Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 2905–2912

BIOORGANIC & MEDICINAL CHEMISTRY

Novel Potent Substance P and Neurokinin A Receptor Antagonists. Conception, Synthesis and Biological Evaluation of Indolizine Derivatives

Régis Millet,[a] Juozas Domarkas,[a] Benoît Rigo,[b] Laurence Goossens,[a] Jean-François Goossens,[a] Raymond Houssin[a] and Jean-Pierre Hénichart[a,*]

[a]*Institut de Chimie Pharmaceutique Albert Lespagnol, Université de Lille 2, EA 2692, 3 rue du Professeur Laguesse, B.P. 83, F-59006 Lille, France*
[b]*Laboratoire d'Ingénierie Moléculaire, Ecole des Hautes Etudes Industrielles, 13 rue de Toul, F-59046 Lille, France*

Received 11 January 2002; accepted 3 May 2002

Abstract—Exploration of SAR around dual NK_1/NK_2 antagonist Cbz-Gly-Leu-Trp-OBzl$(CF_3)_2$ and its derivatives disclosed the essential requirements for more potent dual NK_1/NK_2 binding. We report here the synthesis and the biological properties of a novel series of indolizine including pharmacophoric elements.

Introduction

Substance P (SP), Neurokinin A (NKA) and Neurokinin B (NKB) are members of the tachykinin family (Table 1) and share a common C-terminal amino acid sequence Phe-X-Gly-Leu-Met-NH_2 (Table 1) often referred to as the message moiety of the peptide.[1,2]

The biological actions of tachykinins are mediated through specific G-protein coupled receptor. There are three subtypes of receptor designated NK_1, NK_2 and NK_3. SP mediates its physiological effects[3] mainly by binding to a specific NK_1 receptor whereas NKA and NKB exert their activities via the NK_2 and NK_3 receptors, respectively.

Neurokinin antagonists have been the subject of considerable investigation since the association of their central and peripheral actions with the treatment of chronic diseases like asthma,[4,5] pain,[6] emesis[7,8] and psychiatric disorders.[9,10]

In the pathogenesis of asthma, SP and NKA have been widely studied in animal and human airways. These neuropeptides are localised in sensory airway nerves from which they can be released by a variety of stimuli, including allergen, ozone or inflammatory mediators. Involvement of NK_1 receptors seems to be more important in inflammatory conditions including vasodilatation, mucus secretion, plasma extravasation and leukocyte adhesion-activation, while NK_2 mediates the bronchomotor tone. Taking into account these features, it was hypothesized that dual NK_1/NK_2 antagonism may be useful in the treatment of asthma.[11,12]

We have already reported that the tripeptide Cbz-Gly-Leu-Trp-OBzl$(CF_3)_2$ exhibited dual NK_1 and NK_2 affinities.[13–15] Studies of structure–activity relationships revealed that the C-terminal sequence [Trp-OBzl$(CF_3)_2$] was shown to be favourable for NK_1 recognition and indolylmethyl and Cbz carbamate groups for NK_2 recognition. These results encouraged us to find new potent dual NK_1/NK_2 receptor antagonists by structural modifications of the tripeptide Cbz-Gly-Leu-Trp-OBzl$(CF_3)_2$ (Table 2). Thus, we decided firstly to investigate chemical modifications of C-terminal sequence since a new class of dual NK_1/NK_2 antagonists (Scheme 1) has recently been reported as neurokinin receptor antagonists including the *N*-methylbenzamide group in their structure.[17] For a better understanding of NK_1/NK_2 profile binding and to improve metabolic stability, we went on to replace the ester linkage of Cbz-Gly-Leu-Trp-OBzl$(CF_3)_2$ by several amide groups (**1a**–**c**).

*Corresponding author. Tel.: +33-3-2096-4374; fax: +33-3-2096-4906; e-mail: henicha@phare.univ-lille2.fr

0968-0896/02/$ - see front matter
PII: S0968-0896(02)00144-X

Table 1. Chemical structure of three mammalian tachykinins

Tachykinin	Sequence
SP	Arg-Pro-Lys-Pro-Gln-Gln-*Phe*-Phe-*Gly-Leu-Met-*NH_2
NKA	His-Lys-Thr-Asp-Ser-*Phe*-Val-*Gly-Leu-Met-*NH_2
NKB	Asp-Met-His-Asp-Phe-*Phe*-Val-*Gly-Leu-Met-*NH_2

Amino acids common to carboxyl-terminal are italicized.

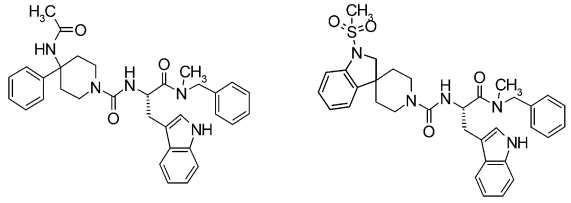
Table 2. Binding affinity and calcium responses in hNK_1- or hNK_2-receptors expressing CHO cells

Compound	hNK_1 K_i[a] (nM)	pA_2[b]	hNK_2 K_i[a] (nM)	pA_2[b]
1a	200±18	6.8±0.4	>3000	n.d.
1b	1580±82	n.d.[c]	>3000	n.d.
1c	100±7	6.7±0.3	79±5	7.1±0.5
2a	79±8	7.0±0.2	>3000	n.d.
2b	158±16	6.9±0.3	398±35	6.2±0.2
2c	100±9	6.7±0.4	100±12	6.7±0.4
Cbz-Gly-Leu-Trp-OBzl$(CF_3)_2$	40±3	n.d.	250±24	n.d.

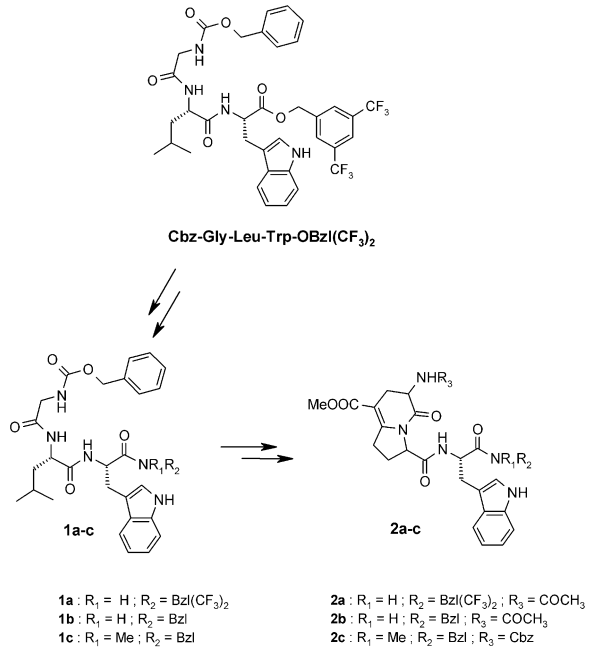
[a]Inhibition of [^{3}H]SP or [^{3}H]NKA binding to membranes of hNK_1- or hNK_2-receptors expressing CHO cells. Each value represents the mean±SD of three independent experiments (triplicate per experiment) performed from a 10^{-11}–10^{-6} M range.
[b]Inhibition of SP- or NKA-induced intracellular $[Ca^{2+}]_i$ increase in hNK_1 or hNK_2 receptors expressing CHO cells loaded with Fura-2. Each value is the mean±SD derived from Schild plot using three independent experiments (triplicate per experiment) in a 10^{-10}–10^{-6} M range for both agonist and antagonist compounds.
[c]n.d., not determined.

In addition, we focused our interest on a rational design of peptidomimetics.[18,19] In previous reports, we described that introducing building units such as spirolactam or lactam led to restricted Cbz-Gly-Leu-Trp-OBzl$(CF_3)_2$ conformations showing high binding affinity and selectivity for NK_1 receptors. To continue our investigation in the design of neurokinin antagonists, we report here (Scheme 2) the synthesis and binding profile of new compounds which include an indolizine moiety in their N-terminal structure (**2a**–**c**) mimicking the Gly-Leu sequence of the tripeptide Cbz-Gly-Leu-Trp-OBzl$(CF_3)_2$. Investigation of such a system should allow an increasing neurokinin binding potency through a decrease in the entropy system.

Furthermore, these chemical modifications led us to explore the importance of the amide function and Cbz carbamate on NK_2 recognition and to improve our knowledge of the bioactive conformation of neurokinin antagonists with a view to creating and optimising dual NK_1/NK_2 antagonists.



Scheme 1. Structure of dual NK_1/NK_2 antagonists.



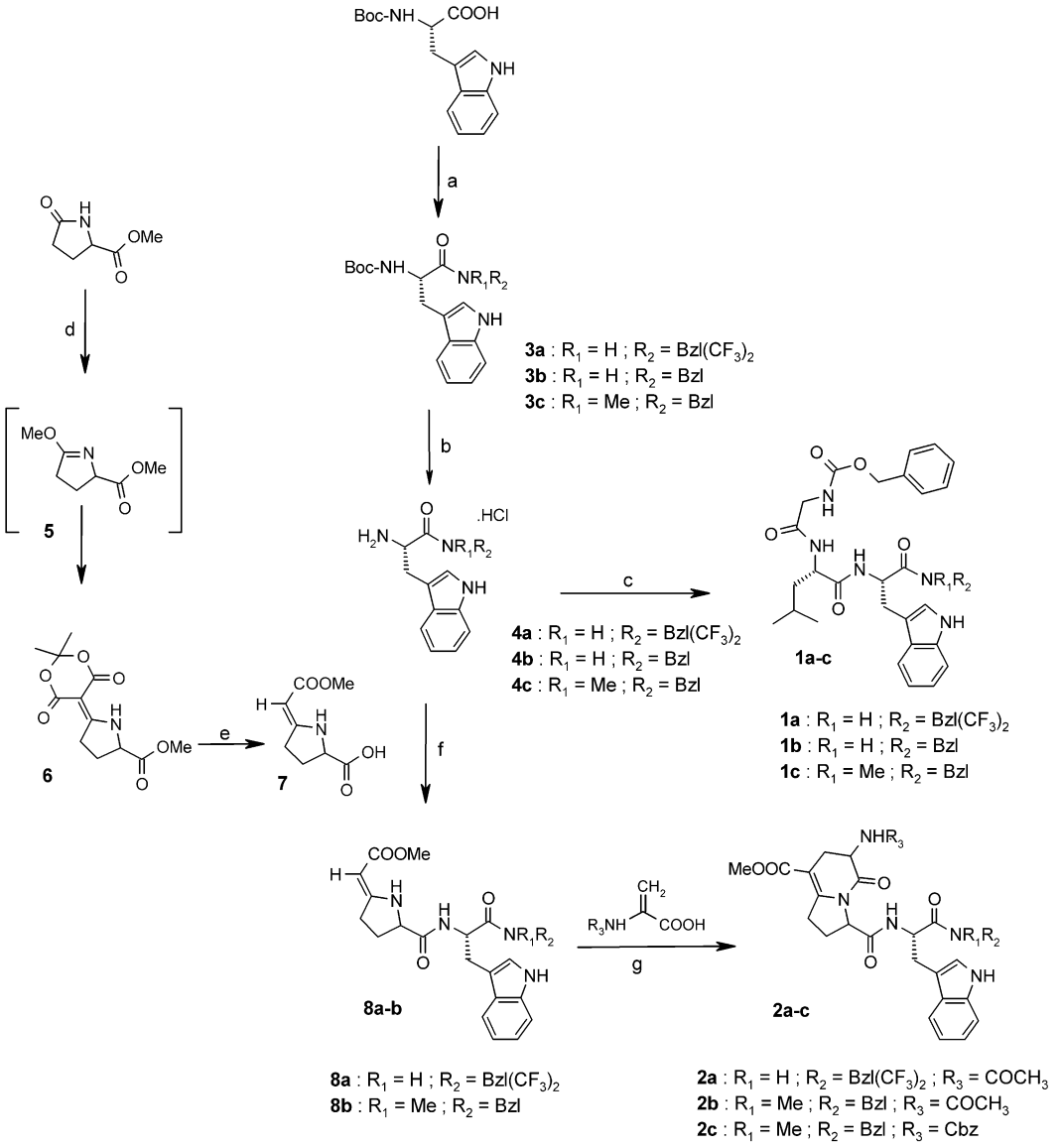
Scheme 2. Drug design of tripeptides **1a**–**c** and indolizines **2a**–**c**.

Results and Discussion

Chemistry

Cbz-Gly-Leu-Trp-N$(R_1)R_2$ peptides (**1a**–**c**) were obtained (Scheme 3) according to classical peptidic methods[20] in solution using Boc as protective group for Trp, and EDCI/HOBt or PyBOP as coupling agents. Boc-Trp-OH was coupled with 3,5-bis(trifluoromethyl) benzylamine, benzylamine, or *N*-methylbenzylamine under EDCI/HOBt conditions (59–83% yield) to afford the amides **3a**–**c**. Removal of the Boc protective group with methanolic HCl solution gave amines **4a**–**c** which were coupled with commercially available Cbz-Gly-Leu-OH by using PyBOP to produce tripeptides **1a**–**c** at 58–64% yields after purification.

The synthesis of 5,6-fused bicyclic skeleton (**2a**–**c**) is outlined in Scheme 3 and represents an extension of the chemistry that we previously described.[21,22] The strategy adopted uses enaminoesters **8a**–**b** and different *N*-substituted aminoacrylic acids[23] in a Michael condensation[24] followed by cyclisation promoted by phosphorous trichloride (30–50% yield). Enaminoesters **8a**–**b** were obtained according to a previously described procedure[25] which used methyl pyroglutamate as its starting point. Conversion of pyroglutamate into iminoether **5** was carried out with dimethyl sulfate and triethylamine and a follow-on condensation with Meldrum's acid afforded enaminoester **6** at 83% yield. Opening of Meldrum's ring and saponification of ester function were carried out with sodium methoxide in methanol followed by acidification with hydrochloric acid to give a *Z*/*E* mixture of carboxylic acid **7** at 70% yield. Coupling reactions with tryptophan amides **4a** or **4c** (PyBOP) were finally completed to generate the



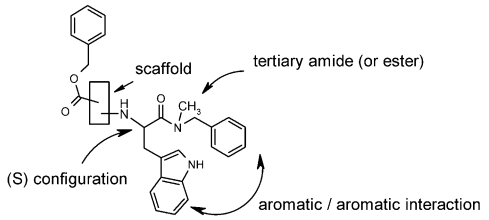
Scheme 3. Reagents and conditions: (a) EDCI, HOBt, amine, DIPEA, CH_2Cl_2, 48 h, rt; (b) MeOH, HCl, 16 h, rt; (c) Cbz-Gly-Leu-OH, PyBOP, DIPEA, CH_2Cl_2, 48 h, rt; (d) (1) $(CH_3)_2SO_4$, 60 °C, 12 h; (2) NEt_3, 0 °C; (3) Meldrum's acid, 24 h, rt; (e) (1) MeONa, MeOH, reflux, 24 h; (2) H_2O, 2 h, rt; (3) HCl, MeOH; (f) PyBOP, DIPEA, CH_2Cl_2, 48 h, rt; (g) PCl_3, dioxane, toluene, reflux, 4 h.

desired enaminoesters **8a**–**b** (70–71% yield) which were directly used in the building of the indolizine ring (**2a**–**c**).

Biological evaluation and structure–activity relationships

The NK_1/NK_2 receptor binding affinity and antagonist activity data are summarized in Table 2. Our peptidic approaches in the design of a dual NK_1/NK_2 antagonist consisted firstly in modifying the ester function of the tripeptide Cbz-Gly-Leu-Trp-OBzl$(CF_3)_2$ by different amide groups to optimize NK_1 and NK_2 affinities and to enhance metabolic stability. Three tripeptides (**1a**–**c**) were prepared and their affinities toward neurokinin receptors were measured. Analysis of the neurokinin binding profile revealed that it was possible to obtain dual NK_1/NK_2 antagonists by substitution of the 3,5-bis(trifluoromethyl)benzyl ester function with *N*-methyl(benzyl)-amide moiety. This observation confirms what we supposed and shows that -Trp-N(Me)Bzl is an interesting pattern providing a good NK_1/NK_2 in binding affinity ratio. Introduction of a primary amide as in compounds **1a**–**b** leads to a significant decrease in binding affinity to NK_2 receptors. Noting these results, we decided to introduce a heterocyclic connector between the previously identified -Trp-N(Me)-Bzl moiety and the Cbz ring of **1c** in an attempt to induce a structural constraint. The indolizine ring was chosen as a scaffold to mimic the Gly-Leu residue of **1c**. Three indolizine compounds (**2a**–**c**) were prepared and their pharmacological results led to two significant observations that enabled us to propose pharmacophoric elements (Scheme 4) of potential dual NK_1/NK_2 receptor antagonists.

Firstly, as it was observed for **1c**-Trp-N(Me)Bzl residues in **2b**,**c** were well tolerated both by NK_1 and NK_2



Scheme 4. Pharmacophoric elements of potential dual NK_1/NK_2 receptor antagonists.

receptors suggesting that these residues are optimal for NK_1/NK_2 recognition. The -Trp-NHBzl$(CF_3)_2$ residue in **2a** saw a significant reduction in NK_2 affinity. Secondly, replacement of the acetamido group in **2b** by Cbz (**2c**) led to an increase in NK_2 affinity. This information is in total agreement with that observed[13] before and led to the conclusion that Cbz carbamate moiety had a direct influence on NK_2 recognition.

All information reported herein and in previous papers[13–16] correlate and result in establishing the essential requirements (Scheme 4) for more potent dual NK_1/NK_2 binding affinity. The C-terminal sequence of tripeptides **1a–c** seems to be responsible for NK_1 recognition—L-tryptophan benzyl esters have already been reported to be selective NK_1 antagonists—whereas indolylmethyl and Cbz moieties proved particularly significant for NK_2 recognition. In addition, the tertiary benzyl amide function of L-Trp has been shown to be an essential requirement for a good NK_1/NK_2 binding ratio.

Furthermore, the in vitro antagonist activities of high affinity selective ligands have been evaluated on all hNK_1- or hNK_2-transfected CHO cells. The ability of compounds to antagonize the calcium mobilization caused by activation of hNK_1- or hNK_2-receptors expressed in CHO cells was studied using Fura-2 fluorescence, as described for hNK_1- or hNK_2-transfected cells.[26–28] SP and NKA stimulate the phosphoinositide-turnover signaling system following binding to its NK_1 and NK_2 receptor, leading to an elevation in intracellular cytosolic Ca^{2+} concentration. Our interest was to examine the effects of blocking the NK_1- or NK_2-receptor with selected compounds ($K_i < 400$ nM) on SP- or NKA-mediated increase in cytosolic Ca^{2+} concentration in CHO cells. SP and NKA induced a maximal elevation of intracellular cytosolic Ca^{2+} concentration from 25 (± 3) to 312 (± 16) nM and from 18 (± 4) to 215 (± 12) nM (mean $\pm$ SEM of 15 separate NK_1 and NK_2-CHO cells. All selected compounds **1a–c** and **2a–c** (10^{-6} M) do not increased intracellular calcium concentration in both cell lines. Inhibition increase in SP- or NKA-induced intracellular calcium concentration was calculated from Schild plot representations, giving pA_2 values indicated in Table 2. Compounds **1a–c** and **2a–c** antagonize the SP- and NKA-induced calcium pathway. Furthermore, for all selected ligands, potent functional antagonistic properties correlate well with the respective specific binding affinities.

Conclusion

For this paper, we prepared dual NK_1/NK_2 antagonists using several pharmacophoric elements proposed in Scheme 4. The development of small molecules -antagonists of SP and NKA—offers a good possibility for the treatment of asthma. Few dual NK_1/NK_2 antagonists have been reported[29–36] and the pharmacophoric elements proposed here open the way for the development of such molecules. Moreover, we have demonstrated that the indolizine ring seems to be an interesting template in the construction of dual NK_1/NK_2 antagonists and easy to use in other drug design peptidic strategies. In our strategy, the introduction of an indolizine scaffold disposed pharmacophoric elements favorably to interact with both the NK_1 and the NK_2 receptors.

Experimental

Chemistry

Melting points were determined on a Büchi 535 capillary melting point apparatus and are uncorrected. Analytical tlc were performed on precoated Kieselgel $60F_{254}$ plates (Merck). The spots were located by uv (254 and 366 nm). Column chromatographies were performed on silica gel 60 230–400 mesh (Merck). IR spectra were determined in potassium bromide with a Bruker Vector 22 spectrophotometer; absorbances are reported in ν (cm^{-1}). ^1H NMR spectra were recorded on a Bruker AC 300 spectrometer (300 MHz) using tetramethylsilane as an internal standard. Chemical shifts were expressed in δ units (ppm) and the splitting patterns were designated as follows: s singlet, bs broad singlet, t triplet, d doublet, dd doublet of doublets, m multiplet, bm broad multiplet. Mass spectra were recorded on a quadripolar Finnigan Mat SSQ 710 instrument in the electron impact (EI) mode. Elemental analyses for C, H, N, were performed by the 'Service Central d'Analyses' (CNRS, Vernaison, France), and are within 0.4% of theory.

General procedure for preparation of *N*-protected amides (3a–c). DIPEA (12.7 mL, 72.9 mmol), EDCI (5.90 g, 30.8 mmol) and HOBt (4.16 g, 30.8 mmol) were added to a solution of Boc-Trp-OH (6.23 g, 20.5 mmol) in CH_2Cl_2 (40 mL) (ice bath). The mixture was stirred for 1 h, then amine (20.5 mmol) was added. The mixture was stirred for 48 h, then the solution was washed with HCl (0.25 N) then $NaHCO_3$ (10%). The organic phase was dried ($MgSO_4$) and evaporated to yield an oil.

***N*-α-*tert*-Butoxycarbonyl-*N*-3,5-bis(trifluoromethyl) benzyltryptophan amide (3a).** The product **3a** was recrystallized from CH_2Cl_2 to give a white solid (6.40 g) at 59% yield: mp 180–182 °C; TLC R_f [hexane/ethyl acetate (5:5)] = 0.55; IR (KBr) ν: 1651 cm^{-1} (CO), 1680 cm^{-1} (CO), 3260 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ: 1.32 (s, 9H), 2.90–3.10 (m, 2H), 4.20–4.59 (m, 3H), 7.00 (t, 1H, $J = 7.5$ Hz), 7.07 (t, 1H, $J = 7.0$ Hz), 7.18 (s, 1H), 7.34 (d, 1H, $J = 8.0$ Hz), 7.63 (d, 1H, $J = 8.0$ Hz), 7.98 (s, 3H), 8.71 (s, 1H), 10.83 (s, 1H); MS (EI) m/z 529 (M^+).

***N*-α-*tert*-Butoxycarbonyl-*N*-benzyltryptophan amide (3b).** The product **3b** was flash chromatographed on a 5×40 cm column using cyclohexane/ethyl acetate (5:5) as the eluting solvent. The product thus obtained crystallized from CH_2Cl_2/hexane (8:2) as a white solid (7.10 g) in 88% yield: mp 136–139 °C; TLC R_f [cyclohexane/ethyl acetate (5:5)] = 0.3; IR (KBr) ν: 1651 cm^{-1} (CO), 1680 cm^{-1} (CO), 3260 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ: 1.32 (s, 9H), 2.89–3.13 (m, 2H), 4.20–4.30 (m, 3H), 6.86 (d, 1H, J = 8.1 Hz), 7.00 (t, 1H, J = 7.2 Hz), 7.09 (t, 1H, J = 7.2 Hz), 7.13 (s, 1H), 7.16–7.29 (m, 5H), 7.34 (d, 1H, J = 8.0 Hz), 7.63 (d, 1H, J = 8.1 Hz), 8.43 (t, 1H, J = 7.5 Hz), 10.83 (s, 1H); MS (EI) m/z 393 (M^+).

***N*-α-*tert*-Butoxycarbonyl-*N*-benzyl-*N*-methyltryptophan amide (3c).** The product **3c** was flash chromatographed on a 5×40 cm column using cyclohexane/ethyl acetate (7:3) as the eluting solvent. The product thus obtained crystallized from diisopropyl ether as a white solid (6.27 g) in 75% yield: mp 144–145 °C; TLC R_f [cyclohexane/ethyl acetate (5:5)] = 0.5; IR (KBr) ν: 1645 cm^{-1} (CO), 1686 cm^{-1} (CO), 3226 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ: 1.30 (s, 3H), 1.33 (s, 6H), 2.72 (s, 1H), 2.78 (s, 2H), 2.89–3.13 (m, 2H), 4.30–4.75 (m, 3H), 7.16–7.29 (m, 8H), 7.34 (d, 1H, J = 8.3 Hz), 7.56 (d, 1H, J = 7.8 Hz), 10.82 (s, 0.33H), 10.88 (s, 0.66H); MS (EI) m/z 407 (M^+).

General procedure for preparation of amides 4a–c. *N*-Protected amide (**3a**–**c**) (10.4 mmol) was dissolved in a mixture of dry tetrahydrofuran (20 mL) and of methanol saturated with hydrochloric acid (30 mL), and the solution was left to stand for 16 h. The solvent was removed in vacuum and the residue was recrystallized.

***N*-3,5-Bis(trifluoromethyl)benzyltryptophanamide hydrochloride (4a).** The product **4a** crystallized from MeOH/CH_2Cl_2 (1:1) as a white solid (4.61 g) in 95% yield: mp 245–247 °C; TLC R_f [CH_2Cl_2/MeOH saturated in NH_3 (9:1)] = 0.6; IR (KBr) ν: 1661 cm^{-1} (CO), 3200–3350 cm^{-1} (NH), 3423 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ: 3.14–3.33 (m, 2H), 4.08 (bs, 1H), 4.41 (dd, 1H, J = 6.5 Hz, J' = 13.0 Hz), 4.56 (dd, 1H, J = 6.4 Hz, J' = 13.1 Hz), 6.97 (t, 1H, J = 7.5 Hz), 7.08 (t, 1H, J = 7.0 Hz), 7.21 (s, 1H), 7.35 (d, 1H, J = 8.0 Hz), 7.68 (d, 1H, J = 7.5 Hz), 7.97 (s, 2H), 8.00 (s, 1H), 8.38 (s, 3H), 9.40 (s, 3H), 11.07 (s, 1H); MS (EI) m/z 429 (M^+).

***N*-Benzyltryptophan amide hydrochloride (4b).** The product **4b** recrystallized from MeOH/acetone (2:8) as a white solid (3.26 g) in 95% yield: mp 228–230 °C; TLC R_f [CH_2Cl_2/MeOH saturated with NH_3 (9:1)] = 0.6; IR (KBr) ν: 1663 cm^{-1} (CO), 3261 cm^{-1} (NH), 3315 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ: 3.14–3.33 (m, 2H), 4.02–4.12 (m, 1H), 4.20–4.34 (m, 2H), 6.99 (t, 1H, J = 7.5 Hz), 7.14–7.30 (m, 7H), 7.38 (d, 1H, J = 8.3 Hz), 7.70 (d, 1H, J = 7.7 Hz), 8.45 (s, 3H), 9.17 (t, 1H, J = 5.8 Hz), 11.16 (s, 1H)); MS (EI) m/z 293 (M^+).

***N*-Benzyl-*N*-methyltryptophan amide hydrochloride (4c).** The product **4c** crystallized from MeOH/ethyl acetate (1:5) as a white solid (3.50 g) at 98% yield: mp 76–77 °C; TLC R_f [CH_2Cl_2/MeOH saturated with NH_3 (9:1)] = 0.6; IR (KBr) ν: 1643 cm^{-1} (CO), 3415 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ: 2.57 (s, 2H), 2.65 (s, 1H), 3.14–3.33 (m, 2H), 4.30–4.70 (m, 3H), 6.97–7.45 (m, 9H), 7.61 (d, 1H, J = 7.7 Hz), 8.51 (s, 3H), 11.18 (s, 1H); MS (EI) m/z 307 (M^+).

General procedure for preparation of tripeptides 1a–c. Amide (**4a**–**c**) (1.00 g, 2.15 mmol), PyBOP (1.23 g, 2.36 mmol) and DIPEA (1.1 mL, 6.44 mmol) were added to a solution of Cbz-Gly-Leu-OH (693 mg, 2.15 mmol) in 40 mL of CH_2Cl_2. The reaction mixture was cooled in an ice bath and stirred for 48 h; then the solvent was removed in vacuum.

***N*-α-Benzyloxycarbonyl-Gly-Leu-*N*-3,5-bis-(trifluoromethyl)benzyltryptophan amide (1a).** The product **1a** was recrystallized from ethyl acetate as a white solid (960 mg) at 61% yield: mp 213–215 °C; TLC R_f [hexane/ethyl acetate (3:7)] = 0.3; IR (KBr) ν: 1640 cm^{-1} (CO), 1718 cm^{-1} (CO), 1723 cm^{-1} (CO), 3250–3400 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ: 0.79–0.83 (m, 6H), 1.30–1.40 (m, 2H), 1.47–1.55 (m, 1H), 3.01–3.17 (m, 2H), 3.65 (d, 2H, J = 5.8 Hz), 4.30–4.53 (m, 4H), 5.03 (s, 2H), 6.97 (t, 1H, J = 7.1 Hz), 7.06 (t, 1H, J = 6.6 Hz), 7.13 (s, 1H), 7.31–7.39 (s, 5H), 7.45–7.49 (m, 1H), 7.55 (d, 1H, J = 7.5 Hz), 7.91 (s, 2H), 7.99 (s, 1H), 8.17 (d, 1H, J = 7.8 Hz), 8.59 (t, 1H, J = 8.0 Hz), 10.82 (s, 1H); MS (CI) m/z 734 (MH^+), 227 $CH_2Ph(CF_3)_2$, 130 (Ind-CH_2^+), 91 Ph-CH_2^+. Anal. calcd for $C_{36}H_{37}F_6N_5O_5$: C, 58.94; H, 5.08; N, 9.55. Found: C, 59.01; H, 5.37; N, 9.42.

***N*-α-Benzyloxycarbonyl-Gly-Leu-*N*-benzyltryptophan amide (1b).** The product **1b** crystallized from CH_2Cl_2 as a white solid (822 mg) at 64% yield: mp 211–212 °C; TLC R_f [hexane/ethyl acetate (3:7)] = 0.3; IR (KBr) ν: 1631 cm^{-1} (CO), 1763 cm^{-1} (CO), 1718 cm^{-1} (CO), 3282 cm^{-1} (NH), 3351 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ: 0.78–0.85 (m, 6H), 1.30–1.40 (m, 2H), 1.47–1.55 (m, 1H), 3.01–3.17 (m, 2H), 3.66 (d, 2H, J = 5.4 Hz), 4.30–4.53 (m, 4H), 5.03 (s, 2H), 6.97 (t, 1H, J = 7.0 Hz), 7.06 (t, 1H, J = 6.8 Hz), 7.13 (s, 1H), 7.20–7.40 (m, 10H), 7.45–7.49 (m, 1H), 7.55 (d, 1H, J = 7.5 Hz), 8.12 (d, 1H, J = 7.8 Hz), 8.41 (t, 1H, J = 8.0 Hz), 10.76 (s, 1H); MS (CI) m/z 598 (MH^+), 130 (Ind-CH_2^+). Anal. calcd for $C_{34}H_{39}N_5O_5$: C, 70.09; H, 6.56; N, 12.57. Found: C, 70.00; H, 6.71; N, 12.44.

***N*-α-Benzyloxycarbonyl-Gly-Leu-*N*-Benzyl-*N*-methyltryptophan amide (1c).** The product was flash chromatographed on a 5×40 cm column using cyclohexane/ethyl acetate (3:7) as the eluting solvent and recrystallized from hexane/CH_2Cl_2 (3:7) to give **1c** as a white solid (763 mg) at 58% yield: mp 92–94 °C; TLC R_f [cyclohexane/ethyl acetate (3:7)] = 0.3; IR (KBr) ν: 1632 cm^{-1} (CO), 1711 cm^{-1} (CO), 3293 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ: 0.79–0.83 (m, 6H), 1.30–1.40 (m, 2H), 1.47–1.55 (m, 1H), 2.66 (s, 1H), 2.68 (s, 2H), 2.92–3.05 (m, 1H), 3.62–3.68 (m, 2H), 4.30–4.53 (m, 4H), 5.05 (s, 2H), 6.90–7.09 (m, 1H), 7.15 (s, 1H), 7.19–7.39 (m, 10H), 7.45–7.49 (m, 1H), 7.57 (d, 1H, J = 8.0 Hz), 7.92 (m, 1H), 8.46 (d, 0.33H, J = 8.0 Hz),

8.53 (d, 0.66H, $J=8.0$ Hz), 10.81 (s, 0.33H), 10.87 (s, 0.66H); MS (CI) m/z 612 (MH^+), 130 (Ind-CH_2^+), 91 (Ph-CH_2^+). Anal. calcd for $C_{35}H_{41}N_5O_5$: C, 68.72; H, 6.75; N, 11.45. Found: C, 68.73; H, 7.01; N, 11.70.

Isopropylidene(methoxycarbonylpyrrolidinylidene)malonate (6). A mixture of dimethyl sulfate (100 mL, 1.06 mol) and methyl pyroglutamate (120 g, 0.85 mol) was stirred at 60 °C for 12 h. The iminoether salt was slowly dropped into an ice well stirred cooled solution of NEt_3 (150 mL, 1.05 mol). Meldrum's acid (123 g, 0.85 mol) was added and the mixture was stirred at room temperature for 24 h. The β-enaminoester **6** was filtered and recrystallized from water as a white solid (189 g) at 83% yield: mp 140–141 °C; TLC R_f [hexane/ethyl acetate (1:1)] = 0.35; IR (KBr) ν: 1720 cm^{-1} (CO), 1745 cm^{-1} (CO), 3300 cm^{-1} (NH); ^1H NMR (300 MHz, $CDCl_3$) δ: 1.70 (s, 6H), 2.20–2.70 (m, 2H), 3.20–3.70 (m, 2H), 3.70 (s, 3H), 4.40–4.90 (m, 1H), 10.10 (s, 1H); MS (EI) m/z 269 (M^+). Anal. calcd for $C_{12}H_{15}NO_6$: C, 53.53; H, 5.61; N, 5.20. Found: C, 53.46; H, 5.59; N, 5.39.

(*E*) and (*Z*)-(*RS*) 5-Methoxycarbonylmethineproline (7). A solution of sodium methoxide (276 mmol) in 140 mL of MeOH was added to a solution of compound **6** (24 g, 88.9 mmol) in MeOH (60 mL). The mixture was refluxed for 24 h then water (1.6 mL, 88.9 mmol) was added. After 2 h, the solution was neutralized with concentrated HCl (23.1 mL, 276 mmol). The inorganic salts were filtered and MeOH was removed under vacuum. The residue obtained was flash chromatographed on a 6×60 cm column using CH_2Cl_2/MeOH (1:1) as the eluting solvent to give **7** as a white solid (11.5 g) at 70% yield: mp 93–95 °C; TLC R_f [CH_2Cl_2/MeOH (1:1)] = 0.5; IR (KBr) ν: 1669 cm^{-1} (CO), 1716 cm^{-1} (CO), 3255 cm^{-1} (NH), 3443 cm^{-1} (OH); ^1H NMR (300 MHz, DMSO-d_6) isomers *E* and *Z* were observed in 1/9 proportion δ: 1.85–1.90 (m, 1H), 2.09–2.16 (m, 1H), 2.49–2.51 (m, 2H), 3.42 (s, 0.3H), 3.48 (s, 2.7H), 3.98 (t, 0.1H, $J=6.9$ Hz), 4.14 (t, 0.9H, $J=6.8$ Hz), 4.34 (s, 0.9H), 4.66 (s, 0.1H), 7.85 (s, 0.1H), 8.05 (s, 0.9H); MS (EI) m/z 185 (M^+). $[\alpha]_{25}^{D}=0°$ (MeOH, $c=1$). Anal. calcd for $C_8H_{11}NO_4$: C, 51.89; H, 5.99; N, 7.56. Found: C, 51.74; H, 6.09; N, 7.69.

General procedure for preparation of β-enaminoesters (8a,b). PyBOP (2.68 g, 3.22 mmol), carboxylic acid **7** (895 mg, 4.83 mmol) and DIPEA (1.7 mL, 9.66 mmol) were added to a solution of tryptophan amide hydrochloride (**4a** or **4c**) (3.22 mmol) in CH_2Cl_2 (50 mL) (ice bath). The solution was stirred for 48 h, then the solvent was evaporated. The residue obtained was dissolved in ethyl acetate and washed successively with saturated $NaHCO_3$ solution, HCl (1 N) and H_2O. The organic phases were dried over $MgSO_4$, filtered then evaporated.

(*E*)- and (*Z*)-5-Methoxycarbonylmethine-(*RS*)-prolyl-*N*-3,5-bis(trifluoromethyl)benzyltryptophan amide (8a). The product **8a** was recrystallized from CH_2Cl_2 as a white solid (1.36 g) at 71% yield: mp 190–194 °C; TLC R_f (ethyl acetate) = 0.5; IR (KBr) ν: 1630 cm^{-1} (CO), 1650 cm^{-1} (CO), 3210 cm^{-1} (NH), 3320 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ: 1.45–1.60 (m, 0.33H), 1.70–1.80 (m, 0.66H), 1.95–2.10 (m, 1H), 2.40–2.50 (m, 2H), 2.95–3.20 (m, 2H), 3.48 (s, 1H), 3.50 (s, 2H), 4.20–4.60 (m, 5H), 6.97 (t, 1H, $J=7.5$ Hz), 7.05 (t, 1H, $J=7.4$ Hz), 7.12 (bs, 1H), 7.32 (d, 1H, $J=8.3$ Hz), 7.57 (d, 1H, $J=7.8$ Hz), 7.92–7.97 (m, 3H), 8.10 (s, 0.33H), 8.14 (s, 0.66H), 8.23 (d, 0.66H, $J=7.8$ Hz), 8.30 (d, 0.33H, $J=7.8$ Hz), 8.70–8.80 (m, 1H), 10.82 (s, 1H); MS (EI) m/z 596 (M^+), 565 (M-OCH_3), 130 (CH_2-Ind^+). Anal. calcd for $C_{28}H_{26}F_6N_4O_4$: C, 56.38; H, 4.39; N, 9.39. Found: C, 56.38; H, 4.43; N, 9.55.

(*E*)- and (*Z*)-5-Methoxycarbonylmethine-(*RS*)-prolyl-*N*-benzyl-*N*-methyltryptophan amide (8b). The product **8b** was flash chromatographed on a 5×40 cm column using ethyl acetate as the eluting solvent. The product was isolated as an unstable oil (1.07 g) at 70% yield and was not purified but utilized directly to synthesize compounds **2a** and **2c**. TLC R_f (ethyl acetate) = 0.2; IR (KBr) ν: 1630 cm^{-1} (CO), 1650 cm^{-1} (CO), 3210 cm^{-1} (NH), 3320 cm^{-1} (NH).

General procedure for preparation of indolizines (2a–c). Acrylic acid (4.21 mmol) and PCl_3 (0.41 mL, 4.63 mmol) were added to a solution of β-enaminoester (**8a**,**b**) (4.21 mmol) in dry dioxane (30 mL) and dry toluene (30 mL). The solution was heated at reflux in an inert atmosphere and stirred for 4 h. The solvents were removed under reduced pressure.

6-Acetamido-5-oxo-8-methoxycarbonyl-1,2,3,5,6,7-hexahydroindolizine-3-carbonyl-3,5-bis(trifluoromethyl)benzyltryptophan amide (2a). The product **2a** was dissolved in boiling acetone. The salts were filtered and the acetone solution was evaporated under reduced pressure to give an oil which was flash chromatographed on a 4×40 cm column using CH_2Cl_2/acetone (1:1) as the eluting solvent. The product obtained was isolated as a white solid (1.49 g) at 50% yield: mp 208–212 °C; TLC R_f [CH_2Cl_2/acetone (1:1)] = 0.4; IR (KBr) ν: 1645 cm^{-1} (CO), 1690 cm^{-1} (CO), 3280 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ: 1.80–1.90 (m, 4H), 2.12–2.40 (m, 2H), 2.65–2.87 (m, 2H), 3.05–3.20 (m, 3H), 3.63 and 3.65 (2s, 3H), 4.25–4.75 (m, 5H), 6.98 (t, 1H, $J=7.5$ Hz), 7.05 (t, 1H, $J=7.4$ Hz), 7.15 and 7.18 (2s, 1H), 7.30–7.35 (m, 1H), 7.55–7.60 (m, 1H), 7.91–7.96 (m, 3H), 8.14–8.27 (m, 1H), 8.50–8.60 (m, 1H), 8.63–8.71 (m, 1H), 10.80–10.85 (m, 1H); MS (EI) m/z 707 (M^+), 648 ($M-CO_2CH_3$). Anal. calcd for $C_{33}H_{31}F_6N_5O_6 \cdot 1/2H_2O$: C, 55.31; H, 4.50; N, 9.77. Found: C, 55.25; H, 4.60; N, 9.90.

6-Acetamido-5-oxo-8-methoxycarbonyl-1,2,3,5,6,7-hexahydroindolizine-3-carbonyl-*N*-benzyl-*N*-methyltryptophan amide (2b). The product **2b** was dissolved in boiling acetone. The salts were filtered and the acetone solution was evaporated under reduced pressure to give an oil which was flash chromatographed on a 4×40 cm column using CH_2Cl_2/acetone (7:3) as the eluting solvent. The product obtained was recrystallized from acetone as a white solid (740 mg) at 30% yield: mp >240 °C; TLC R_f [CH_2Cl_2/acetone (1:1)] = 0.25; IR (KBr) ν: 1634 cm^{-1} (CO), 1697 cm^{-1} (CO), 3303 cm^{-1} (NH), 3373 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ:

1.40–1.60 (m, 1H), 1.87 (s, 3H), 2.02–2.15 (m, 1H), 2.30–2.73 (m, 2H), 2.76–2.80 (m, 4H), 3.00–3.20 (m, 3H), 3.62 (s, 1H), 3.65 (s, 2H), 4.38–5.14 (m, 5H), 6.84–7.37 (m, 9H), 7.59 (d, 1H, $J = 7.6$ Hz), 8.23 (d, 1H, $J = 7.6$ Hz), 8.72 (bs, 1H), 10.81 (s, 0.33H), 10.87 (s, 0.66H); MS (EI) m/z 585 (M^+), 130 (Ind-CH_2^+). Anal. ($C_{32}H_{35}N_5O_6$) C, H, N. Anal. calcd for $C_{32}H_{35}N_5O_6$: C, 65.63; H, 6.02; N, 11.96. Found: C, 65.37; H, 5.83; N, 11.84.

6-Benzyloxycarbonylamino-5-oxo-8-methoxycarbonyl-1,2,3,5,6,7-hexahydroindolizine-3-carbonyl-*N*-benzyl-*N*-methyltryptophan amide (2c). The product **2c** was dissolved in boiling ethyl acetate. The salts were filtered and the ethyl acetate solution was evaporated under reduced pressure to give an oil which was flash chromatographed on a 4×40 cm column using CH_2Cl_2/acetone (75:25) as the eluting solvent. The product obtained was isolated as a white solid (285 mg) at 30% yield: mp 125–135 °C; TLC R_f [CH_2Cl_2/acetone (75:25)] = 0.25; IR (KBr) ν: 1641 cm^{-1} (CO), 1707 cm^{-1} (CO), 3320 cm^{-1} (NH); ^1H NMR (300 MHz, DMSO-d_6) δ: 1.20–2.45 (m, 5H), 2.50–2.82 (m, 4H), 2.85–3.20 (m, 2H), 3.60–3.79 (m, 3H), 4.20–5.20 (m, 7H), 6.80–7.37 (m, 14H), 7.50–7.58 (m, 1H), 8.14–8.17 (m, 1H), 8.79–8.86 (bs, 1H), 10.85–10.93 (4s, 1H); MS (EI) m/z 677 (M^+), 130 (Ind-CH_2^+). Anal. calcd for $C_{38}H_{39}N_5O_7$: C, 67.34; H, 5.80; N, 10.93. Found: C, 66.97; H, 5.92; N, 10.76.

Binding assays

Binding experiments were performed according to standard techniques[37] using clones of Chinese Hamster Ovary (CHO) as the receptor source for both NK_1 and NK_2 subtypes. Crude membranes were prepared and stored in Tris 20 mM, sucrose 250 mM medium, pH 7.4, at −70 °C. Both tritiated radioligands were used with specific activities of 170 Ci/mmol (Amersham). Incubation conditions were the following: Tris 50 mM, Mg 2 mM (final concentrations), pH 7.4 and additional Bacitracine 160 μg/mL at 25 °C for 1 h. The reaction was terminated by rapid vacuum filtration onto glass fiber filters (GF/C Wathman presoaked 2 h in PEI 0.1%): after four 2 mL washes with Tris 50 mM at 4 °C, pH 7.4, the radioactivity trapped onto the filters was counted and binding was calculated. Non-specific binding was determined with additional non-radioactive Substance P 1 μM. Competition curves were fitted according to the Cheng and Prussof equation (Kaleidagraph software, Microsoft for Macintosh).[38]

Intracellular $[Ca^{++}]_i$ measurements

Intracellular calcium concentrations were determined using the fluorescent Ca^{2+} chelating agent Fura-2, as previously described.[39] Cellular suspension (10 million cell/mL) was incubated with permeant fluorescent probe Fura-2 AM (AM = acetyl methyl ester, 5 μM) in Krebs–Ringer buffer (pH 7.4) consisting of NaCl (132 mM), KCl (4 mM), $CaCl_2$ (1 mM), $MgCl_2$ (0.5 mM), glucose (5 mM), HEPES (9.5 mM) at 37 °C for 30 min. Loaded cells were washed free of extracellular dye and centrifuged at 700 rpm during 10 min. After three washes, the cells were resuspended at 1 million/mL in a thermostated quartz cuvette (3 mL). Cells were maintained in suspension by a magnetic stirrer. A spectrofluorometer equipped for dual excitation and mono emission wavelength (Fluorolog, HORIBA group) was used to record the ratio of fluorescence emission intensity at 510 nm resulting for alternative excitation of cells at 340 and 380 nM. The recorded ratios (6 per second) were then automatically converted to cytosolic Ca^{2+} concentration using the established equation described by Grynkiewicz.[40] Schild plots were established from six activity curves with concentrations of selected compounds **1a–c** and **2a–c** (10^{-10}–10^{-6} M) in three independent experiments.

References and Notes

1. Snijdelaar, D. G.; Dirksen, R.; Slappendel, R. *Eur. J. Pain* **2000**, *4*, 121.
2. Gao, Z.; Peet, N. P. *Curr. Med. Chem.* **1999**, *6*, 375.
3. Maggi, C. A.; Pataccini, R.; Rovero, P.; Giachetti, A. *J. Auton. Pharmacol.* **1993**, *13*, 23.
4. Joos, G. F.; Germonpré, P. R.; Pauwels, R. A. *Allergy* **2000**, *55*, 321.
5. Naline, E.; Molimard, M.; Regoli, D.; Emonds-Alt, X.; Bellamy, J. F.; Advenier, C. *Am. J. Physiol.* **1996**, *271*, L763.
6. Hill, R. *Trends. Pharmacol. Sci.* **2000**, *21*, 244.
7. Cocquyt, V.; Van Belle, S.; Reinhardt, R. R.; Decramer, M. L.; O'Brien, M.; Schellens, J. H.; Borms, M.; Verbeke, L.; Van Aelst, F.; De Smet, M.; Carides, A. D.; Eldridge, K.; Gertz, B. J. *Eur. J. Cancer* **2001**, *37*, 835.
8. Diemunsch, P.; Grelot, L. *Drugs* **2000**, *60*, 533.
9. Stout, S. C.; Owens, M. J.; Nemerof, C. B. *Annu. Rev. Pharmacol. Toxicol.* **2001**, *41*, 877.
10. Pacher, P.; Kohegyi, E.; Kecskemeti, V.; Furst, S. *Curr. Med. Chem.* **2001**, *8*, 89.
11. Barnes, P. J. *Respir. Physiol.* **2001**, *125*, 145.
12. Joos, G. F.; Germonpre, P. R.; Pauwels, R. A. *Clin. Exp. Allergy* **2000**, *30*, 60.
13. Millet, R.; Goossens, J.-F.; Bertrand-Caumont, K.; Chavatte, P.; Houssin, R.; Hénichart, J.-P. *Lett. Pept. Sci.* **1999**, *6*, 221.
14. Millet, R.; Goossens, J.-F.; Bertrand-Caumont, K.; Houssin, R.; Hénichart, J.-P. *Lett. Pept. Sci.* **1999**, *6*, 255.
15. Millet, R.; Goossens, L.; Goossens, J.-F.; Chavatte, P.; Bertrand-Caumont, K.; Houssin, R.; Hénichart, J.-P. *J. Pept. Sci.* **2001**, *6*, 323.
16. Millet, R.; Goossens, L.; Bertrand-Caumont, K.; Goossens, J.-F.; Houssin, R.; Hénichart, J.-P. *J. Pharm. Pharmacol.* **2001**, *53*, 929.
17. Qi, H.; Shah, S. K.; Cascieri, M. A.; Sadowski, S. J.; MacCoss, M. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2259.
18. Hanessian, S.; McNaughton-Smith, G.; Lombart, H.-G.; Lubell, W. D. *Tetrahedron Lett.* **1997**, *53*, 12789.
19. Halab, L.; Gosselin, F.; Lubell, W. D. *Biopolymers* **2000**, *55*, 101.
20. Gross, E.; Meienhofer, J. *The Peptides: Analysis, Synthesis, Biology*; Academic Press: New York, 1979–1983; Vols. 1–5.
21. Millet, R.; Rigo, B.; Houssin, R.; Hénichart, J.-P. *J. Heterocycl. Chem.* **1999**, *36*, 1279.
22. Millet, R.; Meulon, E.; Goossens, L.; Rigo, B.; Houssin, R.; Hénichart, J.-P. *J. Heterocycl. Chem.* **2000**, *37*, 1491.
23. Kolar, A. J.; Olsen, R. K. *Synthesis* **1977**, 4757.
24. Capps, N. K.; Davies, G. M.; Loakes, D.; McCabe, R. W.; Young, D. W. *J. Chem. Soc., Perkin Trans. 1* **1991**, 3077.
25. Fasseur, D.; Rigo, B.; Leduc, C.; Cauliez, P.; Couturier, D. *J. Heterocycl. Chem.* **1992**, *29*, 1285.

26. Cascieri, M. A.; Ber, E.; Fong, T. M.; Sadowski, S.; Bansal, A.; Swain, C.; Seward, E.; Frances, B.; Burns, D.; Strader, C. D. *Mol. Pharmacol.* **1992**, *42*, 458.
27. Bradshaw, C. D.; Ceszkowski, K.; Turcatti, G.; Beresford, I. J. M.; Chollet, A. *J. Med. Chem.* **1994**, *37*, 1991.
28. Harrison, T.; Korsgaard, M. P. G.; Swain, C. J.; Cascieri, M. A.; Sadowski, S.; Seabrook, G. R. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1343.
29. Bernstein, P. R.; Aharony, D.; Albert, J. S.; Andisik, D.; Barthlow, H. G.; Bialecki, R.; Davenport, T.; Dedinas, R. F.; Dembofsky, B. T.; Koether, G.; Kosmider, B. J.; Kirkland, K.; Ohnmacht, C. J.; Potts, W.; Rumsey, W. L.; Shen, L.; Shenvi, A.; Sherwood, S.; Stollman, D.; Rusell, K. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2769.
30. Ting, P. C.; Lee, J. F.; Anthes, J. C.; Shih, N.-Y.; Piwinski, J. J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2333.
31. Reichard, G. A.; Ball, Z. T.; Aslanian, R.; Anthes, J. C.; Shih, N.-Y.; Piwinski, J. J. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2329.
32. Gerspacher, M.; Von Sprecher, A.; Mah, R.; Anderson, G. P.; Bertrand, C.; Subramanian, N.; Hauser, K.; Ball, H. A. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1467.
33. Nishi, T.; Ishibashi, K.; Takemoto, T.; Nakajima, K.; Fukazawa, T.; Lio, Y.; Itoh, K.; Mukaiyama, O.; Yamaguchi, T. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1665.
34. Ting, P. C.; Lee, J. F.; Anthes, J. C.; Shih, N.-Y.; Piwinski, J. J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 491.
35. Nishi, T.; Fukazawa, T.; Ishibashi, K.; Nakajima, K.; Sugioka, Y.; Lio, Y.; Kurata, H.; Itoh, K.; Mukaiyama, O.; Satoh, Y.; Yamaguchi, T. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 875.
36. Burkholder, T. P.; Kudlacz, E. M.; Le, T.-B.; Knippenberg, R. W.; Shatzer, S. A.; Maynard, G. D.; Webster, M. E.; Horgan, S. W. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 951.
37. Vollmer, J. Y.; Alix, P.; Chollet, A.; Takeda, K.; Galzi, J. L. *J. Biol. Chem.* **1999**, *53*, 37915.
38. Cheng, Y.; Prussof, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.
39. Le Bourdonnec, B.; Meulon, E.; Yous, S.; Goossens, J. F.; Houssin, R.; Hénichart, J. P. *J. Med. Chem.* **2000**, *43*, 2685.
40. Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. *J. Biol. Chem.* **1985**, *260*, 3440.