCl). After 16 h all volatiles were evaporated, the residue dissolved in CH_2Cl_2, washed with saturated aqueous $NaHCO_3$-solution, water and brine. The solvent was dried over Na_2SO_4 and evaporated, yielding **3** (11.37 g, 27.90 mmol, 91%) as a crude resin. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.75 (m, 2H), 2.00-2.60 (m, ca. 12H), 2.95 (t, 2H), 3.89 (t, 2H), 4.00 (t, 2H), 6.72 (m, 2H), 7.05 (m, 2H), 7.70 (m, 2H), 7.82 (m, 2H). ES-MS: 408 (M+1).

Preparation of 2-[3-(4-{3-[4-(2-benzhydryloxy-ethyl)-piperazine-1-yl]-propyl}-phenoxy)-propyl]-isoindole-1,3-dione (4): A 150°C DMF-solution (300 ml) of **3** (13.96 g, 34.29 mmol), 2-chloroethyl-diphenylmethylether (8.43 g, 34.29 mmol) (prepared according to [4]) and dry potassium carbonate (4.73 g, 34.29 mmol) was heated for 15 h. DMF was removed under high-vacuum, then ice-water was added and the product extracted with AcOEt. The organic layer was washed with brine, dried over Na_2SO_4 and the solvent evaporated, giving **4** (20.12 g, 32.57 mmol, 95%) as crude viscous oil. TLC (SiO_2, CH_2Cl_2-MeOH (19:1)): R_f = 0.20. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.72 (m, 2H), 2.08-2.70 (m, ca. 12H), 3.55-3.70 (m, 4H), 3.89 (t, 2H), 4.00 (t, 2H), 5.35 (s, 1H), 6.71 (m, 2H), 7.02 (m, 2H), 7.20-7.37 (m, 10H), 7.70 (m, 2H), 7.82 (m, 2H). ES-MS: 618 (M+1).

Preparation of 3-(4-{3-[4-(2-benzhydryloxy-ethyl)-piperazine-1-yl]-propyl}-phenoxy)-propyl-amine (5): A solution of 4 (20.12 g, 32.60 mmol) and methyl hydrazine (3.42 ml, 65.21 mmol) was heated for 7 h at 80°C. The cooled mixture was treated with 230 ml of 4N HCl_{aq} for 30 min. then made alkaline by adding KOH-pellets. Extraction with CH_2Cl_2, washing of the organic layer with brine, drying over Na_2SO_4 and solvent evaporation yielded 5 (13.41 g, 27.5 mmol, 85%) as a crude brownish oil. ^{1}H-NMR ($CDCl_3$, 200 MHz): 1.70-1.95 (m, 6H), 2.25-2.60 (m, ca. 8H), 2.68 (t, 2H), 2.90 (t, 2H), 3.59 (t, 2H), 4.01 (t, 2H), 5.37 (s, 1H), 6.71 (m, 2H), 7.09 (m, 2H), 7.20-7.39 (m, 10H). ES-MS: 488 (M+1).

Preparation of 5-([3aS-{3aα, 4β, 6aα}]-2-oxo-hexahydro-thieno[3,4-d]imidazol-6-yl)-pentanoic acid [3-(4-{3-[4-(2-benzhydryl-oxy-ethyl)-piperazine-1-yl]-propyl}-phenoxy)-propyl]-amine (CGP 81077): A CH_2Cl_2-solution (9 ml) of 5 (1.27 g, 2.61 mmol) and (+)-biotin-N-succinimide ester (0.89 g, 2.61 mmol) was stirred at rt for 18 h, then directly crystallized from Et_2O. The crude, crystalline product was chromatographed (SiO_2, CH_2Cl_2-MeOH (9:1) and again crystallized from Et_2O, yielding **CGP 81077** (688 mg, 0.96 mmol, 37%) as yellowish crystals. TLC (SiO_2, CH_2Cl_2-MeOH (19:1)): R_f = 0.20. ^{1}H-NMR ($CDCl_3$, 200 MHz): not interpreted, compatible with expected product. ES-MS: 714 (M+1); $[\alpha]_D$ (MeOH, c=1) +28.8°; Anal. $C_{41}H_{55}N_5O_4S$: C 65.47 (calc. 68.97), H 7.71 (7.76), N 9.85 (9.81). S 4.46 (4.49).

Acknowledgement:

We are grateful for the technical support to Mrs. B. Bohrer, Mrs. C. Marx and Mrs. D. Mathé.

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