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Functionalized Congeners of Tyrosine-Based $P2X_7$ Receptor Antagonists: Probing Multiple Sites for Linking and Dimerization

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Abstract

Chemically funtionalized analogues of antagonists of the $P2X_7$ receptor, an ATP-gated cation channel, were synthesized as tools for biophysical studies of the receptor. These functionalized congeners were intended for use in chemical conjugation with retention of biological potency. The antagonists were L-tyrosine derivatives, related to [*N*-benzyloxycarbonyl-*O*-(4-arylsulfonyl)-L-tyrosyl]benzoylpiperazine (such as MRS2409, **2**). The analogues were demonstrated to be antagonists in an assay of human $P2X_7$ receptor function, consisting of inhibition of ATP-induced K^+ efflux in HEK293 cells expressing the recombinant receptor. The analogues were of the general structure R_1-Tyr(OR_2)-piperazinyl-R_3, in which three positions (R_1–R_3) were systematically varied in structure through introduction of chemically reactive groups. Each of the three positions was designed to incorporate a 3- or 4-nitrophenyl group. The nitro groups were reduced using $NaBH_4$–copper(II) acetylacetonate to amines, which were either converted to the isothiocyanate groups, as potential affinity labels for the receptor, or acylated, as models for conjugation. An alternate route to N^{α}-3-aminobenzyloxycarbonyl functionalization was devised. The various positions of functionalization were compared for effects on biological potency, and the R_2 and R_3 positions were found to be most amenable to derivatization with retention of high potency. Four dimeric permutations of the antagonists were synthesized by coupling each of the isothiocyanate derivatives to either the precursor amine or to other amine congeners. Only dimers linked at the R_2-position were potent antagonists. In concentration–response studies, two derivatives, a 3-nitrobenzyloxycarbonyl derivative **18** and a 4-nitrotoluenesulfonate **26b**, displayed IC_{50} values of roughly 100 nM as antagonists of $P2X_7$ receptor-mediated K^+ flux.

INTRODUCTION

P2X receptors are ligand-gated cation channels activated by ATP (adenosine 5′-triphosphate) and other purine nucleotides. Seven mammalian subtypes of P2X receptors

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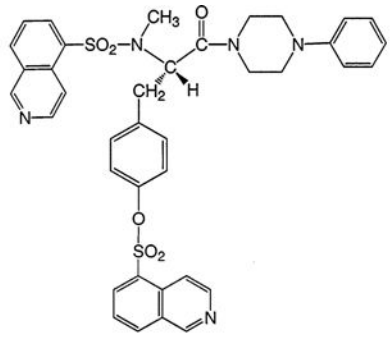
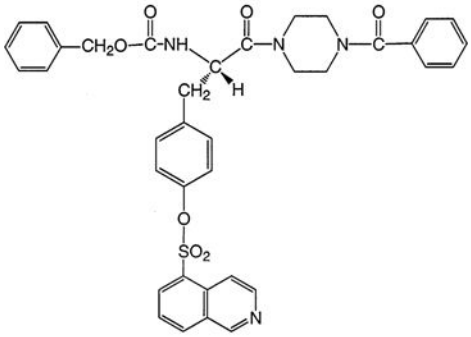
(termed $P2X_1$ through $P2X_7$) have been cloned (1, 2). Each functional ion channel consists of an oligomeric assembly, probably a trimer. Both homo- and heterooligomerization seem to occur commonly; however the $P2X_7$ subtype exists exclusively as a homomer, and the $P2X_6$ subtype always occurs as a homomer. The cationic selectivity of the P2X receptors is Na^+, $K^+ > Ca^{2+}$ (3).

The $P2X_7$ receptor (formerly known as the P_{2Z} receptor) is expressed primarily in the immune/inflammatory system (4–9), i.e., in blood cells (monocytes, macrophages, and lymphocytes) and in the brain on microglial cells, and in the salivary gland (10). Its activation is associated with apoptosis in the immune system and release of inflammatory cytokines, such as IL-1β (8, 9, 11). The $P2X_7$ receptor is distinct from the other P2X subunits in that at high μM concentrations of agonists, it forms or activates a large pore in addition to a cation channel (12, 13). This pore increases permeability indiscriminately to molecules having MW ≤ 900, such as ethidium bromide, which is used as a marker for pore activity. The $P2X_7$ receptor also has a long C-terminal segment which contributes to pore formation (13). 2′- and 3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP) is among the most potent agonists at $P2X_7$ receptors, but also has nanomolar potency at $P2X_1$ receptors (14). Affinity labeling of the $P2X_7$ receptor in mast cells has been carried out using [^{3}H]-BzATP (15).

An objective of the present study was to design antagonist ligand probes based on tyrosine derivatives, to be used for biophysical characterization of the $P2X_7$ receptor. The isoquinoline derivative of tyrosine 1-(*N*,*O*-bis[5-isoquinolinesulfonyl]-*N*-methyl-L-tyrosyl)-4-phenylpiperazine (**1**, KN-62), is a potent noncompetitive antagonist at $P2X_7$ receptors in the submicromolar range (6,7,12). It is also an antagonist of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in the micromolar range (16). We recently reported a series of potent $P2X_7$ receptor antagonists derived from KN-62, of the general structure R_1-Tyr(OR_2)-piperazinyl-R_3, in which three positions (R_1, R_2, and R_3) were systematically varied (17). The derivative MRS 2409 ([N-benzyloxycarbonyl-O-[4-(isoquinolinylsulfonyl)]-L-tyrosyl]benzoylpiperazine), **2**, in which R_1 and R_3 differed from the structure of KN-62, was among the most potent analogues prepared (IC_{50} of 200 nM, for release of K^+ stimulated by 3 mM ATP in HEK293 cells expressing the human $P2X_7$ receptor). We now extend the SAR (structure activity relationship) analysis in this series to include more varied functionalization of congeners (18–20) of MRS 2409, directed toward probing multiple sites on the antagonist molecules for sites of chain attachment that preserve the biological properties. This functionalization is intended for the purpose of cross-linking, either with the receptor and/or with reporter moieties such as fluorescent dyes or radioisotopes. Dimerization of the antagonists through various linkage points has also been carried out in order to explore the spatial relationship between possible multiple binding sites on the large extracellular loop (13, 21) of $P2X_7$ receptor homomers.



1, KN-62



2, MRS 2409

MATERIALS AND METHODS

Chemical Synthesis

Protected tyrosine compounds are from Novabiochem (San Diego, CA). Other chemicals are from Aldrich (Milwaukee, WI). ^{1}H NMR spectra were recorded using a Varian Gemini-300 spectrometer. High-resolution FAB (fast atom bombardment) mass spectra were taken with a JEOL SX102 spectrometer using nitrobenzoic acid as matrix. Elemental analysis was performed by Atlantic Microlab Inc. (Norcross, GA).

General Procedure for the Synthesis of 46, 51, 62

A mixture of Fmoc-Tyr-OH or Cbz-Tyr-OH (3.0 mmol), Boc-piperazine, or benzoyl-piperazine (3.0 mmol), Bis(2-oxo-3-oxoazolidinyl)phosphinic chloride (Bop-Cl) (0.77 g, 3.0 mmol), and CH_2Cl_2 (15 mL) was treated with Et_3N (0.96 mL, 6.8 mmol) and stirred at room temperature for 1 h. The solvent was removed in vacuo, and the crude product was purified with silica gel chromatography eluting with methanol-chloroform (5:95) to furnish a white solid foam (yield 75–85%).

[N-Fmoc-L-tyrosyl]benzoylpiperazine (46)—^{1}H NMR ($CDCl_3$): δ 7.77 (d, J = 7.4 Hz, 2H), 7.59 (d, J = 7.4 Hz, 2H), 7.50–7.28 (m, 5H), 7.05 (d, J = 8.0 Hz, 2H), 6.75 (d, J = 8.0 Hz, 2H), 5.70 (d, J = 8.5 Hz, 1H), 4.84(b, 1H), 4.50–4.25 (m, 2H), 4.25–4.10 (t, J = 7.0 Hz, 1H), 3.80–2.80 (m, 10H).

[N-Fmoc-L-tyrosyl]-Boc-piperazine (51)—^{1}H NMR ($CDCl_3$): δ 7.77 (d, J = 7.3 Hz, 2H), 7.59 (d, J = 7.3 Hz, 2H), 7.40 (t, J = 7.3 Hz, 2H), 7.31 (t, J = 7.3 Hz, 2H), 7.04 (d, J = 7.7 Hz, 2H), 6.74 (d, J = 7.7 Hz, 2H), 5.88 (s, 1H), 5.72 (d, J = 8.4 Hz, 1H), 4.90–4.78 (m, 1H), 4.44–4.28 (m, 2H), 4.24–4.16 (t, J = 7.0 Hz, 1H), 3.60–2.80 (m, 10 H), 1.45 (s, 9H).

[N-Benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl]pip-erazine (62)—^{1}H NMR ($CDCl_3$): δ 7.30–7.40 (m, 5H), 7.02 (d, J = 8.2 Hz, 2H), 6.72 (d, J = 8.2 Hz, 2H), 5.65 (d, J = 8.5 Hz, 1H), 5.45 (br s, 1H), 5.14–5.00 (ABq, J = 12.4 Hz, 2H), 4.90–4.75 (m, 1H), 3.60–2.80 (m, 10H), 1.45 (s, 9H).

General Procedure for the Synthesis of 23a, 23b, 47, 52a, 52b, 55

N-Substituted tyrosine (**46**, **51**, or **62**; 2.0 mmol) was dissolved in 10 mL of anhydrous CH_2Cl_2 and cooled to 0 °C. A solution of benzenesulfonyl chloride or related sulfonyl chloride (4.0 mmol), Et_3N (0.3 mL, 2.2 mmol), DMAP (214 mg, 1.7 mmol), and CH_2Cl_2 (5.0 mL) was added dropwise and stirring continued for 30 min at 0 °C. TLC showed the completion of the reaction. The solvent was removed under reduced pressure. Column chromatography (silica gel, CH_3OH:$CHCl_3$ = 5:95) yielded a white solid foam (70–75% yield).

[N-Benzyloxycarbonyl-O-(3-nitrobenzenesulfonyl)-L-tyrosyl]-Boc-piperazine (23a)—^{1}H NMR ($CDCl_3$): δ 8.71 (t, J = 1.9 Hz, 1H), 8.52 (ddd, J = 1.1, 2.2, 8.2 Hz, 1H), 8.12 (m, 1H), 7.78 (t, J = 8.0 Hz, 1H), 7.40–7.30 (m, 5H), 7.14 (d, J = 8.5 Hz, 1H), 6.93 (m. 1H), 5.58 (d, J = 8.8 Hz, 1H), 5.14–5.00(ABq, J = 12.4 Hz, 2H), 4.88–4.77 (m, 1H), 3.60–2.84 (m, 10H), 1.44 (s, 9H). FAB–HRMS for $C_{32}H_{37}N_4O_{10}S$ (MH^+): calcd 669.2230, found 669.2231. Anal. ($C_{32}H_{36}N_4O_{10}S{\cdot}0.1H_2O$).

[N-Benzyloxycarbonyl-O-(4-nitrobenzenesulfonyl)-L-tyrosyl]-Boc-piperazine (23b)—^{1}H NMR ($CDCl_3$): δ 8.39 (d, J = 9.0 Hz, 2H), 8.02 (d, J = 9.1 Hz, 2H), 7.42–7.28 (m, 5H), 7.13 (d, J = 8.5 Hz, 2H), 6.90 (d, J = 8.5 Hz, 2H), 5.60 (d, J = 8.5 Hz, 1H), 5.14–5.00 (ABq, J = 13.7 Hz, 2H), 4.90–4.77 (m, 1H), 3.60–2.78 (m, 10H), 1.45 (s, 9H). FAB-MS: m/z (relative intensity) 669.1 (M + 1, 6), 613.1 (16), 91 (100). Anal. ($C_{32}H_{36}N_4O_{10}S{\cdot}0.1H_2O$).

[N-Fmoc-O-toluenesulfonyl-L-tyrosyl]benzoylpiperazine (47)—^{1}H NMR ($CDCl_3$): δ 7.86–7.64 (m, 4H), 7.60 (d, J = 7.7 Hz, 2H), 7.50–7.28 (m, 11H), 7.13 (d, J = 8.2 Hz, 2H), 6.84–6.88 (d, J = 8.2 Hz, 2H), 5.66 (d, J = 8.2 Hz, 1H), 4.82 (b, 1H), 4.50–4.25 (m, 2H), 4.25–4.10 (m, 1H), 3.8–2.7 (m, 10H), 2.45 (s, 3H).

[N-Fmoc-O-benzenesulfonyl-L-tyrosyl]-Boc-piperazine (52a)—^{1}H NMR ($CDCl_3$): δ 7.88–7.72 (m, 4H), 7.70–7.60 (m, 2H), 7.46–7.27 (m, 7H), 7.10 (d, J = 8.5 Hz, 2H), 6.90 (d, J = 8.5 Hz, 2H), 5.66 (d, J = 8.2 Hz, 1H), 4.90–4.70 (m, 1H), 4.50–4.30 (m, 2H), 4.25–4.10 (m, 1H), 3.8–2.7 (m, 10H), 1.45 (s, 9H).

[N-Fmoc-O-(4-nitrobenzenesulfonyl)-L-tyrosyl]-Boc-piperazine (52b)—^{1}H NMR ($CDCl_3$): δ 8.38 (d, J = 8.8 Hz, 2H), 8.02 (d, J = 8.8 Hz, 2H), 7.77 (d, J = 7.7 Hz, 2H), 7.56

(d, J = 7.4 Hz, 2H), 7.47–7.27 (m, 4H), 7.14 (d, J = 7.4 Hz, 2H), 6.91 (d, J = 8.5 Hz, 2H), 5.61 (d, J = 8.5 Hz, 1H), 4.90–4.70 (m, 1H), 4.50–4.28 (m, 2H), 4.26–4.10 (m, 1H), 3.58–2.70 (m. 10H), 1.45 (s, 9H).

[N-Fmoc-O-toluenesulfonyl-L-tyrosyl]-Boc-piperazine (55)—^{1}H NMR ($CDCl_3$): δ 7.76 (d, J = 7.1 Hz, 2H), 7.70 (d, J = 8.0 Hz, 2H), 7.57 (d, J = 6.9 Hz, 2H), 7.46–7.27 (m, 6H), 7.10 (d, J = 7.9 Hz, 2H), 6.90 (d, J = 7.8 Hz, 2H), 5.65 (d, J = 8.2 Hz, 1H), 4.85–4.70 (m, 1H), 4.50–4.25 (m, 2H), 4.24–4.15 (m, 1H), 3.80–2.70 (m, 10H), 2.43 (s, 3H), 1.45 (s, 9H).

General Procedure for the Synthesis of 17, 19, 20b, 25, 26a, 26b, 28, 31a, 31b, 33, 53a, 53b, 53c, 53d, 57a, 57b

A tyrosine derivative containing a free amine (**20a**, **24**, **27**, **32**, **52a**, **52b**, **56**, **58a**, **58b**, or **63**; 3.0 mmol) was dissolved in CH_2Cl_2 (8.0 mL). DMAP (300 mg) was added, and the solution was cooled to 0 °C. A solution of a benzoyl chloride (4.2 mmol) (alternatively a benzyl chloroformate or acetyl chloride), Et_3N (0.45 mL), and CH_2Cl_2 (5.0 mL) was added dropwise and stirring continued at 0 °C for 40 min. The solvent was removed, and the residue was washed with petroleum ether three times. The resulting crude product was purified by column chromatography (silica gel, CH_3OH:$CHCl_3$ = 5:95) to yield a white solid foam (80–90% yield).

[N-(4-Nitrobenzyloxycarbonyl)-O-toluenesulfonyl-L-tyrosyl]benzoylpiperazine (17)—^{1}H NMR ($CDCl_3$): δ 8.22 (d, J = 8.8 Hz, 2H), 7.73 (d, J = 8.2 Hz, 2H), 7.60–7.30 (m, 9H), 7.13 (d, J = 8.2 Hz, 2H), 6.93 (d, J = 8.5 Hz, 2H), 5.70 (d, J = 8.5 Hz, 1H), 5.26–5.10 (b, 2H), 4.92–4.70 (b, 1H), 3.90–2.70 (m, 10H), 2.46 (s, 3H). FAB-MS: m/z (relative intensity) 687.2 (M + 1, 35), 391.3 (70), 149.1 (100). Anal. ($C_{35}H_{34}N_4O_9S$·0.4EtOAc).

[N-(3-Nitrobenzyloxycarbonyl)-O-toluenesulfonyl-L-tyrosyl]benzoylpiperazine (19)—^{1}H NMR ($CDCl_3$): δ 8.22–8.16 (m, 2H), 7.72 (d, J = 8.5 Hz, 2H), 7.68–7.62 (m, 1H), 7.54 (t, J = 8.0 Hz, 1H), 7.46–7.30 (m, 7H), 7.13 (d, J = 8.5 Hz, 2H), 6.92 (d, J = 8.5 Hz, 2H), 5.70 (d, J = 8.5 Hz, 1H), 5.22–5.12 (m, 2H), 4.92–4.70 (b, 1H), 4.00–2.70 (m, 10H), 2.43 (s, 3H). FAB–HRMS for $C_{35}H_{35}N_4O_9S$ (MH^+): calcd 687.2125, found 687.2136.

[N-(3-Acetylaminobenzyloxycarbonyl)-O-toluenesulfonyl-L-tyrosyl]benzoylpiperazine (20b)—^{1}H NMR ($CDCl_3$): δ 7.71 (d, J = 8.5 Hz, 2H), 7.52–7.28 (m, 10H), 7.16–6.98 (m, 3H), 6.91 (d, J = 8.5 Hz, 2H), 5.88–5.52 (b, 1H), 5.05 (br s, 2H), 4.88–4.68 (b, 1H), 3.80–2.70 (m, 10H), 2.46 (s, 3H), 2.18 (s, 3H). FAB–HRMS for $C_{37}H_{39}N_4O_8S$ (MH^+): calcd 699.2489, found 699.2507.

[N-Benzyloxycarbonyl-O-(4-acetylamino)benzenesulfonyl-L-tyrosyl]-Boc-piperazine (25)—^{1}H NMR ($CDCl_3$): δ 7.72 (d, J = 8.8 Hz, 2H), 7.63 (d, J = 8.6 Hz, 2H), 7.40–7.28 (m, 5H), 7.10 (d, J = 8.2 Hz, 2H), 6.94 (d, J = 8.6 Hz, 2H), 5.64 (d, J = 8.5 Hz, 1H), 5.20–5.00 (ABq, J = 12.3 Hz, 2H), 4.90–4.70 (m, 1H), 4.00–2.70 (m, 10H), 2.23 (s, 3H), 1.47 (s, 9H). FAB–HRMS for $C_{34}H_{41}N_4O_9S$ (MH^+): calcd 681.2594, found 681.2610. Anal. ($C_{34}H_{40}N_4O_9S$).

[N-Benzyloxycarbonyl-O-(3-nitrobenzenesulfonyl)-L-tyrosyl]benzoylpiperazine (26a)—^{1}H NMR ($CDCl_3$): δ 8.76–8.64 (b, 1H), 8.58–8.30 (dq, J = 8.3, 1.1 Hz, 1H), 8.20–8.14 (m, 1H), 7.80 (t, J = 8.0 Hz, 1H), 7.48–7.28 (m, 10H), 7.16 (d, J = 8.5 Hz, 2H), 7.00–6.92 (m, 2H), 5.57 (d, J = 8.5 Hz, 1H), 5.14–5.02 (m, 2H), 4.92–4.70 (b, 1H), 3.80–2.70 (m, 10H). FAB–HRMS for $C_{34}H_{33}N_4O_9S$ (MH^+): calcd 673.1968, found 673.1981. Anal. ($C_{34}H_{32}N_4O_9S$).

[N-Benzyloxycarbonyl-O-(4-nitrobenzenesulfonyl)-L-tyrosyl]benzoylpiperazine (26b)—^{1}H NMR ($CDCl_3$): δ 8.40 (d, J = 8.8 Hz, 2H), 8.05 (d, J = 8.5 Hz, 2H), 7.48–7.28 (m, 10H), 7.16 (d, J = 8.2 Hz, 2H), 6.92 (d, J = 8.5 Hz, 2H), 5.76 (d, J = 8.5 Hz, 1H), 5.18–5.00 (m, 2H), 4.94–4.72 (b, 1H), 3.70–2.80 (m, 10H). Compound 26b (R_f = 0.85, CH_3OH/$CHCl_3$ = 1: 9) was shown to be pure by TLC.

[N-Benzyloxycarbonyl-O-(4-acetylamino)benzenesulfonyl-L-tyrosyl]benzoylpiperazine (28)—^{1}H NMR ($CDCl_3$): δ 7.72 (q, J = 9.1, 14.8 Hz, 4H), 7.50–7.30 (m, 10H), 7.10 (d, J = 7.7 Hz, 2H), 7.04–6.90 (b, 2H), 5.68–5.52 (b, 1H), 5.16–5.00 (ABq, J = 12.1, 16.2 Hz, 2H), 4.88–4.68 (b, 1H), 3.90–2.70 (m, 10H), 2.17 (s, 3H). FAB-MS: m/z (relative intensity) 685.4 (M + 1, 100), 641.4 (5), 91.1 (70).

[N-Benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl]-(3-nitrobenzoyl) piperazine (31a)—^{1}H NMR ($CDCl_3$): δ 8.34–8.22 (m, 2H), 7.80–7.70 (m, 3H), 7.68–7.36 (m, 1H), 7.42–7.30 (m, 7H), 7.16 (d, J = 8.5 Hz, 2H), 6.96 (d, J = 8.8 Hz, 2H), 5.58 (d, J = 8.8 Hz, 1H), 5.26–5.00 (m, 2H), 4.90–4.70 (b, 1H), 4.00–2.70 (m, 10H), 2.45 (s, 3H). FAB-MS: m/z (relative intensity) 687.2 (M + 1, 40), 643.3 (10), 91.0 (90). FAB–HRMS (NOBA/CsI) for $C_{35}H_{34}N_4O_9SCs$ $[M + Cs]^+$: calcd 819.1101, found 819.1096.

[N-Benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl]-(4-nitrobenzoyl)piperazine (31b)—^{1}H NMR ($CDCl_3$): δ 8.36–8.18 (m, 3H), 7.76 (d, J = 8.2 Hz, 2H), 7.56 (d, J = 8.5 Hz, 2H), 7.42–7.28 (m, 6H), 7.16 (d, J = 8.2 Hz, 2H), 6.96 (d, J = 8.0 Hz, 2H), 5.70–5.56 (m, 1H), 5.18–5.00 (m, 2H), 4.92–4.70 (b, 1H), 4.00–2.70 (m, 10H), 2.48 (s, 3H). FAB-MS: m/z (relative intensity) 687.2 (M + 1, 20), 643.3 (2), 91.0 (50). FAB–HRMS for $C_{35}H_{35}N_4O_9S$ $[MH]^+$: calcd 687.2125, found 687.2139. Anal. ($C_{35}H_{34}N_4O_9S$·0.2EtOAc).

[N-Benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl]-(4-acetylaminobenzoyl) piperazine (33)—^{1}H NMR ($CDCl_3$): δ 7.72 (d, J = 8.2 Hz, 2H), 7.53 (d, J = 8.5 Hz, 2H), 7.45 (s, 1H), 7.40–7.28 (m, 9H), 7.13 (d, J = 8.2 Hz, 2H), 6.91 (d, J = 7.7 Hz, 2H), 5.80–5.56 (b, 1H), 5.20–5.00 (m, 2H), 4.90–4.72 (b, 1H), 3.90–2.70 (m, 10H), 2.45 (s, 3H), 2.19 (s, 3H). FAB-HRMS for $C_{37}H_{39}N_4O_8S$ $[MH]^+$: calcd 699.2489, found 699.2505. Compound **33** (R_f = 0.35, $CH_3OH/CHCl_3$ = 5: 95) was shown to be pure by TLC. Anal. ($C_{37}H_{38}N_4O_8S$·0.5EtOAc).

[N-Fmoc-O-benzenesulfonyl)-L-tyrosyl]benzoylpiperazine (53a)—^{1}H NMR ($CDCl_3$). δ 7.80–7.28 (m, 18H), 7.16 (d, J = 8.2 Hz, 2H), 6.93 (d, J = 8.2 Hz, 2H), 5.64 (d, J = 8.5 Hz, 1H), 4.83 (br s, 1H), 4.50–4.28 (m, 2H), 4.20 (m, 1H), 3.85–2.70 (m, 10H).

[N-Fmoc-O-(4-nitrobenzene)sulfonyl-L-tyrosyl]-(4-nitrobenzoyl)piperazine (53b)—^{1}H NMR ($CDCl_3$): δ 8.42 (d, J = 8.3 Hz, 2H), 8.27 (d, J = 7.7 Hz, 2H), 8.10 (d, J = 8.8 Hz, 2H), 7.78 (d, J = 7.7 Hz, 2H), 7.56 (m, 4H), 7.41 (t, J = 7.4 Hz, 2H), 7.36–7.27(m, 2H), 7.19 (d, J = 8.2 Hz, 2H), 6.98 (d, J = 7.4 Hz, 2H), 5.58 (d, J = 8.8 Hz, 1H), 4.83 (b, 1H), 4.50–4.26 (m, 2H), 4.19(m, 1H), 3.80–2.55 (m, 10H).

[N-Fmoc-O-benzenesulfonyl-L-tyrosyl]-(4-nitrobenzoyl)-piperazine (53c)—^{1}H NMR ($CDCl_3$): δ 8.40–8.16 (b, 2H), 7.90 (d, J = 7.7 Hz, 2H), 7.78 (d, J = 7.4 Hz, 2H), 7.70 (d, J = 7.1 Hz, 1H), 7.64–7.48 (m, 6H), 7.46–7.28 (m, 4H), 7.17 (d, J = 8.2 Hz, 2H), 7.06–6.90 (m, 2H), 5.75–5.50 (b, 1H), 4.90–4.70 (b, 1H), 4.50–4.30 (m, 2H), 4.25–4.10 (m, 1H), 4.00–2.70 (m, 10H).

[N-Fmoc-O-(4-nitrobenzene)sulfonyl-L-tyrosyl]benzoylpiperazine (53d)—^{1}H NMR ($CDCl_3$): δ 8.38 (d, J = 8.5 Hz, 2H), 8.05 (d, J = 8.2 Hz, 2H), 7.77 (d, J = 7.4 Hz, 2H), 7.56 (d, J = 7.4 Hz, 2H), 7.50–7.28 (m, 9H), 7.16 (d, J = 8.2 Hz, 2H), 6.94 (d, J = 8.2 Hz, 2H), 5.63 (d, J = 8.5 Hz, 1H), 4.90–4.47 (b, 1H), 4.50–4.25 (m, 2H), 4.25–4.10 (m, 1H), 3.80–2.70 (m, 10H).

[N-(4-Nitrobenzyloxycarbonyl)-O-toluenesulfonyl-L-tyrosyl]Boc-piperazine (57a)—^{1}H NMR ($CDCl_3$): δ 8.21 (d, J = 8.5 Hz, 2H), 7.76 (d, J = 8.2 Hz, 2H), 7.48 (d, J = 8.5 Hz, 2H), 7.36 (d, J = 8.2 Hz, 2H), 7.18 (d, J = 8.4 Hz, 2H), 7.02 (d, J = 8.5 Hz, 2H), 5.62(d, J = 8.5 Hz, 1H), 5.20 (ABq, J = 12.3, 15.6 Hz, 2H), 4.60–4.40 (m, 1H), 4.00–2.80 (m, 10H), 2.46 (s, 3H), 1.45 (s, 9H).

[N-Benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl]Bocpiperazine (57b)—^{1}H NMR ($CDCl_3$): δ 7.69 (d, J = 7.9 Hz, 2H), 7.42–7.28 (m, 7H), 7.09 (d, J = 7.9 Hz, 2H), 6.88 (d, J = 7.9 Hz, 2H), 5.65 (d, J = 8.5 Hz, 1H), 5.07 (ABq, J = 12.6, 15.4 Hz, 2H), 4.90–4.76 (m, 1H), 3.60–2.70 (m, 10H), 2.45 (s, 3H), 1.44 (s, 9H).

General Procedure for the Synthesis of 3–16

The appropriate tyrosine derivative (**48**, **54a**, **54b**, **54c**, or **54d**; 1.0 mmol), Bop-Cl (1.0 mmol) and appropriate cinnamic acid derivative (1.0 mmol) were dissolved in CH_2Cl_2 (5.0 mL). The solution was treated with Et_3N (2.0 mmol) and stirred at room temperature for 5 h. The solvent was removed, and the residue was purified using silica gel chromatography eluting with methanolchloroform (5:95) to get a solid foam (40–60% yield).

[N-(trans-Cinnamoyl)-O-benzenesulfonyl-L-tyrosyl]benzoylpiperazine (3)—^{1}H NMR ($CDCl_3$): δ 7.85 (d, J = 7.7 Hz, 2H), 7.74–7.60 (m, 2H), 7.60–7.30 (m, 11H), 7.17 (d, J = 8.5 Hz, 2H), 6.94 (d, J = 8.5 Hz, 2H), 6.58–6.46 (m, 1H), 6.40 (d, J = 15.7 Hz, 1H), 5.40–5.10 (b, 1H), 3.90–2.90 (m, 10H). FAB-MS: m/z (relative intensity) 624.3 (M + 1, 40), 434.2 (20), 191.2 (100), 131.1 (100), 105.0 (100). Compound **3** (R_f = 0.60, $CH_3OH/CHCl_3$ = 5: 95) was shown to be pure by TLC.

[N-(trans-2-Methoxycinnamoyl)-O-toluenesulfonyl-L-tyrosyl]benzoylpiperazine (4)—^{1}H NMR ($CDCl_3$): δ 7.72 (d, J = 8.1 Hz, 2H), 7.52–7.32 (m, 10H), 7.16–6.80 (m, 5H),

6.34 (d, J = 8.4 Hz, 1H), 6.00 (d, J = 15.7 Hz, 1H), 5.20–5.00 (m, 1H), 4.00–2.70 (m, 13H), 2.45 (s, 3H).

[N-(cis-2-Methoxycinnamoyl)-O-toluenesulfonyl-L-tyrosyl]-benzoylpiperazine (5)—^{1}H NMR ($CDCl_3$): δ 7.70 (d, J = 7.7 Hz, 2H), 7.50–7.30 (m, 10H), 7.14–6.82 (m, 5H), 6.34 (d, J = 8.5 Hz, 1H), 5.96 (d, J = 12.4 Hz, 1H), 5.20–5.00 (m, 1H), 4.00–2.60 (m, 13H), 2.46 (s, 3H).

[N-(trans-3-Chlorocinnamoyl)-O-toluenesulfonyl-L-tyrosyl]benzoylpiperazine (6)—^{1}H NMR ($CDCl_3$): δ 7.72 (d, J = 8.0 Hz, 2H), 7.61–7.29 (m, 11H), 7.16 (d, J = 8.5 Hz, 2H), 6.94 (d, J = 8.2 Hz, 2H), 6.57 (d, J = 8.3 Hz, 1H), 6.45–6.37 (d, J = 15.7 Hz, 1H), 5.37–5.07 (b, 1H), 4.00–2.70 (m, 10H), 2.46 (s, 3H).

[N-(trans-2,4,5-Trimethoxycinnamoyl)-O-toluenesulfonyl-L-tyrosyl]benzoylpiperazine (7)—^{1}H NMR ($CDCl_3$): δ 7.88 (s, 1H), 7.82 (s, 1H), 7.71 (d, J = 7.7 Hz, 2H), 7.56–7.30 (m, 5H), 7.17 (d, J = 8.2 Hz, 2H), 7.02–6.86 (m, 4H), 6.56 (d, J = 15.9 Hz, 1H), 6.43 (d, J = 8.3 Hz, 1H), 5.40–5.05 (b, 1H), 4.00–2.70 (m, 19H), 2.45 (s, 3H).

[N-(trans-4-Nitrocinnamoyl)-O-benzenesulfonyl-L-tyrosyl]-benzoylpiperazine (8)—^{1}H NMR ($CDCl_3$): δ 8.24 (d, J = 8.5 Hz, 2H), 7.86 (d, J = 8.0 Hz, 2H), 7.76–7.50 (m, 6H), 7.48–7.30 (m, 4H), 7.17 (d, J = 8.2 Hz, 2H), 6.96 (d, J = 8.5 Hz, 2H), 6.64 (d, J = 8.2 Hz, 1H), 6.52 (d, J = 15.9 Hz, 1H), 5.40–5.32 (b, 1H), 3.90–2.60 (m, 10H). FAB-MS: m/z (relative intensity) 669.2 (M + 1, 25), 251.1 (40), 119.0 (100). Compound **8** (R_f = 0.47, $CH_3OH/CHCl_3$ = 5: 95) was shown to be pure by TLC.

[N-(trans-Cinnamoyl)-O-(4-nitrobenzenesulfonyl)-L-tyrosyl]-benzoylpiperazine (9)—^{1}H NMR ($CDCl_3$): δ 8.40 (d, J = 8.5 Hz, 2H), 8.06 (d, J = 8.3 Hz, 2H), 7.70–7.30 (m, 10H), 7.20 (d, J = 8.2 Hz, 2H), 6.95 (d, J = 8.5 Hz, 2H), 6.52 (d, J = 8.2 Hz, 1H), 6.40 (d, J = 15.7 Hz, 1H), 5.40–5.34 (b, 1H), 3.90–2.80 (m, 10H). FAB-MS: m/z (relative intensity) 669.2 (M + 1, 20), 191.2 (85), 131.1 (100), 119.0 (75). Compound **9** (R_f = 0.59, $CH_3OH/CHCl_3$ = 5: 95) was shown to be pure by TLC.

[N-(trans-Cinnamoyl)-O-benzenesulfonyl-L-tyrosyl]-(4-nitrobenzoyl)piperazine (10)—^{1}H NMR ($CDCl_3$): δ 8.26 (d, J = 7.7 Hz, 2H), 7.89 (d, J = 7.7 Hz, 2H), 7.78–7.30 (m, 10H), 7.21 (d, J = 8.5 Hz, 2H), 7.00 (d, J = 7.4 Hz, 2H), 6.60–6.46 (m, 1H), 6.44–6.34 d, J = 15.7 Hz, 1H), 5.40–5.06 (b, 1H), 4.00–2.80 (m, 10H). FAB-MS: m/z (relative intensity) 669.2 (M + 1, 20), 434.2 (35), 131.1 (100). Compound **10** (R_f = 0.56, $CH_3OH/CHCl_3$ = 5: 95) was shown to be pure by TLC.

[N-(trans-4-Nitrocinnamoyl)-O-(4-nitrobenzenesulfonyl)-L-tyrosyl]-benzoylpiperazine (11)—^{1}H NMR ($CDCl_3$): δ 8.41 (d, J = 8.5 Hz, 2H), 8.24 (d, J = 8.5 Hz, 2H), 8.07 (d, J = 8.5 Hz, 2H), 7.74–7.58 (m, 3H), 7.50–7.34 (m, 4H), 7.20 (d, J = 8.5 Hz, 2H), 6.97 (d, J = 8.5 Hz, 2H), 6.64 (d, J = 8.0 Hz, 1H), 6.52 (d, J = 15.7 Hz, 1H), 5.40–5.16 (b, 1H), 3.84–2.80 (m, 10H). FAB-MS: m/z (relative intensity) 714.2 (M + 1, 2), 119.0 (100), 91.0 (50). Compound **11** (R_f = 0.50, $CH_3OH/CHCl_3$ = 5: 95) was shown to be pure by TLC.

[N-(trans-4-Nitrocinnamoyl)-O-benzenesulfonyl-L-tyrosyl]-(4-nitrobenzoyl)piperazine (12)—^{1}H NMR ($CDCl_3$): δ 8.36–8.14 (m, 4H), 7.90 (d, J = 7.4 Hz, 2H), 7.80–7.50 (m, 7H), 7.22 (d, J = 8.5 Hz, 2H), 7.10–6.92 (m, 2H), 6.72–6.40 (m, 2H), 5.40–5.08 (b, 1H), 4.00–2.80 (m, 10H). FAB-MS: m/z (relative intensity) 714.2 (M + 1, 10), 119.0 (100). Compound **12** (R_f = 0.47, $CH_3OH/CHCl_3$ = 5: 95) was shown to be pure by TLC.

[N-(trans-Cinnamoyl)-O-(4-nitrobenzenesulfonyl)-L-tyrosyl]-(4-nitrobenzoyl)piperazine (13)—^{1}H NMR ($CDCl_3$): δ 8.43 (d, J = 8.8 Hz, 2H), 8.28 (d, J = 8.2 Hz, 2H), 8.10 (d, J = 8.8 Hz, 2H), 7.70–7.46 (m, 4H), 7.44–7.34 (m, 3H), 7.23 (d, J = 8.5 Hz, 2H), 7.00 (d, J = 8.0 Hz, 2H), 6.49 (d, J = 8.2 Hz, 1H), 6.40 (d, J = 15.7 Hz, 1H), 5.40–5.16 (b, 1H), 3.90–2.80 (m, 10H). FAB-MS: m/z (relative intensity) 714.2 (M + 1, 5.5), 119.0 (100). Compound **13** (R_f = 0.56, $CH_3OH/CHCl_3$ = 5: 95) was shown to be pure by TLC.

[N-(trans-4-Nitrocinnamoyl)-O-(4-nitrobenzenesulfonyl)-L-tyrosyl]-(4-nitrobenzoyl)piperazine (14)—^{1}H NMR ($CDCl_3$): δ 8.43 (d, J = 8.8 Hz, 2H), 8.34–8.20(m, 4H), 8.10 (d, J = 8.8 Hz, 2H), 7.78–7.50 (m, 4H), 7.24 (d, J = 8.5 Hz, 2H), 7.01 (d, J = 7.7 Hz, 2H), 6.64 (d, J = 8.2 Hz, 1H), 6.54 (d, J = 15.6 Hz, 1H), 5.40–5.10 (b, 1H), 3.90–2.90 (m, 10H). FAB-MS: m/z (relative intensity) 759.2 (M + 1, 1.5), 119.0 (100). Compound **14** (R_f = 0.45, $CH_3OH/CHCl_3$ = 5: 95) was shown to be pure by TLC.

[N-Benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl]-benzoylpiperazine (15)—^{1}H NMR ($CDCl_3$): δ 7.72 (d, J = 8.2 Hz, 2H), 7.48–7.28 (m, 12H), 7.12 (d, J = 8.2 Hz, 2H), 6.90 (d, J = 8.2 Hz, 2H), 5.61 (d, J = 8.5 Hz, 1H), 5.16–4.96 (m, 2H), 4.92–4.74 (m, 1H), 3.80–2.60 (m, 10H), 2.45 (s, 3H). Anal. ($C_{35}H_{35}N_3O_7S \cdot 0.1\ H_2O$).

[N-(2-Chlorobenzyloxycarbonyl)-O-toluenesulfonyl-L-tyrosyl]-benzoylpiperazine (16)—^{1}H NMR ($CDCl_3$): δ 7.72 (d, J = 7.7 Hz, 2H), 7.48–7.30 (m, 11H), 7.13 (d, J = 8.2 Hz, 2H), 6.92 (d, J = 8.2 Hz, 2H), 5.56 (d, J = 8.2 Hz, 1H), 5.28–5.10 (ABq, J = 13.1 Hz, 2H), 4.94–4.74 (m, 1H), 3.90–2.70 (m, 10H), 2.46 (s, 3H). Compound **16** (R_f = 0.64, $CH_3OH/CHCl_3$ = 5: 95) was shown to be pure by TLC.

General Procedure for the Synthesis of 20a, 24, 27, 32

To a solution of an aromatic nitro compound (**19**, **23b**, **26b**, or **31b**; 2.0 mmol) in methanol (10 mL), sodium borohydride (10.0 mmol) was added and the solution stirred at room temperature for 5 min. Then copper(II) acetylacetonate was added and stirring continued for 1 h. TLC showed the completion of the reaction. The solvent was removed, and the residue was washed with petroleum ether two times. Then, the crude product was purified using silica gel chromatography eluting with methanol–chloroform (5:95) to provide a white solid foam (80–85% yield).

[N-(3-Aminobenzyloxycarbonyl)-O-toluenesulfonyl-L-tyrosyl]benzoylpiperazine (20a)—^{1}H NMR ($CDCl_3$): δ 7.72 (d, J = 8.5 Hz, 2H), 7.46–7.28 (m, 7H), 7.18–7.06 (m, 3H), 6.92 (d, J = 8.8 Hz, 2H), 6.74–6.58 (m, 3H), 5.57 (d, J =

9.3 Hz, 1H), 5.04–4.92 (m, 2H), 4.88–4.74 (b, 1H), 3.95–2.70 (m, 10H), 2.45 (s, 3H). FAB–MS: *m*/*z* (relative intensity) 657.3 (M + 1, 45), 658.3 (M + 2, 15), 85 (100). FAB–HRMS for $C_{35}H_{37}N_4O_7S$ $[M + H]^+$: calcd 657.2383, found 657.2385.

[N-Benzyloxycarbonyl-O-(4-aminobenzenesulfonyl)-L-tyrosyl]Boc-piperazine (24)—^{1}H NMR ($CDCl_3$): δ 7.53 (d, J = 8.5 Hz, 2H), 7.40–7.28 (m, 5H), 7.08 (d, J = 8.5 Hz, 2H), 6.92 (d, J = 8.4 Hz, 2H), 6.61 (d, J = 8.8 Hz, 2H), 5.61 (d, J = 8.5 Hz, 1H), 5.02–4.96 (ABq, J = 12.3, 16.0 Hz, 2H), 4.81 (b, 1H), 4.38 (br s, 2H), 3.80–2.60 (m, 10H), 1.45 (s, 9H). FAB-MS (NOBA): *m*/*z* (relative intensity) 639.3 (M + 1, 1), 661.3 (M + Na, 7), 583.2 (8), 539.3 (10). 91.0 (100). FAB-MS (NOBA/LiCl): *m*/*z* (relative intensity) 645.3 (M + Li, 15), 687.3 (M + Li^+_2Cl, 5).

[N-Benzyloxycarbonyl-O-(4-aminobenzenesulfonyl)-L-tyrosyl]benzoylpiperazine (27)—^{1}H NMR ($CDCl_3$): δ 7.55 (d, J = 8.5 Hz, 2H), 7.48–7.28 (m, 10H), 7.10 (d, J = 8.0 Hz, 2H), 6.94 (d, J = 8.0 Hz, 2H), 6.64 (d, J = 8.5 Hz, 2H), 5.63 (d, J = 8.8 Hz, 1H), 5.16–5.00 (ABq, J = 12.4, 15.9 Hz, 2H), 4.92–4.70 (b, 1H), 4.50–4.20 (b, 2H), 4.00–2.80 (m, 10H). FAB-MS: *m*/*z* (relative intensity) 643.4 (M + 1, 24), 391.4 (20), 251.2 (60), 91.1 (100). FAB–HRMS for $C_{34}H_{35}N_4O_7S$ $[M + H]^+$: calcd 643.2226, found 643.2244.

[N-Benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl]-(4-aminobenzoyl)piperazine (32)—^{1}H NMR ($CDCl_3$): δ 7.70 (d, J = 8.2 Hz, 2H), 7.40–7.30 (m, 7H), 7.23 (d, J = 8.2 Hz, 2H), 7.12 (d, J = 8.5 Hz, 2H), 6.90 (d, J = 8.5 Hz, 2H), 6.70 (d, J = 8.4 Hz, 2H), 5.61 (d, J = 8.5 Hz, 1H), 5.12–5.02 (ABq, J = 12.3, 15.1 Hz, 2H), 4.88–4.76 (m, 1H), 3.89 (s, 2H), 3.90–2.80 (m, 10H), 2.45 (s, 3H). FAB-MS: *m*/*z* (relative intensity) 657.2 (M + 1, 95), 679.2 (M+ Na, 25), 120.1 (100), 91.0 (85). FAB–HRMS for $C_{35}H_{37}N_4O_7S$ $[M + H]^+$: calcd 657.2383, found 657.2396. Anal. ($C_{35}H_{36}N_4O_9S \cdot 0.1H_2O$).

General Procedure for the Synthesis of 21, 29, 34

An amine compound (**20a**, **27**, or **32**; 0.08 mmol) was mixed with chloroform (2.0 mL), water (1.0 mL), and sodium bicarbonate (20 mg. 0.24 mmol) and stirred vigorously for 10 min. Thiophosgene (2.0 mmol) was added and stirring continued for 1 h. The solvent was removed, and the residue was washed two times with petroleum ether. The crude product was purified with a preparative TLC plate to provide a white solid foam (70–85% yield).

[N-(3-Isothiocyanato)benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl]benzoylpiperazine (21)—^{1}H NMR ($CDCl_3$): δ 7.72 (d, J = 8.8 Hz, 2H), 7.46–7.30 (m, 8H), 7.24–7.08 (m, 5H), 6.92 (d, J = 8.8 Hz, 2H), 5.64 (d, J = 8.5 Hz, 1H), 5.05 (s, 2H), 4.90–4.72 (b, 1H), 4.00–2.70 (m, 10H), 2.45 (s, 3H). FAB–HRMS for $C_{36}H_{35}N_4O_7S_2$ $[M + H]^+$: calcd 699.1947, found 699.1948. Anal. ($C_{36}H_{34}N_4O_7S_2$).

[N-Benzyloxycarbonyl-O-(4-isothiocyanato)benzenesulfonyl-L-tyrosyl]benzoylpiperazine (29)—^{1}H NMR ($CDCl_3$): δ 7.82 (d, J = 8.5 Hz, 2H), 7.48–7.28 (m, 12H), 7.14 (d, J = 8.5 Hz, 2H), 6.92 (d, J = 8.5 Hz, 2H), 5.59 (d, J = 8.5 Hz, 1H),

5.16–5.00 (ABq, J = 12.1, 14.8 Hz, 2H), 4.92–4.72 (b, 1H), 3.80–2.70 (m, 10H). FAB-HRMS for $C_{35}H_{33}N_4O_7S_2$ $[M + H]^+$: calcd 685.1791, found 685.1810.

[N-Benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl]-(4-isothiocyanatobenzoyl)piperazine (34)—^{1}H NMR ($CDCl_3$): δ 7.42 (d, J = 8.5 Hz, 2H), 7.40–7.30 (m, 9H), 7.24 (d, J = 8.2 Hz, 2H), 7.14 (d, J = 8.5 Hz, 2H), 6.93 (d, J = 8.5 Hz, 2H), 5.60 (d, J = 8.5 Hz, 1H), 5.16–5.00 (ABq, J = 12.4, 15.4 Hz, 2H), 4.90–4.72 (b, 1H), 3.80–2.60 (m, 10H), 2.47 (s, 3H). FAB-MS (NOBA): m/z (relative intensity) 699.2 (M + 1, 22), 721.2 (M+ Na, 14), 91.1 (100). FAB–HRMS (CsI) for $C_{36}H_{34}N_4O_7S_2Cs$ $[M + Cs]^+$: calcd 831.0923, found 831.0895. Anal. ($C_{36}H_{34}N_4O_7S_2$·0.4EtOAc).

General Procedure for the Synthesis of 22, 30, 35a, 35b

1,4-Diaminobutane (100 mg, 1.1 mmol) or 1,2-diaminoethane (100 mg, 1.7 mmol) was added in the flask. Then, a solution of isothiocyanate compound (21 or 29 or 34) (0.01 mmol) and CH_2Cl_2 (0.1 mL) was added dropwise and stirring continued at room temperature for 1 h. The solvent was removed and the residue was washed two times with petroleum ether. The crude product was purified on a preparative TLC plate using methanol–chloroform (1:9) as mobile phase to afford a white solid foam (40–45% yield).

[N-3-{3-(4-Aminobutyl)thioureido}benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl]benzoylpiperazine (22a)—^{1}H NMR ($CDCl_3$): δ 7.71 (d, J = 8.2 Hz, 2H), 7.48–7.30 (m, 10H), 7.18–7.10 (m, 2H), 7.08–7.00 (m, 1H), 6.90 (d, J = 8.2 Hz, 2H), 5.40–4.60 (m, 4H), 3.80–2.80 (m, 14H), 2.45 (s, 3H), 1.30–1.20 (m,4H). FAB-HRMS for $C_{40}H_{47}N_6O_7S_2$ $[M + H]^+$: calcd 787.2948, found 787.2957.

[N-Benzyloxycarbonyl-O-4-{3-(4-aminobutyl)thioureido} benzenesulfonyl-L-tyrosyl]benzoylpiperazine (30)—^{1}H NMR ($CDCl_3$): δ 7.80–7.56 (m, 4H), 7.54–7.30 (m, 10H), 7.20–6.90 (m, 4H), 5.64 (d, J = 8.5 Hz, 1H), 5.18–5.00 (ABq, J = 12.4, 15.0 Hz, 2H), 4.88–4.72 (b, 1H), 3.80–2.25 (m, 14H), 1.30–1.20 (m, 4H). FAB-HRMS (CsI) for $C_{39}H_{44}N_6O_7S_2Cs$ $[M + Cs]^+$: calcd 905.1767, found 905.1771.

[N-Benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl](4-{3-[(2-aminoethyl)thioureido})benzoylpiperazine (35a)—^{1}H NMR ($CDCl_3$): δ 7.71 (d, J = 8.0 Hz, 2H), 7.52–7.28 (m, 11H), 7.13 (d, J = 8.0 Hz, 2H), 6.91 (d, J = 8.0 Hz, 2H), 5.70 (d, J = 7.7 Hz, 1H), 5.16–4.86 (ABq, J = 12.4, 17.0 Hz, 2H), 4.90–4.72 (b, 1H), 3.84–2.56 (m, 14H), 2.45 (s, 3H). FAB-HRMS (CsI) for $C_{38}H_{42}N_6O_7S_2Cs$ $[M + Cs]^+$: calcd 891.1611, found 891.1641.

[N-Benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl](4-{3-[(4-aminobutyl)thioureido})benzoylpiperazine (35b)—^{1}H NMR ($CDCl_3$): δ 7.73 (d, J = 8.5 Hz, 2H), 7.48–7.28 (m, 11H), 7.15 (d, J = 8.2 Hz, 2H), 6.93 (d, J = 8.5 Hz, 2H), 5.61 (d, J = 9.1 Hz, 1H), 5.14–5.02 (ABq, J = 12.4, 15.9 Hz, 2H), 4.92–4.74 (b, 1H), 3.80–2.60 (m, 14H), 2.43 (s, 3H), 1.40–1.20 (m, 4H). FAB–HRMS (CsI) for $C_{40}H_{47}N_6O_7S_2$ $[M + H]^+$: calcd 787.2948, found 787.2919.

General Procedure for the Synthesis of 64

A solution of isothiocyanate compound **21** (20 mg, 0.029 mmol) in dry DMF (0.5 mL) was stirred under nitrogen for 4 days at 80 °C. The solvent was removed with nitrogen stream, and the residue was purified with preparative TLC (methanol: chloroform = 3:97) to afford a white solid (4.5 mg, 10% yield).

***1,3-Bis{*[3-N-benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl]benzoylpiperazinethiourea (64)—**^{1}H NMR ($CDCl_3$): δ 8.02 (b, 2H), 7.70 (d, J = 8.0 Hz, 4H), 7.50–7.30 (m, 20H), 7.22–7.02 (m, 6H), 6.90 (d, J = 8.2 Hz, 4H), 5.88 (b, 2H), 5.18–4.94 (m, 4H), 4.81 (b, 2H), 3.80–2.80 (m, 20H), 2.45 (s, 6H).). FAB-MS: m/z (relative intensity) 1377.3 (M + Na, 15), 1355.3 (M + H, 5), 261.2 (100). FAB-HRMS (CsI) for $C_{71}H_{70}N_8O_{14}S_3Cs$ $[M + Cs]^+$: calcd 1487.3228, found 1487.3258. FAB-HRMS (CsI) for $C_{71}H_{69}N_8O_{14}S_3Cs_2$ $\{[(M - H)Cs]Cs\}^+$: calcd 1619.2204, found 1619.2222.

General Procedure for the Synthesis of 65, 66, 67

Mixing equimolar amounts of isothiocyanate and amine component (Scheme 7) in dry DMF, and following the same synthetic procedure as for compound **64**, the correspondence dimer was obtained in 5–10% yield.

***1,3-Bis{*[N-benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl]-(benzo-4-yl)piperazine}thiourea (65)—**^{1}H NMR ($CDCl_3$): δ 8.38 (b, 2H), 7.72 (d, J = 8.0 Hz, 4H), 7.50–7.28 (m, 20H), 7.24–7.06 (m, 6H), 6.94 (d, J = 8.2 Hz, 4H), 5.68–5.58 (m, 2H), 5.14–5.00 (m, 4H), 4.90–4.74 (b, 2H), 3.80–2.80 (m, 20H), 2.45 (s, 6H). FAB-MS: m/z (relative intensity) 1355.41 (M + H, 7), 1356.41 (M + 2H, 5.5), 1377.38 (M + Na, 1), 1393.35 (M + K, 2.5), 119.0 (100). FAB–HRMS (CsI) for $C_{71}H_{70}O_{14}N_8S_3Cs$ $[M + Cs]^+$, calcd; 1487.3228; found, 1487.3210.

1-[(N-Benzyloxycarbonyl-O-(benzenesulfon-4-yl)-L-tyrosyl)benzoylpiperazine]-3-[(N-benzyloxycarbonyl-O-toluenesulfonyl-L-tyrosyl)(benzo-4-yl)piperazine]thiourea (66)—^{1}H NMR ($CDCl_3$): δ 7.80 (b, 2H), 7.72 (d, J = 8.2 Hz, 4H), 7.50–7.28 (m, 10H), 7.22–7.28 (m, 6H), 6.93 (d, J = 8.0 Hz, 4H), 5.60 (b, 2H), 5.16–4.98 (m, 4H), 4.80 (b, 2H), 3.80–2.80 (m, 20H), 2.45 (s, 3H). FAB-MS: m/z (relative intensity) 1341.4 (M + H, 5), 119.0 (100). FAB–HRMS (CsI) for $C_{70}H_{68}O_{14}N_8S_3Cs$ $[M + Cs]^+$, calcd; 1473.3071; found, 1473.3105.

***1,3-Bis{*[N-benzyloxycarbonyl-O-(benzenesulfon-4-yl)-L-tyrosyl]benzoylpiperazine}thiourea (67)—**^{1}H NMR ($CDCl_3$): δ 9.30 (b, 2H), 7.90–7.55 (m, 8H), 7.50–7.30 (m, 20H), 7.20–6.80 (m, 8H), 5.65 (d, J = 8.5 Hz, 2H), 5.25–4.90 (m, 4H), 4.80 (b, 2H), 3.80–2.50 (m, 20H). FAB–HRMS (CsI) for $C_{69}H_{66}N_8O_{14}S_3Cs$ $[M + Cs]^+$: calcd 1459.2915, found 1459.2919.

Pharmacological Analysis. $P2X_7$ Receptor Channel Activation

All experiments were performed using adherent HEK293 cells stably transfected with cDNA encoding the human $P2X_7$ receptor. Adherent cells on 12-well polylysine-coated plates were incubated at 37 °C in 1 mL of physiological salt solution (125 mM NaCl, 5 mM KCl, 1 mM

$MgCl_2$, 1.5 mM $CaCl_2$, 25 mM NaHEPES (pH 7.5), 10 mM D-glucose, 1 mg/mL BSA). Antagonists were added from 1000× stock solutions dissolved in DMSO. Cells were preincubated with antagonists for 15 min prior to stimulation for 10 min with 3 mM ATP (final concentration). Reactions were terminated by rapid aspiration of the extracellular medium in each well. The adherent cells in each well were then extracted overnight with 1 mL 10% HNO_3. K^+ content in these nitric acid extracts was assayed by atomic absorbance spectrophotometry. Duplicate or triplicate wells were run for all test conditions in each separate experiment, and the measured K^+ contents were averaged. Antagonist function was measured by the percent inhibition of the K^+ release triggered by 3 mM ATP in paired wells in the absence of antagonist.

RESULTS

Chemical Synthesis

The analogues consisted of L-tyrosine derivatives, of the general structure R_1-Tyr-(OR_2)-piperazinyl-R_3 (Table 1), in which three variable regions were systematically varied in structure (compounds **3**–**35**) through facile acylation reactions and other procedures. The lead compound MRS 2409 (17), **2**, contained an isoquinolinyl sulfonate at the tyrosyl hydroxyl group. For the present study, this group was first substituted with a phenyl sulfonate ester, a structural simplification known to preserve affinity for the receptor (17). Each of the three regions (R_1–R_3) was probed systematically for possbile attachment sites, through synthesis alternating with biological evaluation, which consisted of screening at a single concentration (initially 3 μM). This process led to the identification and partial optimization of potent, functionalized $P2X_7$ antagonists.

The synthetic routes for systematic substitution at three positions on the molecule are shown in Schemes 1–5. Subsequent functional group replacement beginning with nitro groups is shown in Scheme 6. Synthetic yields are listed in Materials and Methods. General probing of the SAR at each position was carried out. Scheme 1 indicates substitutions made at the R_1 position, including both *N*-cinnamoyl (4–7) and *N*-benzyloxycarbonyl (15 and 16) derivatives. Each of the three ring-substitution positions shown in Table 1 (R_1–R_3) was designed for the initial incorporation of a nitro group (at the meta-position for R_1, meta- or para-positions for R_2 and R_3). For a series of *N*-cinnamoyl derivatives (Scheme 2), multiple nitro groups (11–14) were also included. At R_2 and R_3 the nitro groups were introduced as shown in Schemes 3 and 4.

The synthesis of *N*-(nitrobenzyloxycarbonyl)-L-tyrosine derivatives (Scheme 5) required a modification of the original route based on simple reduction of 4-nitro groups. Since the *N*-(4-aminobenzyloxycarbonyl)tyrosine derivative of **17** proved to be unstable, we sought to develop an alternate synthetic method, aimed instead at the 3-nitro derivative, **18**. Usually, benzyl chloroformates are synthesized through the reaction of benzyl alcohol with phosgene (22). Here, an interesting alternative for the incorporation of *N*-(3-nitrobenzyloxycarbonyl) into tyrosine was the transformation of the α-amine of the tyrosyl moiety into an isocyanate group first with the aid of 1,1′-carbonyldiimidazole (23) to yield **49**, and subsequent addition of the alcohol to yield **18**.

The nitro groups at R_1, R_2, and R_3 were directly reduced to amines (Scheme 6), which were either converted to the isothiocyanate groups, in **21**, **29**, and **34**, as potential affinity labels for the receptor, or acylated, as models for conjugation. The isothiocyanate groups also reacted with alkyl diamines to produce the primary amine congeners **22**, **30**, and **35**. Several methods were compared for reduction of the aromatic nitro compounds. Many reducing reagents have been used to reduce aromatic nitro compounds (24) with the most classic being zinc, tin, or iron in the presence of an acid. Other reagents include hydrazine, $TiCl_4$–dialkyl telluride and sulfide (25). However, most of them lack the desired chemoselectivity over the functional groups that are present in our compounds such as amide, *tert*-butoxycarbonyl (Boc), 9-fluorenylmethoxycarbonyl (Fmoc), and benzyloxycarbonyl (Cbz). Though aromatic nitro compounds are seldom reduced by sodium borohydride in alcoholic solution, we found $NaBH_4$–copper(II) acetylacetonate (26) to be the most effective and a mild catalyst for the reduction of nitro groups on these tyrosine derivatives. This reaction occurred nearly quantitatively as judged using TLC.

Four dimer permutations (**64**–**67**) were created by coupling each of the isothiocyanate derivatives to either the precursor amine or to other amine congeners (Scheme 7). These coupling reactions required elevated temperature and were carried out in moderate yield.

Biological Activity

The effects of substitution at R_1, R_2, and R_3 on inhibition of $P2X_7$ receptor-mediated ion flux (Table 1) were compared. Experiments were performed using adherent HEK293 cells stably transfected with cDNA encoding the human $P2X_7$ receptor. Cells were preincubated with each antagonist at a fixed concentration (3 μM) prior to stimulation for 10 min with 3 mM ATP. The percent inhibition of the K^+ release was the parameter used to indicate antagonist function.

The three structural regions for conjugation were compared in their effects on biological potency. Among *N*-cinnamoyl derivatives, more favorable potency was associated with the unsubstituted derivative **9** than with methoxy substitution. Multiple methoxy substitution, e.g., **7**, eliminated the antagonistic effect at the $P2X_7$ receptor. The 4-nitro substitution of the cinnamoyl group also reduced potency (cf. **14** vs **13**). The Cbz derivative **15** was a potent antagonist of the human $P2X_7$ receptor, and 4- or 3-nitro substitution preserved that property, as in **18** and **19**. However, reduction and acetylation of the 3-nitro group, **20a** and **20b**, reduced potency. The corresponding isothiocyanate **21** and amine congener **22** were inactive or weakly potent, respectively.

Nitro subsitution of the tyrosyl side chain (R_2 substituent), at the 3- or 4-position, as in benzoylpiperazine (at R_3) derivatives **26a** and **26b**, respectively, maintained potency at the human $P2X_7$ receptor. There was no difference in biological potency between 3- and 4-nitro derivatives. The corresponding t-Boc derivatives (at the R_3 position, e.g., **23a** and **23b**) similarly antagonized the human $P2X_7$ receptor. This antagonism was preserved when the 4-nitro group was reduced and acetylated (e.g. **24** and **25**; **27** and **28**). A primary amine congener linked at the R_2 4-position, **30**, was a potent antagonist of the human $P2X_7$

receptor. The corresponding isothiocyanate **29** displayed considerable potency as a $P2X_7$ receptor antagonist and warranted examination as an irreversible receptor inhibitor.

For the R_3 substituent, 3- or 4-nitrobenzoyl subsitution, **31a** and **31b**, respectively, preserved antagonistic properties at the human $P2X_7$ receptor. As for the R_2 substituent, there was no difference in biological potency between 3- and 4-nitro derivatives. The $P2X_7$ receptor antagonism was increased when the 4-nitro group was reduced but diminished upon acetylation (e.g., **32** and **33**). An isothiocyanate **34** only weakly antagonized the effects of ATP at the $P2X_7$ receptor. A primary amine congener containing four methylenes linked at the R_2 4-position, **35b**, was a potent antagonist of the human $P2X_7$ receptor, while a shorter homologue **35a** (two methylenes) antagonized weakly.

Full concentration–response curves provided a more precise means of comparison among some selected, potent compounds, including the 3-nitro-Cbz derivative **18**, a 4-nitrotoluenesulfonate **26b**, a primary amine congener **30**, and 4-aminobenzoyl derivative **32** (Figure 1). Compounds **18** and **26b**, were roughly as potent as **1** as $P2X_7$ receptor antagonists, and appeared to have IC_{50} values in the 0.1 μM range. The other compounds appeared to have IC_{50} values in the 0.3 μM range (**30**) or > 0.3 μM (**32**).

Among antagonist dimers, only the R_2–R_3- and R_2–R_2-linked dimers, **66** and **67**, respectively, displayed significant antagonistic properties at the $P2X_7$ receptor.

DISCUSSION

The binding site for KN-62, **1**, resides within the first 335 residues of the human $P2X_7$ receptor, likely within the large extracellular loop (13, 21). While it is not feasible to model this binding interaction, due to lack of a high-resolution template for this ion channel and uncertainty about the oligomeric nature of the channel (27), chemical probes of the receptor may be very useful in studying the structure. The stoichiometry of association of subunits in P2X receptors has been studied suggesting a trimeric organization (2, 27, 28), but little is known about the correspondence of antagonist binding sites to the overall oligomeric ion channel structure. By functionalizing derivatives (17) of the widely used $P2X_7$ antagonist KN-62 it may be possible to locate the binding site (in conjunction with mutagenesis studies). Mutagenesis studies of the $P2X_7$ receptor aimed at structural and functional elucidation have already been carried out (29, 32). Peptide mapping of receptor digests (30) using electrophoretic or mass spectroscopic methods following affinity labeling of the receptor would also be useful in this regard.

Since there may be multiple binding sites on the $P2X_7$ receptor homomer, it may be possible to detect the preferred orientation of covalently linked antagonist dimers that may occupy two distinct binding sites, based on divergent biological potencies for a variety of linkage combinations. Since the dimerization of the antagonists in the present study through the tyrosyl side chain, alone, provided retention of antagonist properties, it is likely that this region of the antagonist occurs in a portion of the binding site that is somewhat relaxed in its steric requirements. Whether the dimers **66** and/or **67** are able to bridge two putative binding sites on the $P2X_7$ homomer will be the object of further studies. The species selectivity of

the new functionalized congeners also should be investigated, since differences have been noted for KN-62 (33).

Receptor ligands that bridge two subunits of a multimeric receptor may also potentially be of use for tissue selectivity, if there are differences between tissues in the organization of subunits. Such differences have been demonstrated for native $P2X_7$ receptors (34), which in peritoneal macrophage and bone marrow cells exist as a strongly bound multimeric complex, and in brain glia and/or astrocytes appear to form only as monomeric subunits.

In conclusion, we have systematically varied substituent groups in a series of tyrosyl analogues, resulting in analogues, among which are antagonists that are equipotent to the lead structure **1** and also contain chemical functional groups for linking purposes. Such functionalized congeners have been used effectively for the structural investigations of other purine receptors (18, 19, 31), including adenosine A_1, A_{2A}, and A_3 receptors. Suitable sites and chemical approaches to cross-linking these antagonists have been found on the tyrosyl side chain (R_2) and on the CR-substituent (R_3). The more versatile region for functionalization appears to be the R_2 substituent, since even dimerization through this substituent preserves the receptor binding property. We have prepared several amine functionalized congeners, that may potentially be conjugated to fluorescent probes for receptor characterization, or immobilized on a solid support for isolation of the receptor by affinity chromatography. Among P2X receptors, only the $P2X_1$ subtype has so far been isolated in this manner (35).

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Abbreviations

Bop-Cl	bis(2-oxo-3-oxazolidinyl)phosphinic chloride
Boc	*tert*-butyloxycarbonyl
BSA	bovine serum albumin
Cbz	benzyloxycarbonyl
DMAP	4-(dimethylamino)pyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
EtOAc	ethyl acetate
FAB	fast atom bombardment
Fmoc	9-fluorenylmethyloxycarbonyl
HEK	human embryonic kidney

HEPES	*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)
HRMS	high-resolution mass spectrometry
KN-62	1-[*N*,*O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine
MRS 2409	[*N*-benzyloxycarbonyl-*O*-[4-(isoquinolinylsulfonyl)]-L-tyrosyl]benzoylpiperazine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin-layer chromatography

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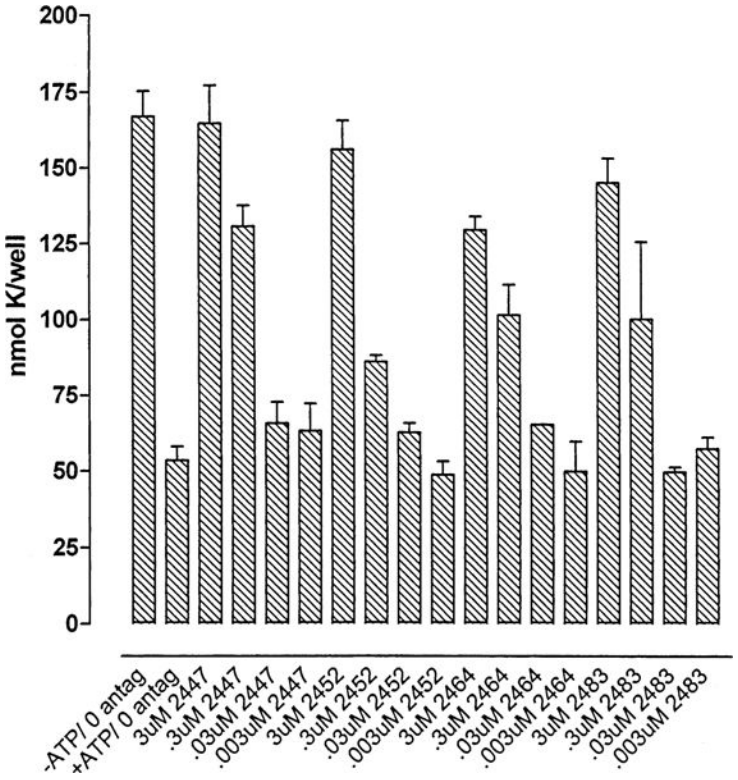
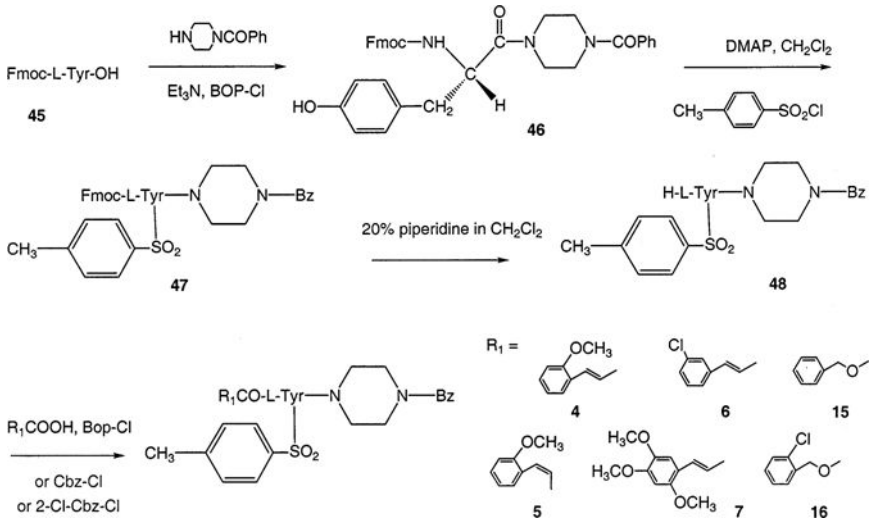


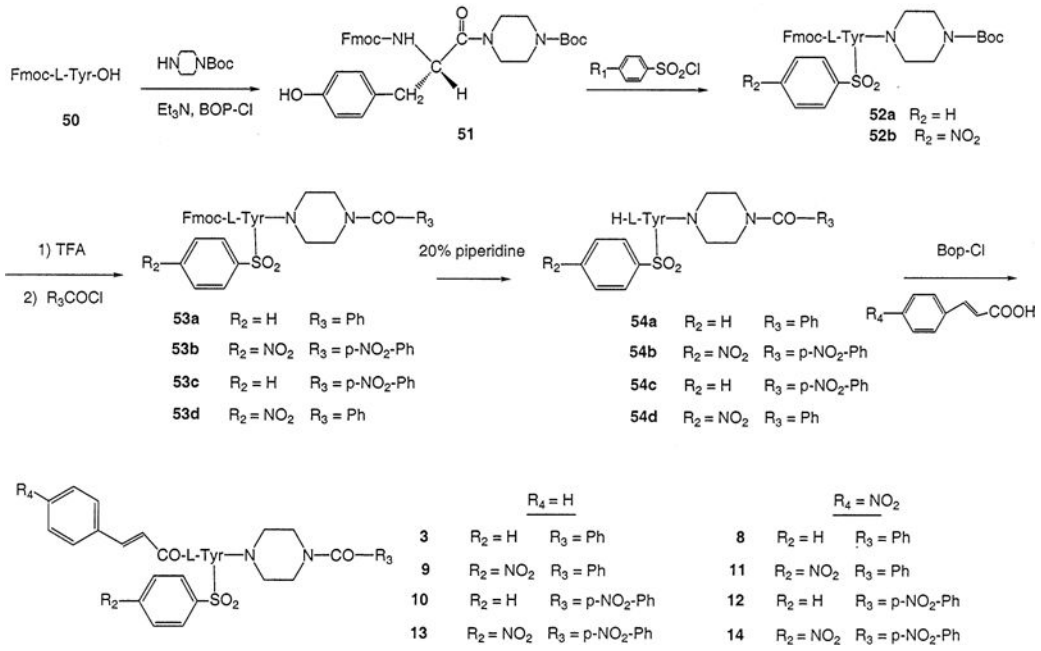
Figure 1.

Effects of tyrosyl derivatives on $P2X_7$ receptor-activation in $hP2X_7$–HEK cells. Cells were preincubated with or without the indicated concentrations of selected antagonists (**26b** MRS2447; **32** MRS2452; **18** MRS2464; and **30** MRS2483) prior to stimulation with 3 mM ATP. Data points represent the mean (± SD) K^+ contents from triplicate wells in a single experiment. IC_{50} values mentioned in the text are rough estimates from visual inspection of the concentration–response relationships. Hill coefficients were not determined, since previous studies have shown that the lead compound (KN-62) represses $P2X_7$ receptor function via complex mechanisms that are not readily amenable to standard ligand-binding analyses.



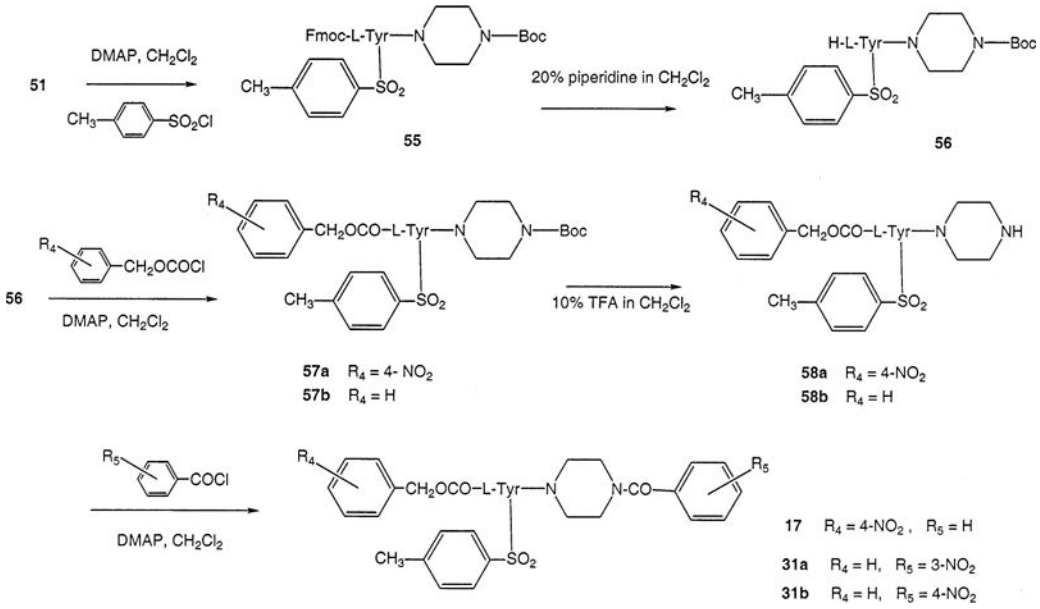
Scheme 1.

Synthesis of $P2X_7$ Receptor Antagonists Consisting of L-Tyrosine Derivatives Differing in the Cinnamoyl or Urethane Substituent Present at the Nα Position



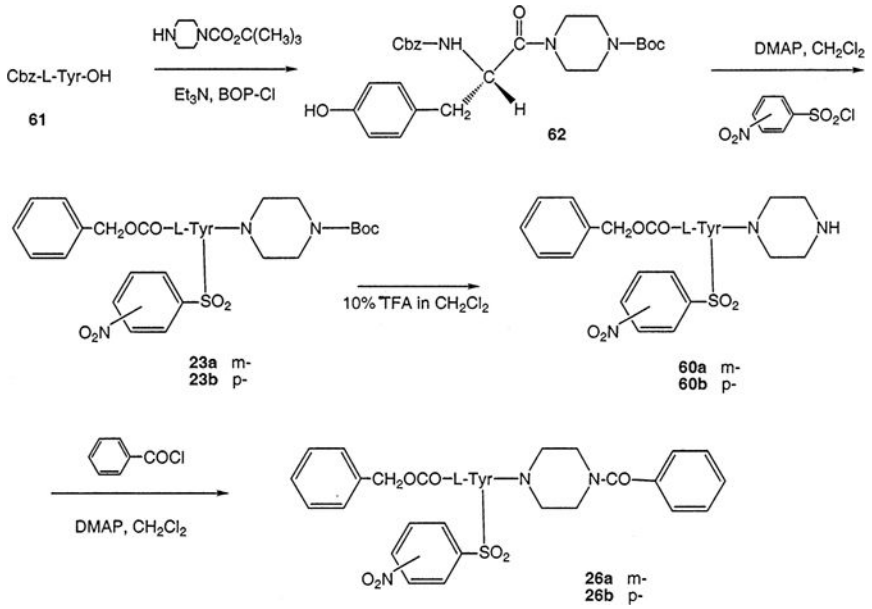
Scheme 2.

Synthesis of $P2X_7$ Receptor Antagonists Consisting of L-Tyrosine Derivatives Containing a Nitro Substitution at Either the *N*α-Cinnamoyl Substituent, the Aryl Sulfonate Group Present on the Tyrosyl Side Chain, or the *C*α-Benzoylpiperazine Substituent



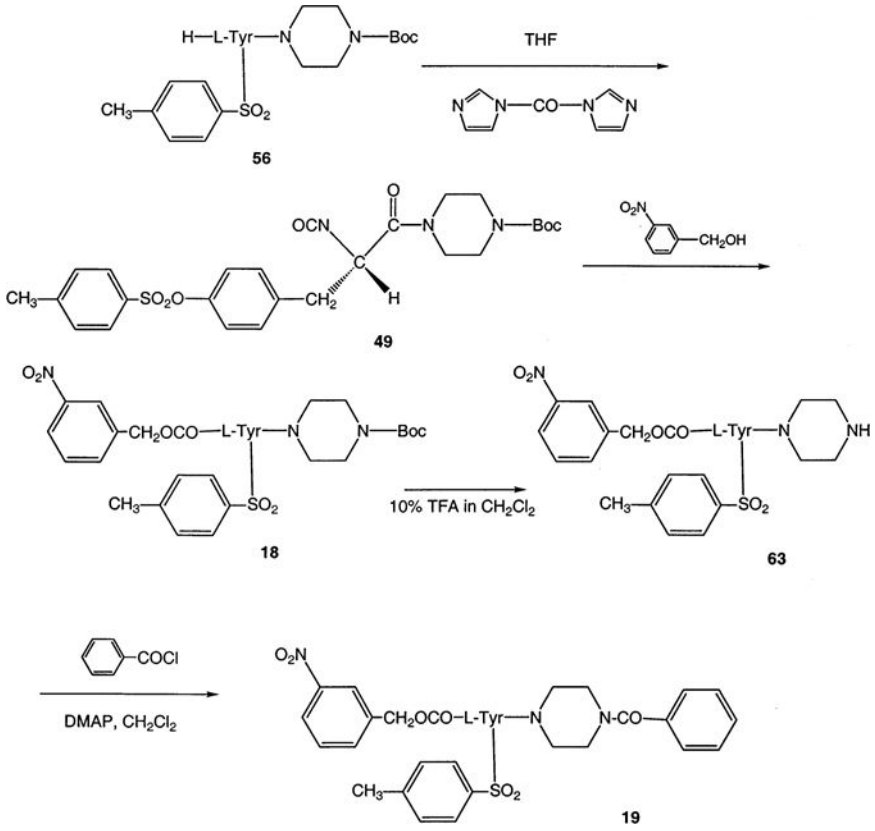
Scheme 3.

Synthesis of P2X$_7$ Receptor Antagonists Consisting of L-Tyrosine Derivatives Containing a Nitro Substitution at the *N*α-Benzyloxycarbonyl Substituent or of the *C*α-Benzoylpiperazine Substituent



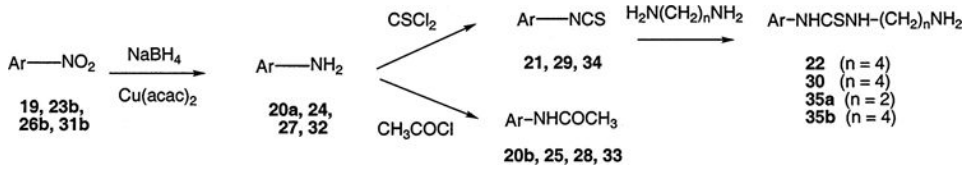
Scheme 4.

Synthesis of $P2X_7$ Receptor Antagonists Consisting of L-Tyrosine Derivatives Containing a Nitro Substitution at the Aryl Sulfonate Group Present on the Tyrosyl Side Chain



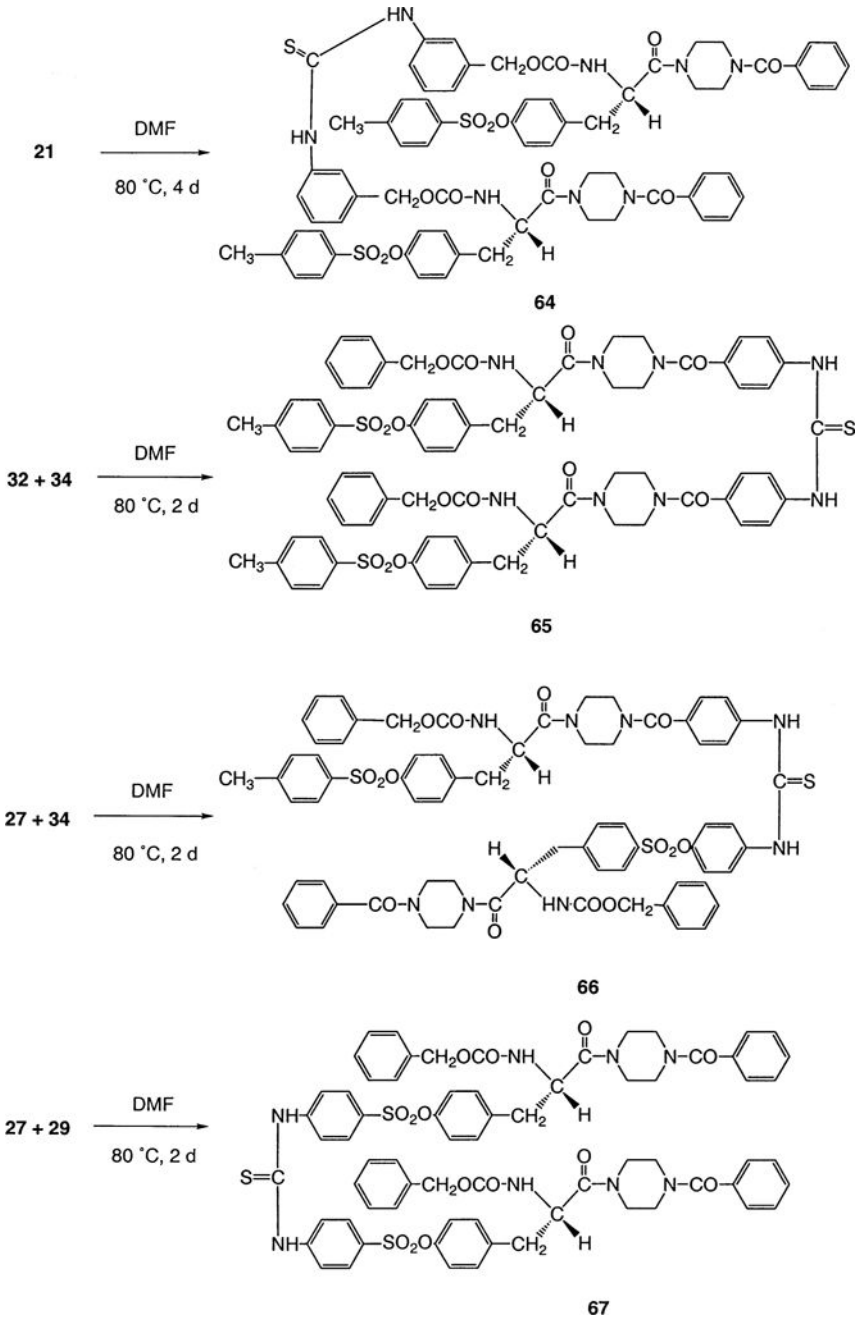
Scheme 5.

Synthesis of $P2X_7$ Receptor Antagonists Consisting of L-Tyrosine Derivatives Containing 3-Nitro Substitution at the *N*α-Benzyloxycarbonyl Substituent



Scheme 6.

Functional Group Replacement in $P2X_7$ Receptor Antagonists

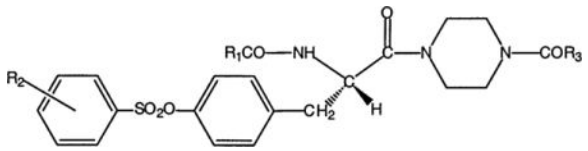


Scheme 7.

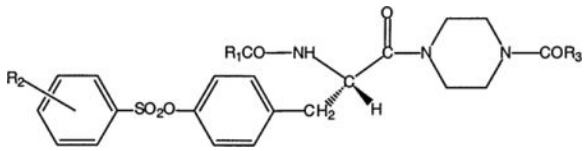
Dimerization of $P2X_7$ Receptor Antagonists through Formation of Thiourea Linkages

Table 1

Antagonistic Effects of Tyrosine Derivatives on Function of Human $P2X_7$ Receptors Expressed in HEK293 Cells[a]



compound	R_1	R_2	R_3	% inhibition	*n*
		Monomeric			
3	Ph-CH=CH (trans)	H	Ph	62 ± 4	2
4	2-OCH_3-Ph-CH=CH (trans)	4-CH_3	Ph	27 ± 2	2
5	2-OCH_3-Ph-CH=CH (cis)	4-CH_3	Ph	17 ± 1	2
6	3-Cl-Ph-CH=CH (trans)	4-CH_3	Ph	57 ± 5	2
7	2,4,5-$(OCH_3)_3$-Ph-CH=CH (trans)	4-CH_3	Ph	−2 ± 6	2
8	4-NO_2-Ph-CH=CH (trans)	H	Ph	43 ± 20	2
9	Ph-CHdCH (trans)	4-NO_2	Ph	72 ± 4	3
10	Ph-CHdCH (trans)	H	Ph-4-NO_2	41 ± 1	2
11	4-NO_2-Ph-CH=CH (trans)	4-NO_2	Ph	58 ± 1	2
12	4-NO_2-Ph-CH=CH (trans)	H	Ph-4-NO_2	30 ± 2	2
13	Ph-CH=CH (trans)	4-NO_2	Ph-4-NO_2	43 ± 14	3
14	4-NO_2-Ph-CH=CH (trans)	4-NO_2	Ph-4-NO_2	4 ± 9	2
15	Ph-CH_2O	4-CH_3	Ph	71 ± 21	2
16	2-Cl-Ph-CH_2O	4-CH_3	Ph	43 ± 17	3
17	4-NO_2-Ph-CH_2O	4-CH_3	Ph	69 ± 25	3
18	3-NO_2-Ph-CH_2O	4-CH_3	O-$C(CH_3)_3$	94 ± 28	3
19	3-NO_2-Ph-CH_2O	4-CH_3	Ph	72 ± 6	2
20a	3-NH_2-Ph-CH_2O	4-CH_3	Ph	50 ± 6	2
20b	3-CH_3CONH–Ph-CH_2O	4-CH_3	Ph	9 ± 13	2
21	3-SCN-Ph-CH_2O	4-CH_3	Ph	−4 ± 5	2
22	3-[$H_2N(CH_2)_4NH$-CSNH]-Ph-CH_2O	4-CH_3	Ph	17 ± 4	2



compound	R_1	R_2	R_3	% inhibition	*n*
23a	Ph-CH_2O	3-NO_2	O-$C(CH_3)_3$	74 ± 2	2
23b	Ph-CH_2O	4-NO_2	O-$C(CH_3)_3$	30 ± 28	2
24	Ph-CH_2O	4-NH_2	O-$C(CH_3)_3$	64 ± 17	2
25	Ph-CH_2O	4-$NHCOCH_3$	O-$C(CH_3)_3$	64 ± 27	2
26a	Ph-CH_2O	3-NO_2	Ph	75 ± 10	2
26b	Ph-CH_2O	4-NO_2	Ph	78 ± 18	3
27	Ph-CH_2O	4-NH_2	Ph	56 ± 9	2
28	Ph-CH_2O	4-$NHCOCH_3$	Ph	31 ± 41	2
29	Ph-CH_2O	4-NCS	Ph	72 ± 1	2
30	Ph-CH_2O	4-[$H_2N(CH_2)_4NH$-CSNH]-	Ph	80 ± 11	3
31a	Ph-CH_2O	4-CH_3	Ph-3-NO_2	60 ± 16	2
31b	Ph-CH_2O	4-CH_3	Ph-4-NO_2	61 ± 6	2
32	Ph-CH_2O	4-CH_3	Ph-4-NH_2	93 ± 3	2
33	Ph-CH_2O	4-CH_3	Ph-4-$NHCOCH_3$	45 ± 21	2
34	Ph-CH_2O	4-CH_3	Ph-4-NCS	30 ± 16	2
35a	Ph-CH_2O	4-CH_3	Ph-4-NHCS-$NH(CH_2)_2NH_2$	14 + 19	2
35b	Ph-CH_2O	4-CH_3	Ph-4-NHCS-$NH(CH_2)_4NH_2$	63 ± 16	2
		Dimeric			
	linkage				
64	R_1–R_1			−5 ± 4	2
65	R_3–R_3			26	1
66	R_2–R_3			61 ± 29	2
67	R_2–R_2			72 ± 41	2

[a]All experiments were performed using adherent HEK293 cells stably transfected with cDNA encoding the human P2X7 receptor. Cells were preincubated with antagonists (3 μM final concentration) for 15 min prior to stimulation for 10 min with 3 mM ATP (final concentration). Antagonist function was measured by the percent inhibition of the K^+ release triggered by 3 mM ATP in paired wells in the absence of antagonist. Data points represent the mean ± SD of values obtained. **15**, MRS 2427; **18**, MRS 2464; **26b**, MRS 2447; **30**, MRS 2483; **32**, MRS 2452; **35b**, MRS 2484; **66**, MRS 2454; **67**, MRS 2455.