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Synthesis and Receptor Binding Affinity of New Selective GluR5 Ligands

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Abstract—Two hybrid analogues of the kainic acid receptor agonists, 2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid (ATPA) and (2*S*,4*R*)-4-methylglutamic acid ((2*S*,4*R*)-4-Me-Glu), were designed, synthesized, and characterized in radioligand binding assays using cloned ionotropic and metabotropic glutamic acid receptors. The (*S*)-enantiomers of *E*-4-(2,2-dimethylpropylidene)glutamic acid ((*S*)-**1**) and *E*-4-(3,3-dimethylbutylidene)glutamic acid ((*S*)-**2**) were shown to be selective and high affinity GluR5 ligands, with K_i values of 0.024 and 0.39 μM, respectively, compared to K_i values at GluR2 of 3.0 and 2.0 μM, respectively. Their affinities in the [^{3}H]AMPA binding assay on native cortical receptors were shown to correlate with their GluR2 affinity rather than their GluR5 affinity. No affinity for GluR6 was detected ($IC_{50} > 100$ μM).

Introduction

Receptors for the major excitatory amino acid neurotransmitter (*S*)-glutamic acid ((*S*)-Glu) are divided into two major classes, the ionotropic and the metabotropic Glu receptors.[1–3] The ionotropic Glu receptors mediate fast excitatory responses through cation channels, whereas the metabotropic receptors mediate slower signals through G-proteins and second messenger systems. The class of ionotropic Glu receptor consists of *N*-methyl-D-aspartic acid (NMDA), 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) and kainic acid (KAIN) receptors, and the class of metabotropic Glu receptor comprises three groups named Group I, II and III.

Receptors containing NMDA subunits (NR1, NR2a–d, and NR3) or AMPA subunits (GluR1–4) have been extensively characterized pharmacologically, whereas only a limited number of ligands are available for studies of receptors containing KAIN receptor subunits (GluR5-7 and KA1-2).[4,5] Two agonists have, however, been useful tools for investigation of the KAIN receptor subunits. Thus, (2*S*,4*R*)-4-methylglutamic acid ((2*S*,4*R*)-4-Me-Glu) has shown some selectivity for KAIN receptor subunits,[6,7] and 2-amino-3-(5-*tert*-butyl-3-hydroxy-4-isoxazolyl)propionic acid (ATPA), which was originally described as a weak AMPA receptor agonist,[8] has been shown to be a highly potent and selective agonist at GluR5.[9,10]

We have used the structures of (2*S*,4*R*)-4-Me-Glu and ATPA for the design of new hybrid structures as potential ligands at GluR5. The syntheses of the enantiomers of *E*-4-(2,2-dimethylpropylidene)glutamic acid, (*S*)-**1** and (*R*)-**1**, and *E*-4-(3,3-dimethylbutylidene)glutamic acid, (*S*)-**2** and (*R*)-**2** (Fig. 1), are described in this paper. Their receptor selectivity has been determined in binding assays on native receptors, on cloned AMPA and KAIN receptor subunits expressed as homomers in stable cell lines, and on cloned metabotropic Glu receptors.

Results

Chemistry

The synthesis of the enantiomers of **1** and **2** commenced with the preparation of the two enantiomers of **3** prepared from commercially available (*S*)- and (*R*)-Glu (Scheme 1). Generation of the kinetic enolate of (*S*)- and (*R*)-**3** followed by addition of the appropriate aldehyde[11] gave aldol adducts **4** and **5** in 85–90% isolated yield as

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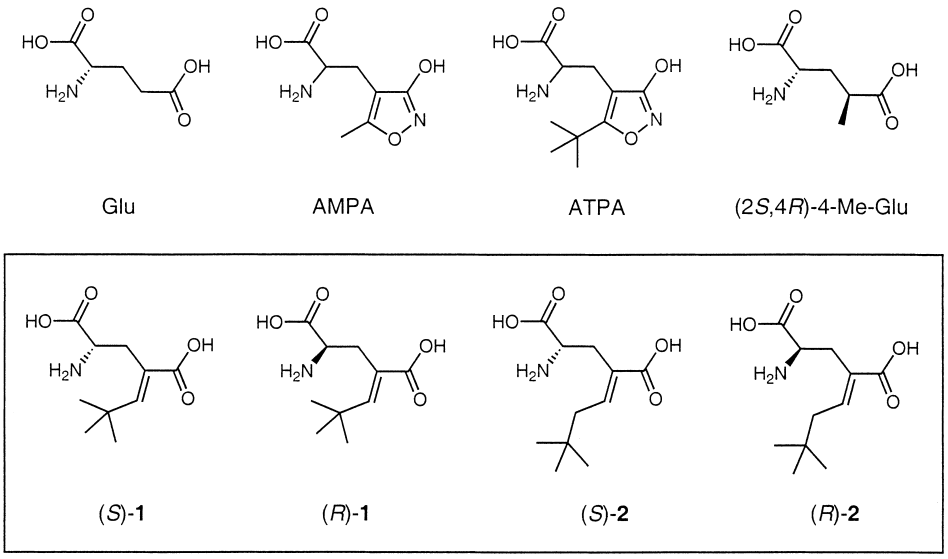
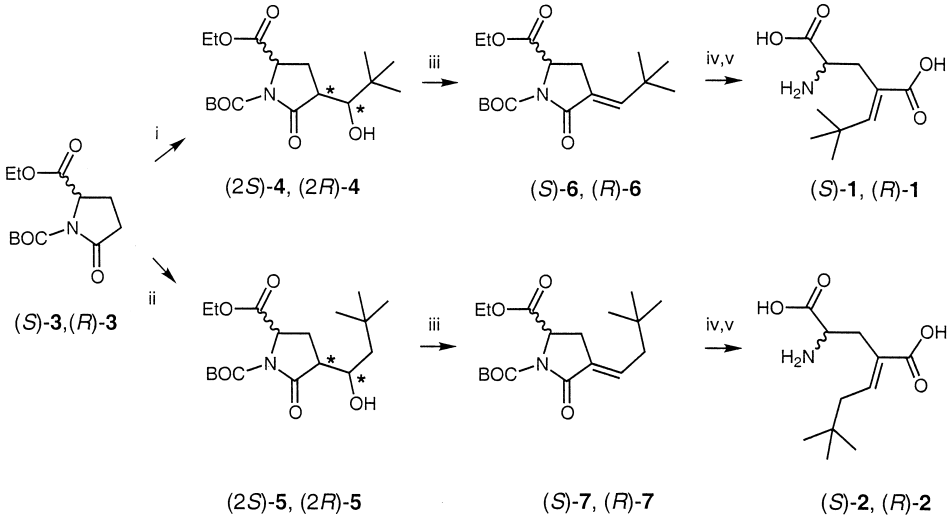


Figure 1.



Scheme 1. (i) (a) LHMDS, THF, −78 °C; (b) $(CH_3)_3CHO$, $Et_2O{\cdot}BF_3$, −78 °C; (ii) (a) LHMDS, THF, −78 °C; (b) $(CH_3)_3CH_2CHO$, $Et_2O{\cdot}BF_3$, −78 °C; (iii) MsCl, DMAP, Et_3N, CH_2Cl_2; (iv) LiOH, H_2O, THF; (v) HCl/EtOAc.

mixtures of diastereomers. Further exploration of the diastereomeric composition of **4** and **5** was considered redundant since both of the newly created chiral centers were removed in the following step. Elimination of the β-hydroxy groups to give the α,β-unsaturated imides **6** and **7** was achieved via formation of the mesylate derivatives. For all compounds, only the *E* isomer was observed, which is in agreement with earlier reported studies.[11,12] Hydrolysis of the imide and ester functionalities with LiOH/THF[11] gave, together with the expected *N*-BOC protected diacid, 5–10% of the pyroglutamate derivative resulting from hydrolysis of the carbamate functionality. This ratio of *endo*/*exo*-cyclic cleavage of the imide group under basic conditions in carbamate protected pyroglutamate systems has previously been addressed,[13] and in agreement with these findings the undesired *exo* cleavage could not be avoided. Flash chromatography gave pure *N*-BOC protected diacid which, upon removal of the *N*-BOC protecting group under acidic conditions, provided the enantiomers of the 4-alkylidene substituted Glu analogues **1** and **2** [94–99% enantiomeric excess (ee) as determined by chiral HPLC analysis]. The HCl salts of the enantiomers of **1** and **2** could be recrystallized directly or converted into their zwitterions upon treatment with propylene oxide and then recrystallized.

In vitro pharmacology

The affinity of the new compounds, (*S*)-**1**, (*R*)-**1**, (*S*)-**2** and (*R*)-**2**, and of the reference compounds AMPA, KAIN, ATPA, and (2*S*,4*R*)-Me-Glu for native AMPA, KAIN and NMDA receptors was determined using the ligands [^{3}H]AMPA,[14] [^{3}H]KAIN[15] and [^{3}H]CPP,[16] respectively (see Table 1). None of the new compounds showed detectable affinity ($IC_{50} > 100\,\mu M$) in the [^{3}H]KAIN or the [^{3}H]CPP binding assays, whereas (*S*)-**1** and (*S*)-**2** showed moderate affinity for the [^{3}H]AMPA binding site. Similar affinity to the [^{3}H]AMPA binding site was observed for ATPA (Table 1), in agreement with earlier findings.[8]

Table 1. Affinities for native and cloned ionotropic Glu receptors

	IC_{50} (μM)			K_i (μM)		
	[^{3}H]AMPA	[^{3}H]KAIN	[^{3}H]CPP	GluR2	GluR5	GluR6
(*S*)-**1**	1.95±0.13	>100	>100	3.0±0.5	0.024±0.005	>100
(*R*)-**1**	>100	>100	>100	101±31	0.78±0.16	>100
(*S*)-**2**	6.9±0.8	>100	>100	2.0±0.2	0.39±0.09	>100
(*R*)-**2**	>100	>100	>100	>100	42±6	>100
ATPA	3.9±0.6	31±1	>100	2.3±0.2	0.0076±0.0021	>100
AMPA	0.04±0.02	>100	>100	0.0082±0.0032	1.9±0.2	>100
KAIN	4.0±1.2	0.007±0.002	>100	nt	0.083±0.006	0.021±0.010
(2*S*,4*R*)-4-Me-Glu[a]	26.6±3.6	0.032±0.003	5.9±0.9[b]	nt	nt	0.0019±0.002

Values represent mean±SEM, $n \geq 3$.
Nt: not tested.
[a]ref 7.
[b]IC_{50} value determined using [^{3}H]CGP39653 as the radioligand.

The affinity for cloned homomeric GluR2, GluR5 and GluR6 expressed in HEK-293 cells was also determined. ATPA had the highest affinity for GluR5, $K_i = 0.0076\,\mu M$, compared to 0.024 μM for (*S*)-**1** and 0.39 μM for (*S*)-**2**. (*R*)-**1** (94% ee) showed some affinity for GluR5, probably reflecting the contamination by (*S*)-**1**, but was virtually without affinity for other receptors. ATPA, (*S*)-**1** and (*S*)-**2** all showed moderate affinity for GluR2, but no affinity for GluR6 (Table 1).

None of the compounds showed significant activity as agonists or antagonists (up to 1000 μM) at the metabotropic receptors mGluR1, 2 or 4, representing Group I, II and III, respectively.[17]

Discussion

Apart from KAIN and other kainoids, two Glu analogues have previously been described as KAIN receptor agonists, (2*S*,4*R*)-4-Me-Glu and ATPA, the former as a non-selective Glu6 agonist,[7,18] whereas ATPA is a very potent and selective GluR5 agonist.[9,10] Using these two analogues as leads two new potential GluR5 agonists were designed and synthesized in their enantiomeric forms, (*S*)-**1**, (*R*)-**1**, (*S*)-**2** and (*R*)-**2** (Fig. 1). Through protected (*S*)- and (*R*)-pyroglutamate and enolate formation, the appropriate substituents were introduced affording the desired compounds after deprotection and ring opening (Scheme 1). The compounds were obtained with different degrees of enantiomeric purity (ee 94–>99%).

Pharmacological characterization showed the *S*-forms to be potent inhibitors at GluR5, (*S*)-**1** having the highest affinity ($K_i = 0.024\,\mu M$) and (*S*)-**2** a somewhat lower affinity ($K_i = 0.39\,\mu M$). Both compounds showed moderate affinity for GluR2 and no affinity for GluR6 (Table 1), (*S*)-**1** showing much higher selectivity for GluR5 than (*S*)-**2**. In contrast to the affinity determined for (*S*)-**1** and (*S*)-**2** at GluR5, no affinity was observed in the [^{3}H]KAIN binding assay and only moderate affinity in the [^{3}H]AMPA binding assay. This indicates that the [^{3}H]KAIN binding assay is of very limited value for detection of affinity for GluR5 receptors, whereas the moderate affinity observed in the [^{3}H]AMPA binding assay on native receptors appears to correlate with GluR2 binding. These findings are in agreement with the binding data for ATPA, which also shows moderate affinity for the [^{3}H]AMPA binding site and very high affinity for GluR5 (Table 1).

In conclusion, (*S*)-**1** and (*S*)-**2** are both selective ligands for GluR5, (*S*)-**1** showing the highest affinity and selectivity. In contrast to (2*S*,4*R*)-4-Me-Glu, they show no affinity for GluR6. (*S*)-**1** shows a receptor binding profile similar to that of ATPA, although ATPA has slightly higher affinity for GluR5.

Experimental

General methods. All reagents were obtained from commercial suppliers. All reactions were carried out in a nitrogen atmosphere. ^{1}H NMR spectra were obtained on a Varian 300 MHz spectrometer with $CHCl_3$ as reference. Analytical HPLC for determination of ee was performed using a Sumichiral OA-5000 column (4.6×150 mm). The column was kept at 60 °C and eluted at 1.5 mL/min with a mobile phase consisting of 85% of an aqueous solution (50 mM NH_4OAc and 0.1 mM $Cu(OAc)_2$, pH adjusted to 4.7 using AcOH), and 15% isopropanol. The column was connected to a TSP HPLC system (Bie & Berntsen A/S, Copenhagen, Denmark) consisting of a P4000 pump, an AS3000 autoinjector and an SM5000 PDA detector.

Ethyl (*S*)-*E*-1-(*tert*-butoxycarbonyl)-4-(2,2-dimethylpropylidene)pyroglutamate ((*S*)-6). To HMDS (3.58 mL; 17.2 mmol) in dry THF (100 mL) at −78 °C was added dropwise *n*-BuLi (6.92 mL, 2.5 M in hexane; 17.2 mmol). The reaction mixture was then allowed to stir at −78 °C for 15 min. (*S*)-**3** (4.0 g; 15.6 mmol) dissolved in dry THF (10 mL) was added dropwise and the reaction mixture stirred for 1 h at −78 °C. Pivalaldehyde (1.87 mL; 17.2 mmol) was added followed by freshly distilled $Et_2O{\cdot}BF_3$ (2.18 mL; 17.2 mmol). Stirring was continued at −78 °C for 3 h, then quenched with saturated NH_4Cl and the aqueous layer extracted with Et_2O. The collective organic layers were washed with saturated NaCl, dried (Na_2SO_4) and evaporated to give crude (*S*)-**4**, which was submitted to flash chromatography (heptane/EtOAc, 7:3) to give (*S*)-**4** (4.75 g; 89%) as a mixture of diastereomers.

To (*S*)-**4** (3.40 g; 9.91 mmol) and DMAP (1.33 g; 10.9 mmol) dissolved in dry CH_2Cl_2 and cooled to 0 °C, was added Et_3N (6.90 mL; 49.55 mmol) followed by MsCl (0.84 mL; 10.90 mmol). The cooling bath was removed and stirring was continued for 30 h at rt. The reaction mixture was diluted with CH_2Cl_2 and the organic layer washed with 1 M $NaHCO_3$, 1 M HCl, saturated NaCl, dried (Na_2SO_4), and evaporated to give crude (*S*)-**6**. Flash chromatography (heptane/EtOAc, 8:2) gave (*S*)-**6** (2.55 g; 80%) as a colorless solid. Crude (*S*)-**4** was also used directly in this step with comparable results. ^{1}H NMR ($CDCl_3$) δ 6.68 (1H, br t, J = 2.7 Hz), 4.54 (1H, dd, J = 10.3, 3.6 Hz), 4.19 (2H, q, J = 7.1 Hz), 3.10 (1H, ddd, J = 17.3, 10.3, 3.0 Hz), 2.75 (1H, ddd, J = 17.3, 3.6, 2.5 Hz), 1.48 (9H, s), 1.24 (3H, t, J = 7.1 Hz), 1.11 (9H, s); ^{13}C NMR ($CDCl_3$) δ 171.47, 167.33, 150.03, 148.79, 124.67, 83.38, 61.54, 55.82, 33.62, 29.22, 27.76, 26.06, 13.97.

Ethyl (*R*)-*E*-1-(*tert*-butoxycarbonyl)-4-(2,2-dimethylpropylidene)pyroglutamate ((*R*)-6). The preparation of (*R*)-**6** was carried out according to the procedure for (*S*)-**6** to give 71% (two steps) as a colorless solid. ^{1}H NMR and ^{13}C NMR were identical to the spectra of (*S*)-**6**.

Ethyl (*S*)-*E*-1-(*tert*-butoxycarbonyl)-4-(3,3-dimethylbutylidene)pyroglutamate ((*S*)-7). The preparation of (*S*)-**7** was carried out according to the procedure for (*S*)-**6** to give 69% (two steps) as a colorless solid. ^{1}H NMR ($CDCl_3$) δ 6.78 (1H, br tt, J = 8.2, 2.7 Hz), 4.58 (1H, dd, J = 10.2, 3.6 Hz), 4.18 + 4.16 (2H, q + q, J = 7.1 Hz), 2.92 (1H, dddt, J = 17.3, 10.2, 3.0, 1.7 Hz), 2.58 (1H, dddt, J = 17.3, 3.6, 2.5, 1.2 Hz), 1.99 (2H, br d, J = 8.2 Hz), 1.48 (9H, s), 1.23 (3H, t, J = 7.1 Hz), 0.89 (9H, s); ^{13}C NMR ($CDCl_3$) δ 171.37, 166.02, 150.13, 137.17, 129.80, 83.41, 61.54, 55.80, 43.44, 32.08, 29.16, 27.76, 25.78, 13.95.

Ethyl (*R*)-*E*-1-(*tert*-butoxycarbonyl)-4-(3,3-dimethylbutylidene)pyroglutamate ((*R*)-7). The preparation of (*R*)-**7** was carried out according to the procedure for (*S*)-**6**, to give 67% (two steps) as a colorless solid. ^{1}H NMR and ^{13}C NMR were identical to the spectra of (*S*)-**7**.

(*S*)-*E*-4-(2,2-Dimethylpropylidene)glutamic acid ((*S*)-1) hydrochloride. To (*S*)-**6** (570 mg; 1.75 mmol) dissolved in THF (13.2 mL) was added LiOH (12.6 mL, 2.5 M; 31.5 mmol). The two-phase reaction mixture was stirred vigorously for 4 h at rt and then cooled to 0 °C. pH was adjusted to 2 with 1 M HCl and the aqueous layer extracted repeatedly with Et_2O. The collective organic layers were washed with saturated NaCl, dried (Na_2SO_4) and evaporated to give crude diacid. Flash chromatography (CH_2Cl_2/MeOH/AcOH, 100:5:2) gave pure diacid.

The diacid was dissolved in saturated HCl/EtOAc (20 mL) and stirred at rt for 1 h. The solvent was removed under reduced pressure and the remaining solid triturated with Et_2O to give crude (*S*)-**1**,HCl (330 mg), which was recrystallized (AcOH/EtOAc) to give (*S*)-**1**,HCl (271 mg; 63%): 94% ee. Mp 168–172 °C. Elemental analysis ($C_{10}H_{18}ClNO_4$): calcd 47.72% C, 7.21% H, 5.56% N, found 47.91% C, 7.07% H, 5.49% N. ^{1}H NMR (D_2O) δ 7.09 (1H, br s), 4.20 (1H, t, J = 8 Hz), 3.20 (1H, dd, J = 14.3, 8 Hz), 2.98 (1H, dd, J = 14.3, 8 Hz), 1.18 (9H, br s).

(*R*)-*E*-4-(2,2-Dimethylpropylidene)glutamic acid ((*R*)-1) hydrochloride. The preparation of (*R*)-**1** was carried out according to the procedure for (*S*)-**1** to give (*R*)-**1**,HCl (316 mg; 59%) as a colourless solid; 94% ee. Mp 169–172 °C. Elemental analysis ($C_{10}H_{18}ClNO_4$): calcd 47.72% C, 7.21% H, 5.56% N, found 47.85% C, 7.15% H, 5.45% N. ^{1}H NMR was identical to the spectrum of (*S*)-**1**.

(*S*)-*E*-4-(3,3-Dimethylbutylidene)glutamic acid ((*S*)-2). The preparation of (*S*)-**2** was carried out according to the procedure for (*S*)-**1** to give 380 mg (69%) of the crude HCl salt, which was converted into its corresponding zwitterion by treatment with 10 equiv propylene oxide in 70% *i*-PrOH–H_2O at rt for 1 h. Re-crystallization (H_2O) gave (*S*)-**2** (151 mg, 39%) as a colorless solid: > 99% ee. Mp 180–183 °C. Elemental analysis ($C_{11}H_{19}NO_4$, 1/8 H_2O): calcd 57.06% C, 8.38% H, 6.05% N, found 57.17% C, 8.24% H, 5.88% N. ^{1}H NMR (D_2O) δ 6.67 (1H, br t, J = 8 Hz), 3.36 (1H, br dd, 9, 4.5 Hz), 2.74 (1H, dd, 13.5, 4.5 Hz), 2.51 (1H, dd, 13.5, 9 Hz), 2.10 (2H, m), 0.91 (9H, br s).

(*R*)-*E*-4-(3,3-Dimethylbutylidene)glutamic acid ((*R*)-2). The preparation of (*R*)-**2** was carried out according to the procedure for (*S*)-**2** to give 240 mg (37%) as a colorless solid: > 99% ee. Mp 180–183 °C. Elemental analysis ($C_{11}H_{18}NO_4$, 1/4 H_2O): calcd 56.52% C, 8.41% H, 5.99% N, found 56.77% C, 8.24% H, 5.83% N. ^{1}H NMR was identical to the spectrum of (*S*)-**2**.

In vitro pharmacology

Affinity for AMPA, KAIN and NMDA receptor sites was determined using the respective ligands [^{3}H]AMPA, [^{3}H]KAIN and [^{3}H]CPP, and the experimental procedures previously described.[14–16] Effects at the mGlu receptor subtypes, mGlu1α, mGlu2 and mGlu4a were studied following previously described procedures.[17]

Transfected cell lines

HEK-293 cell lines stably expressing $GluR2_i$, GluR5-1a, and GluR6 were established by transfection with NeuroSearch designed expression vectors derived from pcDNA3 (InVitrogen) incorporating cDNA for human GluRs using either zeocin or G418 as selection.

Ligand binding studies

HEK-293 cells with stable expression of GluRs were harvested and washed once with Tris–HCl buffer (50 mM pH 7.1) and stored at −80 °C until the day of the experiment. The membrane pellets were washed once with Tris–HCl buffer (50 mM pH 7.1) to remove endogenous (*S*)-Glu (resuspension in > 100 volumes of ice-cold buffer and centrifugation at 27,000×*g* for 10 min) and the final pellets were resuspended in assay buffer and used for binding experiments. All procedures were performed at 0–4 °C.

GluR2 receptors were labelled with 5 nM [^{3}H]AMPA in Tris–HCl buffer (30 mM pH 7.1) containing 2.5 mM $CaCl_2$ and 100 mM KSCN at 25–30 μg protein/assay. The samples were incubated in triplicate for 30 min at 2 °C. Binding to GluR5 receptors was performed at 3 nM [^{3}H]ATPA (previously described as a GluR5 radioligand[19]) in Tris–HCl buffer (50 mM pH 7.1) at 67–125 μg protein/assay. The samples were incubated in triplicate for 60 min at 2 °C. GluR6 receptors were labelled with [^{3}H]KAIN (5 nM) in Tris–HCl buffer (50 mM pH 7.1) at 22–27 μg protein/assay and incubated for 60 min at 2 °C. In all assays non-specific binding was determined in the presence of 0.6 mM (*S*)-Glu and binding was terminated by rapid filtration over Whatman GF-C glass fiber filters. The amount of radioactivity on the filters was determined by conventional liquid scintillation counting using a Tri-carb™ liquid scintillation analyzer (model 1600CA; Packard, USA) with a counting efficiency of 58%. In all assays the specific binding was > 78%. All estimates of binding parameters was performed by non-linear regression analysis using Graph-Pad Prism Software ver. 2.01.

Acknowledgements

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