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Old Molecules for New Receptors: Trp(Nps) Dipeptide Derivatives as Vanilloid TRPV1 Channel Blockers

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Dedicated to Professor Joaquín Plumet on the occasion of his 60th birthday

*The transient receptor potential vanilloid member 1 (TRPV1), an integrator of multiple pain-producing stimuli, is regarded nowadays as an important biological target for the discovery of novel analgesics. Here, we describe the first experimental evidence for the behavior of an old family of analgesic dipeptides, namely Xaa-Trp(Nps) and Trp(Nps)-Xaa (Xaa = Lys, Arg) derivatives, as potent TRPV1 channel blockers. We also report the synthesis and biological investigation of a series of new conformationally restricted Trp(Nps)-dipeptide derivatives with improved TRPV1/NMDA selectivity. Compound **15b**, which incorporates an N-terminal 2S-azetidine-derived Arg residue, was the most selective compound in this series. Collectively, a new family of TRPV1 channel blockers emerged from our results, although further modifications are required to fine-tune the potency/selectivity/toxicity balance.*

Introduction

TRPV1 is a nonselective cation channel with high permeability to calcium. It belongs to the superfamily of transient receptor potential (TRP) channels, which is characterized by six transmembrane domains.[1,2] The TRPV1 receptor is activated by exogenous agonists, such as capsaicin (the pungent principle of hot peppers) and resiniferatoxin, as well as by physical and chemical noxious stimuli, such as heat ($>42\,^{\circ}C$) and protons (pH 5).[3,4] Moreover, this receptor is activated by a series of inflammatory mediators, including bradikinin[5] and lipoxygenase products,[6] and by the endocannabinoid anandamide.[7,8] It is also recognized that TRPV1 is up-regulated under several pathological conditions, from inflammatory and diabetic pain to cancer of the cervix.[9–11] Two separate studies by Caterina and Davis have demonstrated a reduction in thermal hyperalgesia in different pain models in mice lacking the TRPV1 gene.[12,13] All these data suggest a role for TRPV1 as an integrator of multiple pain-producing stimuli and, therefore, this receptor is considered a biological target of leading interest for the discovery of novel analgesics.

Treatment with TRPV1 agonists, such as capsaicin, resiniferatoxin, olvanil, and related compounds, led to decreased sensitivity to painful stimuli and, consequently, had therapeutic applications against inflammatory hyperalgesia, emesis, and urinary incontinence.[14,15] However, due to the aversive and often dose-limiting side effects of vanilloid agonists, industrial and academic efforts, since the cloning of TRPV1 in 1997,[3] have been focused on developing antagonists for this receptor.[16–19] The TRPV1 antagonists reported to date can be subdivided into two families: capsaicin-competitive TRPV1 antagonists and noncompetitive channel blockers. Within the first family, we find compounds related to vanilloid agonists, such as capsazepine and the 5-iodinated resiniferatoxin,[20,21] as well as diverse small molecules, structurally unrelated to vanilloids, which have emerged from high-throughput screening (HTS) programs. Potent TRPV1 antagonists, obtained after optimization of the initial HTS hits, mainly included di- and trisubstituted urea and thiourea derivatives,[22,23] *N*-aryl cinnamides,[25] and piperazine-1-benzimidazoles.[27] Some of these small molecules were highly active in animal models of chronic and inflammatory pain, including chronic pain states associated with bone cancer metastasis, and are currently under preclinical or clinical trials for a range of conditions.[26–27]

The nonselective ruthenium red can be considered to be the most classical noncompetitive vanilloid antagonist.[16] This polycationic inorganic dye is able to block agonist-evoked Ca^{2+} currents by interacting with a region of TRPV1 different from the capsaicin-binding site. Mutation of Asp646 of TRPV1 to

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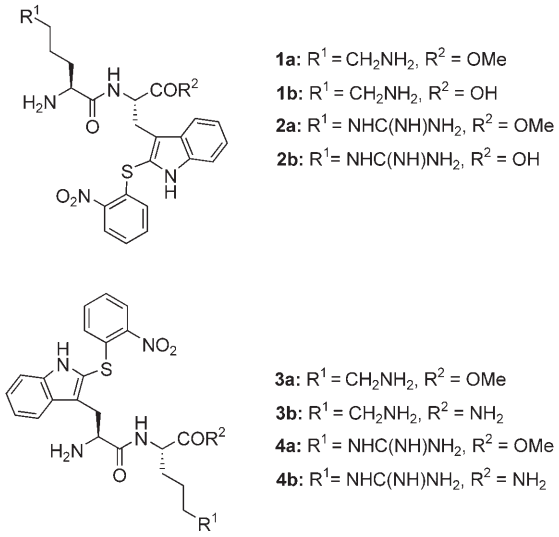
Asn led to a tenfold decrease in ruthenium red's blockade efficacy; this suggested a possible interaction of this antagonist at the pore-region level of the receptor, where this residue is located.[28] Similarly, natural and synthetic Arg-rich peptides, which display analgesic activity, were reported to block heterologously expressed TRPV1 channels through their binding to a site located near the entryway of the aqueous pore.[29] Screening of a library of *N*-alkylglycine peptoids resulted in the discovery of new TRPV1 channel blockers. These compounds, which appear to be noncompetitive TRPV1 antagonists that recognize a receptor site distinct from that of capsaicin, attenuated in vivo thermal nociception and hyperalgesia, and suppressed capsaicin-evoked pain.[30] More recently, some perhydro-3-oxo-1,4-diazepinium derivatives have also been identified as novel TRPV1 channel blocker hits for further structure–activity improvement.[31]

In the mid-1980s, we described a series of Trp(Nps) dipeptide derivatives, exemplified by compounds **1**–**4** (Scheme 1), showing potent and naloxone-sensitive antinociceptive effects after i.c.v. administration in rodents.[32] While these compounds



Scheme 1. Structure of the Trp(Nps)-containing dipeptides used in the initial study.

did not induce analgesia by acting directly on opioid receptors, a combination of weak peptidase-inhibiting activity and moderate enkephalin-releasing properties may account for their opioidlike behavioral effects. However, until now, the biological target responsible for the marked analgesia elicited by these dipeptide derivatives has remained elusive. The structure–activity relationships for this series of compounds indicated the need for a basic amino acid residue (Orn, Lys, Arg) and an absolute requirement for the Trp residue substituted at position 2 with the *o*-nitrophenylsulfenyl or the related *o*-methoxycarbonylphenylsulfenyl moiety.[32,33] However, the relative position of the amino acid residues within the peptide sequence had no perceptible influence on the observed antinociceptive effects.[34]

A comparative examination of the organic noncompetitive TRPV1 antagonists described to date and our Trp(Nps) dipeptides showed some common structural elements, namely one or two aromatic moieties and at least one basic amino or guanidino group. Considering that these groups could be pharmacophores for the interaction with the TRPV1 receptor, and taking into account that this receptor is unambiguously expressed in the central nervous system,[2] we hypothesized that the i.c.v. analgesic activity of the Trp(Nps)-derived compounds could be due, at least in part, to their interaction with this molecular integrator of pain-related noxious stimuli.

Our first aim was to address this hypothesis by evaluating the inhibition of Ca^{2+} influx through the TRPV1 channel by known Xaa-Trp(Nps) and Trp(Nps)-Xaa (Xaa = Lys, Arg) dipeptide derivatives. After these preliminary studies, we obtained experimental evidence for potent TRPV1 blockade and also for modest inhibition of the glutamate NMDA receptor. Since it is well known that the incorporation of conformational restrictions into peptides can increase affinity and pharmacological selectivity as well as improve pharmacokinetic properties,[35] we next prepared and evaluated two series of conformationally constrained Trp(Nps) dipeptide analogues incorporating azetidine-derived amino acids and a tetrahydro-β-carboline moiety (THBC). In the first series, the basic amino acid Xaa, in Xaa-Trp(Nps) and Trp(Nps)-Xaa dipeptides, was replaced by the corresponding nonproteinogenic azetidine-restricted amino acid. This modification introduces a constraint at the ϕ torsion angle level, as a consequence of the N^{α},C^{α}-closing to the azetidine ring, and also reduces the flexibility of the basic side chain. In the THBC derivatives, the mobility of the *o*-nitrophenyl group is considerably restricted with respect to the phenylsulfenyl moiety due to cyclization with the Trp α-NH group.

Results and Discussion

Trp(Nps)-containing dipeptides as TRPV1 channel blockers

Among the series of our previously reported Trp(Nps) derivatives,[32–34] compounds **1a, b**, **2a, b**, **3b** and **4b** were selected as representative models for resynthesis and study (Scheme 1). These dipeptide derivatives were assayed for blockade potency on the capsaicin-induced channel activity of TRPV1 heterogously expressed in *Xenopus* oocytes (Figure 1). Since the homomeric TRPV1 permeation properties closely resemble those described for the glutamate NMDA receptor, and to assess the selectivity of the Trp(Nps) derivatives, the evaluation was also performed on this receptor.

As shown in Figure 1, the selected Trp(Nps)-containing dipeptides were able to block the capsaicin-evoked ionic current by $\geq$40% in a rapid and reversible manner. Two compounds, H-Arg-Trp(Nps)-OMe (**2a**) and H-Trp(Nps)-Arg-NH_2 (**4b**), inhibited TRPV1 channel activity by $>$80% at 10 μM. In general, the blockade potency was higher for Arg derivatives than for Lys analogues, and an ester or a carboxamide moiety at the C terminus is preferred over the corresponding free carboxylic acid. The order of the peptide sequence has no apparent influence on the activity. Notably, commercial dipeptides H-Lys-Trp-OH

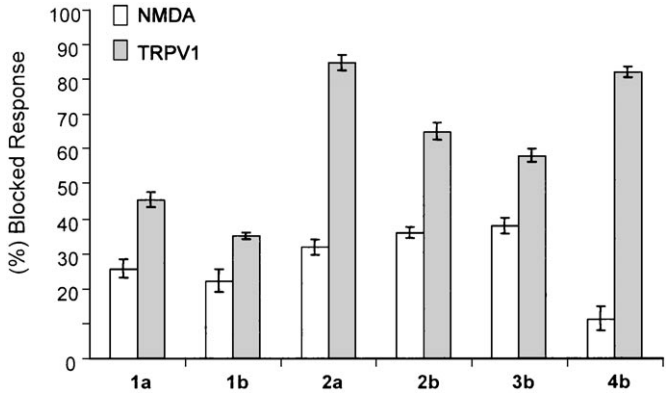


Figure 1. Blockade profile of Xaa-Trp(Nps) and Trp(Nps)-Xaa dipeptides, at 10 μM, in the capsaicin-induced TRPV1 channel activity and in the glutamate-evoked NMDA channel activity.

and H-Arg-Trp-OH, which lack the Nps aromatic moiety, were completely ineffective in the TRPV1 blockade assay (data not shown). The structure–activity relationships at the TRPV1 level were in agreement with those previously found in the in vivo antinociceptive assays.[32–34] Since TRPV1 is also expressed in the brain,[2] the SAR data points to TRPV1 as the plausible biological target that mediates the central analgesic activity of Trp(Nps) dipeptide derivatives. The 10–20% reduction in the blockade activity observed on changing the C-terminal methyl ester (in **1a** and **2a**) to a free carboxylate (in **1b** and **2b**) suggests binding of these dipeptide derivatives to the carboxylate-rich zone of the pore-forming region of TRPV1, as with other noncompetitive antagonists. Although the Trp(Nps)-derived dipeptides preferentially block TRPV1 channels, a modest inhibition of NMDA receptors was also seen; the selectivity was higher for Arg than for Lys derivatives, and derivative **4b** was the most selective compound.

Synthesis of azetidine-containing dipeptide derivatives

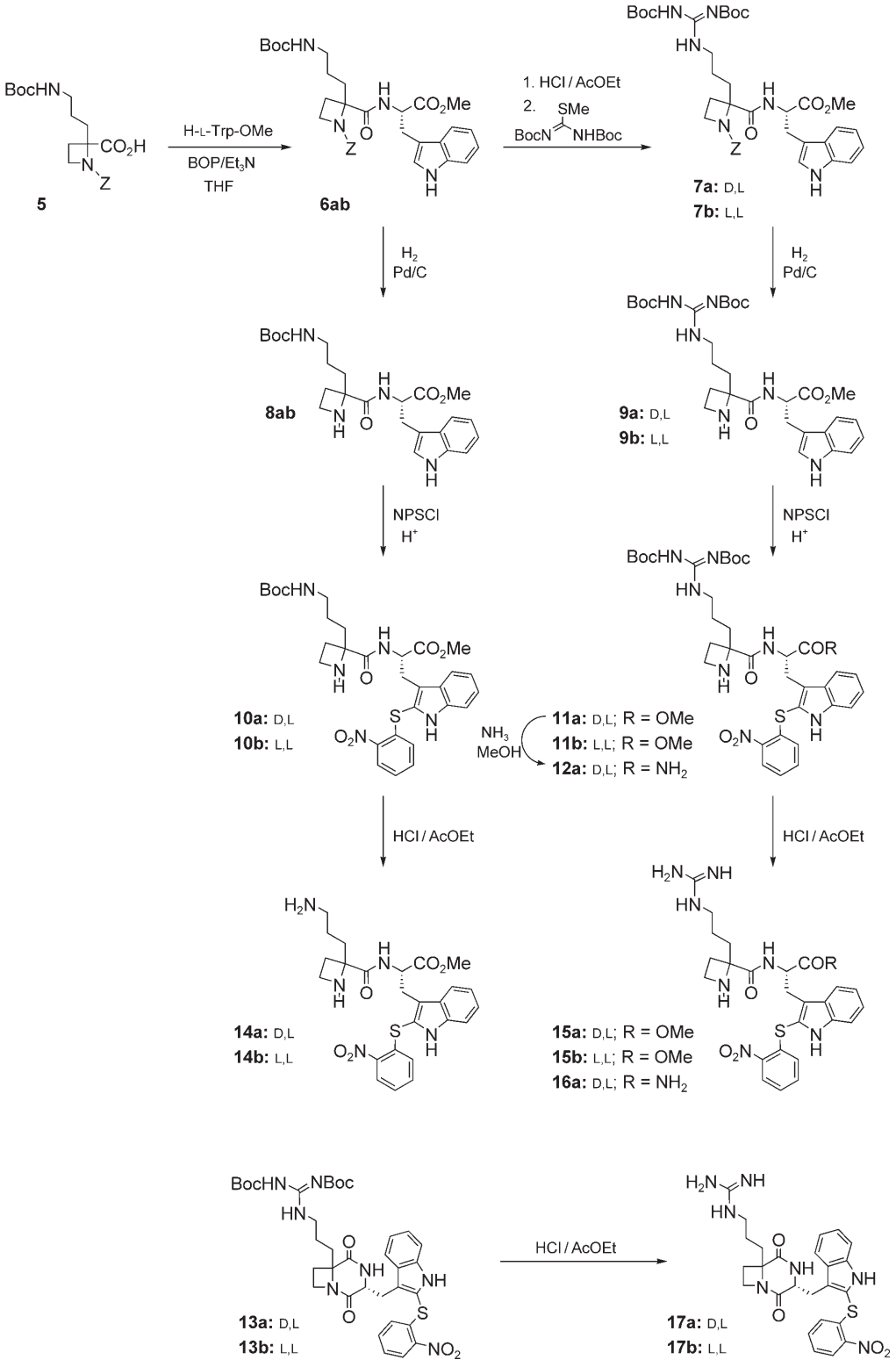
The synthesis of the azetidine-containing Azx-Trp(Nps) derivatives started with the preparation of dipeptide **6ab** by direct coupling of the Orn-derived azetidine **5** [Z-Azo(Boc)-OH][36] to H-L-Trp-OMe, by using BOP as the coupling agent (Scheme 2). Removal of the Boc group from **6ab**, followed by guanidylation, afforded the diastereoisomeric Azr-derived dipeptides **7a** and **7b**, which were easily separated by column chromatography. Removal of the Z protecting group from **6ab**, **7a** and **7b** and subsequent incorporation of the Nps moiety at position 2 of the indole ring by treatment with NpsCl in acidic media, provided compounds **10ab**, **11a** and **11b**. At this point, the Azo-derived isomeric mixture **10ab** could be chromatographically separated into its corresponding diastereoisomers **10a** and **10b**. Finally, treatment of Boc-protected derivatives with HCl/EtOAc afforded the expected *N*-deprotected analogues **14a**, **14b**, **15a** and **15b**. The carboxamide derivative **16a** was obtained by treatment of the corresponding methyl ester **11a** with NH_3/MeOH to give **12a**, followed by removal of the Boc groups. During the ammonolysis process, the formation of the corresponding diketopiperazine (DKP) was an important side-reaction, especially in the case of the *S,S* diastereoisomer, which resulted in the exclusive formation of the cyclic dipeptide derivative **13b**. Diketopiperazines **13a** and **13b** were also treated with HCl/EtOAc to remove Boc groups from the Azr side-chain (Compounds **17a** and **17b**; Scheme 2).

The reverse sequence azetidine-restricted derivatives Trp-(Nps)-Azx were prepared by a synthetic route that started from the coupling of Z-L-Trp-OH to the corresponding Orn azetidine methyl ester (H-Azo(Boc)-OMe, **18**). As shown in Scheme 3, the expected Azo-Trp(Nps) derivatives **26a** and **26b** were obtained from the dipeptide derivatives **19a** and **19b**, following a sequence of reactions involving removal of the Z group, introduction of the Nps moiety, and Boc deprotection. Removal of the side-chain Boc group from compound **19**, followed by guanidylation and the above-indicated sequence of treatments, allowed the preparation of the Trp(Nps)-Azr analogues **27a** and **27b**. Again, the amide derivatives **28a** and **28b** were obtained by ammonolysis of the corresponding protected methyl ester derivatives **24** to amides **25,** and subsequent acid deprotection. As expected, all Trp(Nps)-Azx derivatives existed in solution as mixtures of *cis*/*trans* rotamers around the CON(Azx) amide bond. The main rotamers were assigned as *trans* by comparison of the chemical shifts of C-2 and C-4 azetidine carbon atoms in the ^{13}C NMR spectra by analogy with related Xaa-Pro sequences.[37–40]

By following the rules developed for dipeptide derivatives,[34,41,42] assignment of the configuration at the azetidine C-2 stereogenic center in Azx-Trp(Nps) and Trp(Nps)-Azx dipeptides was performed on the basis of the chemical shifts and the HPLC retention times of the different diastereoisomeric pairs. The heterochiral derivative in a given pair was assigned as that having more shielded 2′-H protons and longer retention times.

Synthesis of tetrahydro-β-carboline derivatives

THBC-derived compounds **32** and **33** were prepared by using solid-phase methodologies. Although several reports indicate that Wang-type resins are suitable for the Pictet–Spengler cyclization with low (1%) TFA concentrations,[43,44] attempts to use these conditions for THBC ring formation on a Rink amide resin resulted in incomplete reaction and premature cleavage. To overcome these inconveniences, the Pictet–Spengler transformation was performed on a base-labile HMBA resin; this allows highly acidic conditions.[45] As indicated in Scheme 4, removal of the Boc group from dipeptide **29**, condensation with excess of benzaldehyde or (*o*-nitro)benzaldehyde and treatment of the corresponding imine intermediate with 25% TFA/DCM afforded the expected resin-bound tetrahydro-β-carboline derivatives **30** and **31**. Final compounds **32** and **33** were obtained as C-terminal methyl esters, after Fmoc removal and cleavage by heating in MeOH/TEA (9:1). Two diastereoisomers, in an approximately 1:3 *cis*/*trans* ratio, were formed in each case, as evidenced by ^1H NMR. This is consistent with previous results indicating about a 1:1 *cis*/*trans* ratio during the solid-phase Pictet–Spengler reaction, followed by epimerization of the *cis* isomer.[46]

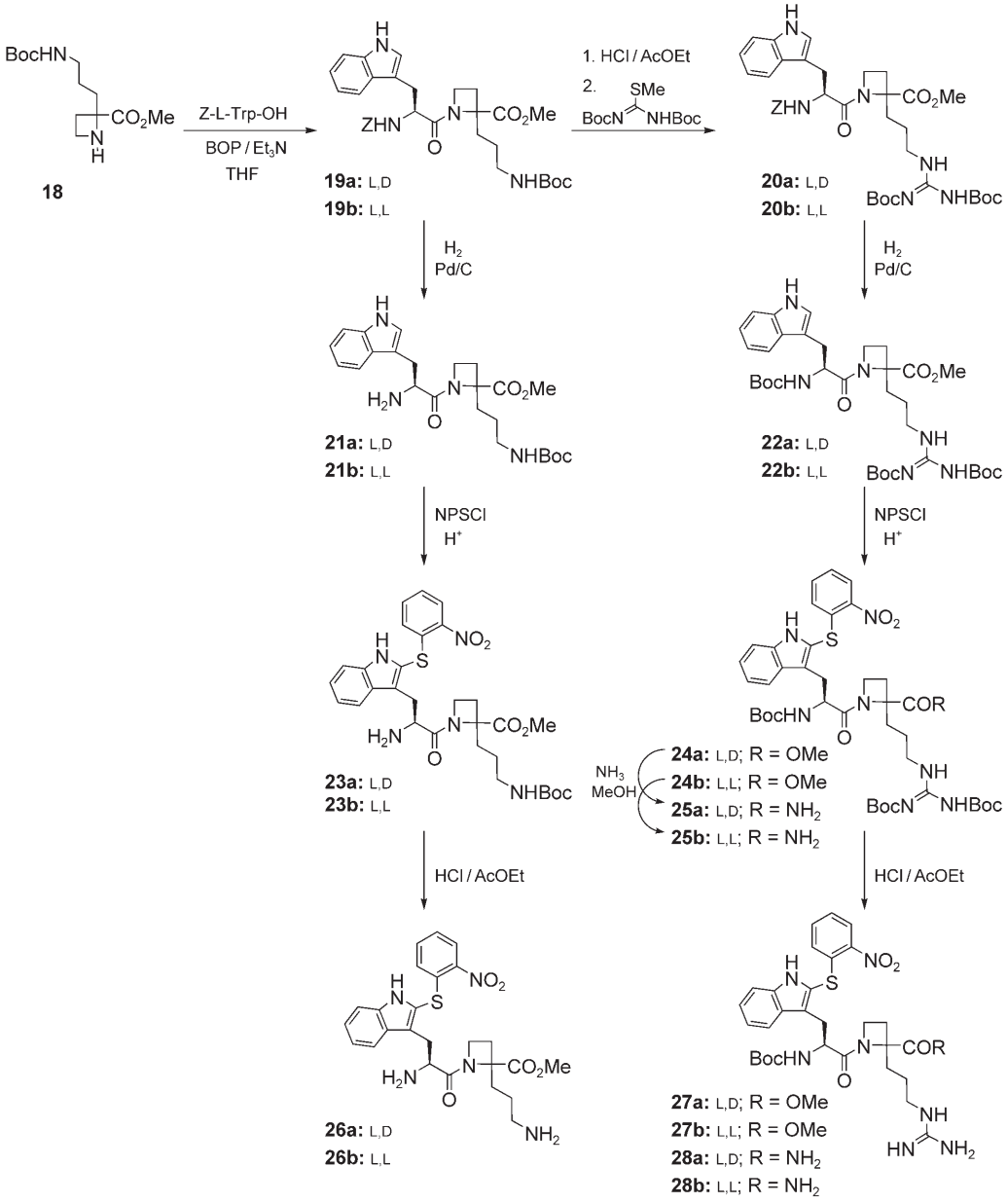


Scheme 2. Synthesis of restricted Azx-Trp(Nps) derivatives.

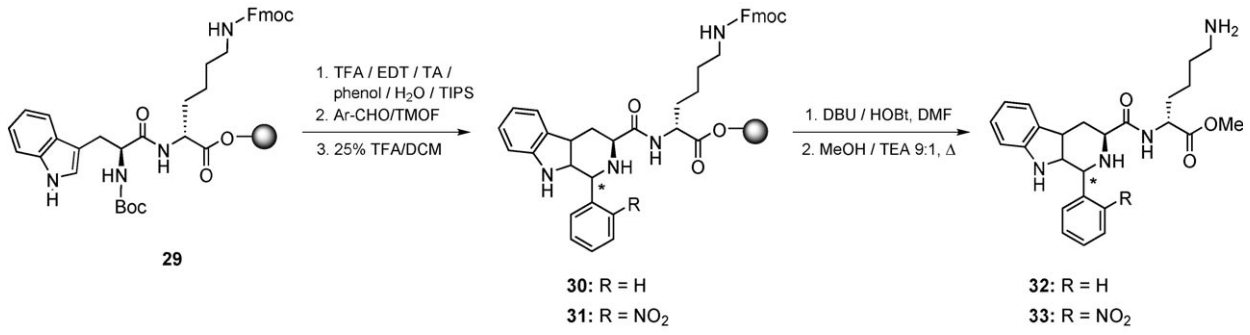
TRPV1 and NMDA channel blockade by restricted dipeptide derivatives

All of the conformationally restricted dipeptide derivatives were screened at a 10 μM concentration for blockade activity of recombinant TRPV1 and NMDA channels, heterologously expressed in *Xenopus* oocytes. The results were compared to those obtained for model compounds **1**–**4** (Figures 2 and 3).

Interestingly, in the Azx-Trp(Nps) series, dipeptide derivatives **10** and **11**, with a Boc-protection at the Azx side-chain, did not



Scheme 3. Synthesis of restricted Trp(Nps)-Azx derivatives.



Scheme 4. Synthesis of restricted THBC derivatives.

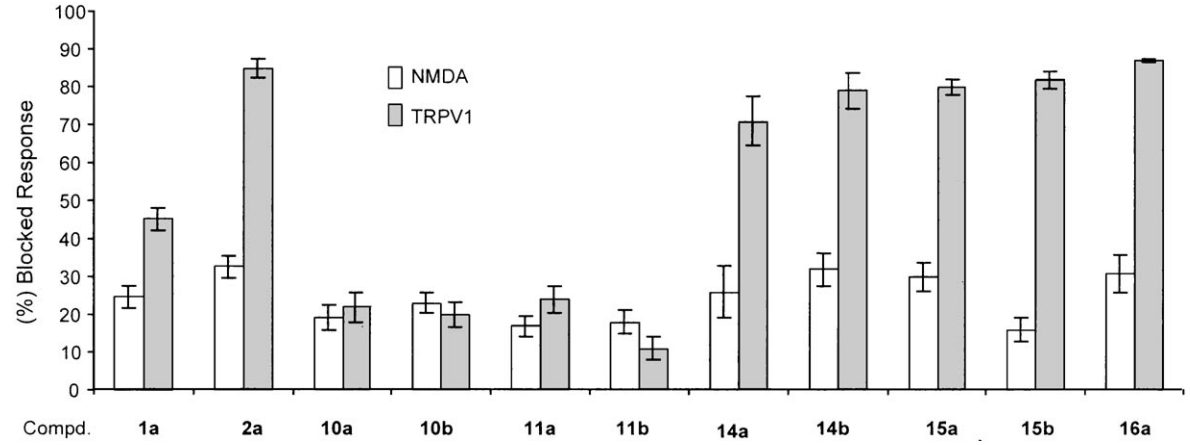


Figure 2. Blockade profile of restricted Azx-Trp(Nps) analogues, at 10 μM, in the capsaicin-induced TRPV1 channel activity and in the glutamate-evoked NMDA channel activity.

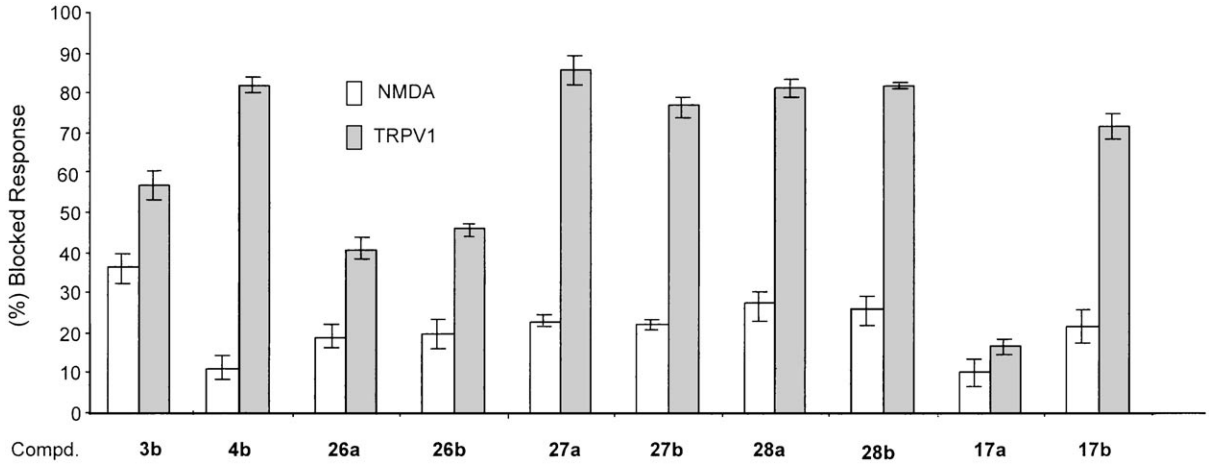


Figure 3. Blockade profile of restricted Trp(Nps)-Azx analogues and DKPs, at 10 μM, in the capsaicin-induced TRPV1 channel activity and in the glutamate-evoked NMDA channel activity.

significantly block either TRPV1 or NMDA receptors; this underlines the crucial importance of the free basic group (amino or guanidino). Compound **14a**, with an unprotected Orn-derived azetidine moiety, showed higher TRPV1 blockade than model compound **1a**, with an *N*-terminal Lys residue. This result could suggest that the conformational restriction induced by the Azo residue favored the interaction of **14a** with the receptor. However, the similar activities of **14a** and its diastereoisomer **14b** indicate a certain flexibility of the TRPV1 ion channel to locate the Trp(Nps)-derived compounds. Concerning Azr derivatives, the incorporation of the azetidine restriction maintained the blockade potency compared with the unconstrained Arg analogue (Figure 2, compounds **2a, 15a**, **15b**, and **16b**). In this series, compound **15b** was appreciably more selective than parent compound **2a** due to the diminished blockade of the NMDA receptor.

As illustrated in Figure 3, Trp(Nps)-Azo dipeptide derivatives **26a** and **26b** exhibited lower potency than the reference compound **3b**, and the reverse sequence analogues **14a** and **14b**. In contrast, Trp(Nps)-Azr derivatives **27** and **28** were roughly equipotent to model compound **4b** and to their Azr-Trp(Nps) counterparts **15** and **16** (blockade $\geq$80%). Nevertheless, the encountered selectivity was, in general, lower than that observed for the nonrestricted compound **4b**.

Overall, significant influence on the blockade activity of either the azetidine C-2 configuration or the C-terminal substituent (methyl ester or amide) was not observed. However, the configurational factor was much more important in the highly restricted diketopiperazine analogues **17**. Thus, while the *S*,*S* compound **17b** was able to maintain high TRPV1 blockade (72%), its *S*,*R* diastereoisomer **17a** only reached a 17% blocked response. This suggests that, due to the restricted flexibility, the relative spatial disposition between the guanidine group and the aromatic indole-Nps cluster should be fairly different in the diastereoisomeric diketopiperazines, while, in the linear analogues, they might adopt similar tridimensional arrangements. A comparable strong dependence on the absolute configuration was previously demonstrated in the i.c.v. analgesic activity of related cyclo[Trp(Nps)-Arg] derivatives.[33]

The restriction of the mobility of the *o*-nitrophenyl ring by formation of a tetrahydro-β-carboline, resulted in derivative **33**, which was unable to block either the TRPV1 or the NMDA receptor (data not shown). This large difference between the activity of **33** and the corresponding unrestricted dipeptides could be explained by an inappropriate relative disposition between the indole and *o*-nitrophenyl rings in the THBC system. In fact, compound **33** did not show the characteristic shielding of the Nps H-6 proton in the ^{1}H NMR spectra of Trp(Nps) derivatives, which appeared at approximately 6.5 ppm.[34] This particular chemical shift value, associated with a conformation in which the H-6 phenyl proton is above or below the plane of the indole ring and is therefore shielded due to the indole ring current effect, was correlated to active compounds in the tail-flick test.[34] This conformation seems not to be present in the THBC derivatives, since phenyl- and *o*-nitrophenyl-substituted compounds, **32** and **33**, showed very close proton patterns in the aromatic region. Additionally, participation of the sulfur lone pairs of the Nps moiety in the molecular recognition process could not be totally discarded.

Dipeptide derivatives **15a**, **15b**, **27a** and **27b** were selected among the most potent compounds in order to address whether these TRPV1 antagonists might reduce pain perception after intraplantar injection of capsaicin in mice.[30] Unfortunately, when injected i.p., these compounds were not able to produce any analgesic activity in vivo. A possible explanation for this lack of activity could be the acute toxicity displayed by our dipeptide derivatives in a toxicology animal model, which resulted in convulsive symptoms which killed the animals after short periods of time (~1 h).

Conclusions

In this paper we report for the first time that the analgesic Trp-(Nps)-containing dipeptides possessing a basic amino acid behave as TRPV1 channel blockers. This provides experimental evidence for the vanilloid receptor as the possible molecular integrator of the central analgesic activity displayed by these compounds.

The inhibition of the Ca^{2+} influx through the TRPV1 channel by a series of related azetidine-restricted dipeptide analogues was neither significantly modified by changes in the C-terminal carboxylate group (methyl ester or amide), nor by the absolute configuration at C-2 of the azetidine core, nor by the order of residues in the dipeptide sequence. This blockade potency was selective, as evidenced by the modest inhibition of the glutamate NMDA receptor. Notably, the incorporation of an *N*-terminal (2*S*)-azetidine, as in **15b**, resulted in a more selective derivative than the reference linear compound **2a**. Due to its non-proteinogenic azetidine residue, compound **15b** could be considered to be an advantageous lead compound for further investigation. However, it was unable to produce significant analgesic activity in vivo, mostly due to high toxicity. New modifications of the Trp(Nps) derivatives, directed to reduce the intrinsic toxicity, are being performed and will be published in due course.

Experimental Section

Chemistry: Compounds **1**–**4** were prepared as previously described.[32–34] General procedures, synthesis and characterization of all starting materials and intermediates are reported in the Supporting Information.

Removal of tert-*butoxycarbonyl groups*: The corresponding Boc-protected dipeptide derivative (0.52 mmol) was treated with a 3.2 M EtOAc/HCl solution (5 mL) and stirred at room temperature for 4 h. Then the solution was evaporated to dryness by coevaporating several times with CH_2Cl_2. The resulting residue was purified on reversed-phase SPE cartridges (Discovery DSC-18LT) with the solvent system specified in each case.

*(2R,1″S) and (2S,1″S)-2-[1″-Methoxycarbonyl-2′-[2-(o-nitrophenylsulfenyl)indole-3-yl]ethyl]carbamoyl-2-(3′-amino)propylazetidine dihydrochloride [H-D- and H-L-Azo-L-Trp(NPS)-OMe·2 HCl] (**14a** and **14b**):*

*Isomer (2R,1″S) (**14a**):* From **10a**; yield: 85%; yellow solid; m.p. 165–167 °C (isopropanol/diethyl ether). $[\alpha]_D = +12.0$ ($c = 0.42$, MeOH); ^{1}H NMR (300 MHz, D_2O): $\delta = 8.09$ (d, ^3J(H,H) = 8.3, 1H, 3H-NPS), 7.53–7.14 (m, 6H, Ar), 6.52 (d, ^3J(H,H) = 8.0, 1H, 6H-NPS), 4.76 (m, 1H, 1″-H), 3.68 (m, 1H, 4-H), 3.46 (s, 3H, OMe), 3.37 (m, 1H, 4-H), 3.29 (dd, ^3J(H,H) = 6.5, 14.7, 1H, 2″-H), 3.15 (dd, ^3J(H,H) = 8.8, 14.7, 1H, 2″-H), 2.77 (t, ^3J(H,H) = 7.8, 2H, 3′-H), 2.36 (m, 1H, 3-H), 2.14 (m, 1H, 3-H), 2.02 (m, 2H, 1′-H), 1.30 ppm (m, 2H, 2′-H); ^{13}C NMR (75 MHz, D_2O): $\delta = 172.88$ (1″-CO), 170.02 (2-CO), 144.33–112.06 (14C, Ar), 71.28 (2-C), 53.76 (1″-C), 53.24 (OMe), 40.94 (4-C), 38.65 (3′-C), 32.65 (1′-C), 28.59 (2″-C), 26.34 (3-C), 20.59 ppm (2′-C); ESI-MS: *m/z*: 512.2 $[M+1]^+$; elemental analysis calcd (%) for $C_{25}H_{29}N_5O_5S \cdot 2HCl$: C 51.37, H 5.35, N 11.98; found C 51.25, H 5.54, N 11.71.

*Isomer (2S,1″S) (**14b**):* From **10b**; yield: 86%; yellow solid; m.p. 156–157 °C (isopropanol/diethyl ether). $[\alpha]_D = -32.8$ ($c = 0.47$, MeOH); ^{1}H NMR (300 MHz, D_2O): $\delta = 8.10$ (d, ^3J(H,H) = 7.8, 1H, 3H-NPS), 7.56–7.14 (m, 6H, Ar), 6.47 (d, ^3J(H,H) = 8.0, 1H, 6H-NPS), 4.71 (m, 1H, 1″-H), 3.69 (m, 1H, 4-H), 3.52 (s, 3H, OMe), 3.27 (dd, ^3J(H,H) = 5.1, 14.7, 1H, 2″-H), 3.11 (dd, ^3J(H,H) = 8.8, 14.7, 1H, 2″-H), 2.42 (m, 4H, 3-H, 3′-H), 1.98 (m, 1H, 1′-H), 1.65 (m, 1H, 1′-H), 1.08 (m, 1H, 2′-H), 0.51 ppm (m, 1H, 2′-H); ^{13}C NMR (75 MHz, D_2O): $\delta =$ 170.50 (1″-CO), 168.36 (2-CO), 142.25–110.04 (14C, Ar), 69.25 (2-C), 51.89 (1″-C), 51.23 (OMe), 39.09 (4-C), 36.40 (3′-C), 30.55 (1′-C), 26.68 (2″-C), 23.88 (3-C), 18.33 ppm (2′-C); ESI-MS: *m/z*: 512.2 $[M+1]^+$; elemental analysis calcd (%) for $C_{25}H_{29}N_5O_5S \cdot 2HCl$: C 51.37, H 5.35, N 11.98; found C 51.63, H 5.14, N 12.12.

*(2R,1″S) and (2S,1″S)-2-[1″-Methoxycarbonyl-2′-[(2-o-nitrophenylsulfenyl)indole-3-yl]ethyl]carbamoyl-2-(3′-guanidinyl)propylazetidine dihydrochloride [H-D- and H-L-Azr-L-Trp(NPS)-OMe·2 HCl] (**15a** and **15b**):*

*Isomer (2R,1″S) (**15a**):* From **11a**. Eluent: CH_3CN/H_2O (10:90); yield: 76%; yellow solid; m.p. 190–192 °C (isopropanol/diethyl ether). $[\alpha]_D = +15.3$ ($c = 0.33$, MeOH); ^{1}H NMR (300 MHz, D_2O): $\delta = 8.16$ (d, ^3J(H,H) = 8.2, 1H, 3H-NPS), 7.57–7.18 (m, 6H, Ar), 6.56 (d, ^3J(H,H) = 8.2, 1H, 6H-NPS), 4.77 (m, 1H, 1″-H), 3.72 (m, 1H, 4-H), 3.50 (s, 3H, OMe), 3.46 (m, 1H, 4-H), 3.33 (dd, ^3J(H,H) = 6.4, 14.7, 1H, 2″-H), 3.22 (dd, ^3J(H,H) = 8.6, 14.7, 1H, 2″-H), 2.95 (t, ^3J(H,H) = 6.9, 2H, 3′-H), 2.36 (m, 1H, 3-H), 2.24 (m, 1H, 3-H), 2.00 (m, 2H, 1′-H), 1.14 ppm (m, 2H, 2′-H); ^{13}C NMR (75 MHz, D_2O): $\delta = 172.79$ (1″-CO), 170.21 (2-CO), 156.77 (C=N), 144.44–112.03 (14C, Ar), 71.45 (2-C), 53.76 (1″-C), 53.15 (OMe), 40.89 (4-C), 40.28 (3′-C), 32.88 (1′-C), 28.54 (2″-C), 26.20 (3-C), 21.77 ppm (2′-C); ESI-MS: *m/z*: 554.1 $[M+1]^+$; elemental analysis calcd (%) for $C_{26}H_{31}N_7O_5S \cdot 2HCl$: C 49.84, H 5.31, N 15.65; found C 49.96, H 5.12, N 15.78.

*Isomer (2S,1″S) (**15b**):* From **11b**. Eluent: CH_3CN/H_2O (10:90); yield: 60%; yellow solid; m.p. 185–187 °C (isopropanol/diethyl ether). $[\alpha]_D = -11.7$ ($c = 0.19$, MeOH); ^1H NMR (300 MHz, D_2O): $\delta = 8.15$ (d, $^3J(H,H) = 8.0$, 1H, 3H-NPS), 7.60–7.18 (m, 6H, Ar), 6.48 (d, $^3J(H,H) = 7.9$, 1H, 6H-NPS), 4.88 (m, 1H, 1″-H), 3.72 (m, 2H, 4-H), 3.58 (s, 3H, OMe), 3.33 (dd, $^3J(H,H) = 4.6$, 14.8, 1H, 2″-H), 3.22 (dd, $^3J(H,H) = 11.3$, 14.8, 1H, 2″-H), 2.57 (m, 2H, 3′-H), 2.39 (m, 2H, 3-H), 1.95 (m, 1H, 1′-H), 1.62 (m, 1H, 1′-H), 0.87 (m, 1H, 2′-H), 0.10 ppm (m, 1H, 2′-H); ^{13}C NMR (75 MHz, D_2O): $\delta = 172.70$ (1″-CO), 170.10 (2-CO), 156.53 (C=N), 144.71–112.00 (14C, Ar), 71.21 (2-C), 53.38 (1″-C), 53.32 (OMe), 40.76 (4-C), 40.21 (3′-C), 33.05 (1′-C), 28.77 (2″-C), 26.15 (3-C), 21.26 ppm (2′-C); ESI-MS: *m/z*: 554.1 $[M+1]^+$; elemental analysis calcd (%) for $C_{26}H_{31}N_7O_5S \cdot 2HCl$: C 49.84, H 5.31, N 15.65; found C 49.62, H 5.54, N 15.53.

*(2R,1″S)-2-[1″-Carbamoyl-2″-[(2-o-nitrophenylsulfenyl)indole-3-yl]ethyl]-carbamoyl-2-(3′-guanidinyl)propylazetidine dihydrochloride [H-D-Azr-L-Trp(NPS)-NH_2·2HCl] (**16a**):*

From **12a**. Eluent: CH_3CN/H_2O (0:100); yield: 78%; yellow solid; m.p. 176–178 °C (isopropanol/diethyl ether). $[\alpha]_D = +38.1$ ($c = 0.46$, MeOH); ^1H NMR (300 MHz, D_2O): $\delta = 8.12$ (d, $^3J(H,H) = 8.0$, 1H, 3H-NPS), 7.60–7.04 (m, 6H, Ar), 6.53 (d, $^3J(H,H) = 7.8$, 1H, 6H-NPS), 4.67 (m, 1H, 1″-H), 3.73 (m, 1H, 4-H), 3.49 (m, 1H, 4-H), 3.19 (m, 2H, 2″-H), 2.92 (m, 2H, 3′-H), 2.35 (m, 2H, 3-H), 1.99 (m, 2H, 1′-H), 1.14 ppm (m, 2H, 2′-H); ^{13}C NMR (75 MHz, D_2O): $\delta = 174.93$ (1″-CO), 172.50 (2-CO), 156.50 (C=N), 144.63–112.03 (14C, Ar), 70.40 (2-C), 54.16 (1″-C), 40.81 (4-C), 40.22 (3′-C), 32.98 (1′-C), 28.62 (2″-C), 26.90 (3-C), 21.74 ppm (2′-C); ESI-MS: *m/z*: 539.3 $[M+1]^+$; elemental analysis calcd (%) for $C_{25}H_{30}N_8O_4S \cdot 2HCl$: C 49.10, H 5.27, N 18.32; found C 49.04, H 5.45, N 18.13.

*(2R,2″S) and (2S,2″S)-2-Methoxycarbonyl-2-(3′-amino)propyl-1-[2-(o-nitrophenylsulfenyl)-L-tryptophyl]azetidine dihydrochloride [H-L-Trp-(NPS)-D- and H-L-Trp(NPS)-L-Azo-OMe·2HCl] (**26a** and **26b**):*

*Isomer (2R,2″S) (**26a**):* From **23a**. Eluent: CH_3CN/H_2O (10:90); yield: 5%; yellow solid; m.p. 179–181 °C (isopropanol/diethyl ether). $[\alpha]_D = +7.1$ ($c = 1.09$, MeOH); ^1H NMR (400 MHz, D_2O): $\delta = 8.16$ (d, $^3J(H,H) = 8.1$, 1H, 3H-NPS), 7.58–7.10 (m, 6H, Ar), 6.50 (d, $^3J(H,H) = 8.0$, 1H, 6H-NPS), 4.22 (t, $^3J(H,H) = 7.9$, 1H, α-Trp), 3.87 (q, $^3J(H,H) = 9.1$, 15.0, 1H, 4-H), 3.60 (s, 3H, OMe), 3.24 (d, $^3J(H,H) = 7.9$, 2H, β-Trp), 2.89 (q, $^3J(H,H) = 9.0$, 15.0, 1H, 4-H), 2.74 (m, 2H, 3′-H), 2.15 (m, 1H, 3-H), 1.81 (m, 1H, 3-H), 1.66 (m, 1H, 1′-H), 1.51 (m, 1H, 1′-H), 1.37 (m, 1H, 2′-H), 1.12 ppm (m, 1H, 2′-H); ^{13}C NMR (100 MHz, D_2O): $\delta = 172.59$ (2-CO), 168.22 (1″-CO), 144.66–112.29 (14C, Ar), 71.13 (2-C), 53.45 (OMe), 49.72 (α-Trp), 47.57 (4-C), 39.45 (3′-C), 31.22 (1′-C), 26.01 (β-Trp), 25.06 (3-C), 21.84 ppm (2′-C); ESI-MS: *m/z*: 512.3 $[M+1]^+$; elemental analysis calcd (%) for $C_{25}H_{29}N_5O_5S \cdot 2HCl$: C 51.37, H 5.35, N 11.98; found C 51.25, H 5.52, N 11.64.

*Isomer (2S,2″S) (**26b**):* From **23b**. Eluent: CH_3CN/H_2O (10:90); yield: 70%; yellow solid; m.p. 182–184 °C (isopropanol/diethyl ether). $[\alpha]_D = -52.8$ ($c = 1.09$, MeOH); ^1H NMR (500 MHz, D_2O): $\delta = 8.13$ (d, $^3J(H,H) = 8.0$, 1H, 3H-NPS), 7.60–7.19 (m, 6H, Ar), 6.49 (d, $^3J(H,H) = 8.0$, 1H, 6H-NPS), 4.17 (t, $^3J(H,H) = 7.6$, 1H, α-Trp), 3.88 (q, $^3J(H,H) = 8.8$, 15.4, 1H, 4-H), 3.57 (s, 3H, OMe), 3.34 (q, $^3J(H,H) = 9.0$, 15.4, 1H, 4-H), 3.22 (dd, $^3J(H,H) = 7.6$, 14.7, 1H, β-Trp), 3.15 (dd, $^3J(H,H) = 7.6$, 14.7, 1H, β-Trp), 2.89 (t, $^3J(H,H) = 7.3$, 2H, 3′-H), 2.14 (m, 1H, 3-H), 1.98 (m, 2H, 1′-H), 1.68 (m, 1H, 2′-H), 1.53 ppm (m, 1H, 2′-H); ^{13}C NMR (100 MHz, D_2O): $\delta = 172.62$ (2-CO), 167.87 (1″-CO), 144.54–112.33 (14C, Ar), 71.62 (2-C), 53.66 (OMe), 50.40 (α-Trp), 47.59 (4-C), 39.37 (3′-C), 30.29 (1′-C), 25.66 (β-Trp), 24.40 (3-C), 21.27 ppm (2′-C); ESI-MS: *m/z*: 512.3 $[M+1]^+$; elemental analysis calcd (%) for $C_{25}H_{29}N_5O_5S \cdot 2HCl$: C 51.37, H 5.35, N 11.98; found C 51.46, H 5.29, N 12.12.

*(2R,2″S) and (2S,2″S)-2-Methoxycarbonyl-2-(3′-guanidinyl)propyl-1-[2-o-nitrophenylsulfenyl)-L-tryptophyl]azetidine dihydrochloride [H-L-Trp-(NPS)-D- and H-L-Trp(NPS)-L-Azr-OMe·2HCl] (**27ab**):*

*Isomer (2R,2″S) (**27a**):* From **24a**. Eluent: CH_3CN/H_2O (10:90); yield: 92%; yellow solid; m.p. 187–190 °C (isopropanol/diethyl ether). $[\alpha]_D = +14.3$ ($c = 0.31$, MeOH); ^1H NMR (300 MHz, D_2O): $\delta = 8.20$ (d, $^3J(H,H) = 8.0$, 1H, 3H-NPS), 7.61–7.12 (m, 6H, Ar), 6.52 (d, $^3J(H,H) = 8.0$, 1H, 6H-NPS), 4.30 (t, $^3J(H,H) = 8.0$, 1H, α-Trp), 3.94 (m, 1H, 4-H), 3.63 (s, 3H, OMe), 3.28 (d, $^3J(H,H) = 8.0$, 2H, β-Trp), 3.00 (m, 1H, 4-H), 2.88 (m, 2H, 3′-H), 2.17 (m, 1H, 3-H), 1.84 (m, 1H, 3-H), 1.64 (m, 1H, 1′-H), 1.37 (m, 2H, 1′-H, 2′-H), 0.86 ppm (m, 1H, 2′-H); ^{13}C NMR (75 MHz, D_2O): $\delta = 172.90$ (2-CO), 168.01 (1″-CO), 156.77 (C=N), 144.64–112.21 (14C, Ar), 71.39 (2-C), 53.42 (OMe), 49.50 (α-Trp), 47.74 (4-C), 41.16 (3′-C), 30.85 (1′-C), 26.03 (β-Trp), 24.67 (3-C), 22.29 ppm (2′-C); ESI-MS: *m/z*: 554.3 $[M+1]^+$; elemental analysis calcd (%) for $C_{26}H_{31}N_7O_5S \cdot 2HCl$: C 49.84; H 5.31; N 15.65; found C 49.56, H 5.14, N 15.32.

*Isomer (2S,2″S) (**27b**):* From **24b**. Eluent: CH_3CN/H_2O (10:90); yield: 92%; yellow solid; m.p. 186–188 °C (isopropanol/diethyl ether). $[\alpha]_D = +22.2$ ($c = 0.31$, MeOH); ^1H NMR (300 MHz, D_2O): $\delta = 8.12$ (d, $^3J(H,H) = 8.2$, 1H, 3H-NPS), 7.56–7.08 (m, 6H, Ar), 6.44 (d, $^3J(H,H) = 7.9$, 1H, 6H-NPS), 4.16 (t, $^3J(H,H) = 7.7$, 1H, α-Trp), 3.81 (m, 1H, 4-H), 3.50 (s, 3H, OMe), 3.32 (m, 1H, 4-H), 3.23 (m, 1H, β-Trp), 3.13 (m, 1H, β-Trp), 3.00 (t, $^3J(H,H) = 6.9$, 2H, 3′-H), 2.06 (m, 2H, 3-H), 1.87 (m, 2H, 1′-H), 1.50 (m, 2H, 2′-H), 1.36 ppm (m, 1H, 2′-H); ^{13}C NMR (100 MHz, D_2O): $\delta = 172.85$ (2-CO), 167.76 (1″-CO), 156.86 (C=N), 144.60–112.29 (14C, Ar), 71.95 (2-C), 53.59 (OMe), 50.29 (α-Trp), 47.57 (4-C), 40.89 (3′-C), 30.38 (1′-C), 25.67 (β-Trp), 24.31 (3-C), 22.29 ppm (2′-C); ESI-MS: *m/z*: 554.3 $[M+1]^+$; elemental analysis calcd (%) for$C_{26}H_{31}N_7O_5S \cdot 2HCl$: C 49.84, H 5.31, N 15.65; found C 49.95, H 5.23, N 15.86 .

*(2R,2″S) and (2S,2″S)-2-Carbamoyl-2-(3′-guanidinyl)propyl-1-[2-(o-nitrophenylsulfenyl)-L-tryptophyl]azetidine dihydrochloride [H-L-Trp-(NPS)-D- and H-L-Trp(NPS)-L-Azr-NH_2·2HCl] (**28ab**):*

*Isomer (2R,2″S) (**28a**):* From **25a**; yield: 99%; yellow solid; m.p. 196–199 °C (isopropanol/diethyl ether). $[\alpha]_D = +94.5$ ($c = 0.44$, MeOH); ^1H NMR (300 MHz, D_2O): $\delta = 8.19$ (d, $^3J(H,H) = 7.8$, 1H, 3H-NPS), 7.63–7.20 (m, 6H, Ar), 6.50 (d, $^3J(H,H) = 8.8$, 1H, 6H-NPS), 4.23 (m, 1H, α-Trp), 3.84 (q, $^3J(H,H) = 9.5$, 15.5, 1H, 4-H), 3.26 (m, 2H, β-Trp), 2.98 (q, $^3J(H,H) = 9.5$, 15.5, 1H, 4-H), 2.87 (m, 2H, 3′-H), 2.18 (m, 1H, 3-H), 1.73 (m, 1H, 3-H), 1.64 (m, 1H, 1′-H), 1.28 (m, 2H, 1′-H, 2′-H), 0.91 ppm (m, 1H, 2′-H); ^{13}C NMR (100 MHz, D_2O): $\delta = 176.36$ (2-CO), 169.09 (1″-CO), 156.76 (C=N), 144.61–112.22 (14C, Ar), 73.68 (2-C), 49.78 (α-Trp), 46.80 (4-C), 40.98 (3′-C), 31.96 (1′-C), 26.09 (β-Trp), 24.87 (3-C), 22.24 ppm (2′-C); ESI-MS: *m/z*: 539.3 $[M+1]^+$; elemental analysis calcd (%) for $C_{25}H_{30}N_8O_4S \cdot 2HCl$: C 49.10, H 5.27, N 18.32; found C 49.34, H 5.04, N 18.54.

*Isomer (2S,2″S) (**28b**):* From **25b**; yield: 99%; yellow solid; m.p. 199–201 °C (isopropanol/diethyl ether). $[\alpha]_D = +18.4$ ($c = 0.30$, MeOH); ^1H NMR (300 MHz, D_2O): $\delta = 8.12$ (d, $^3J(H,H) = 7.8$, 1H, 3H-NPS), 7.53–7.05 (m, 6H, Ar), 6.46 (d, $^3J(H,H) = 7.8$, 1H, 6H-NPS), 4.09 (m, 1H, α-Trp), 3.73 (m, 1H, 4-H), 3.19 (d, $^3J(H,H) = 7.8$, 2H, β-Trp), 2.99 (m, 2H, 3′-H), 2.83 (m, 1H, 4-H), 2.04 (m, 1H, 3-H), 1.91 (m, 3H, 3-H, 1′-H), 1.35 ppm (m, 2H, 2′-H); ^{13}C NMR (75 MHz, D_2O): $\delta = 176.09$ (2-CO), 169.11 (1″-CO), 156.84 (C=N), 144.46–112.31 (14C, Ar), 74.81 (2-C), 50.70 (α-Trp), 46.73 (4-C), 40.76 (3′-C), 31.93 (1′-C), 25.85 (β-Trp), 23.92 (3-C), 22.27 ppm (2′-C); ESI-MS: *m/z*: 539.3 $[M+1]^+$; elemental analysis calcd (%) for $C_{25}H_{30}N_8O_4S \cdot 2HCl$: C 49.10, H 5.27, N 18.32; found C 49.02, H 5.45, N 18.14.

*(3S,6R)- and (3S,6S)-3-[2-(o-Nitrophenylsulfenyl)indole-3-yl]methyl-6-(3′-guanidinyl)propyl-1,4-diazabicyclo[4.2.0]octan-2,5-dione hydrochloride (**17a** and **17b**):*

*Isomer (3S,6R) (**17a**):* From **13a**. Eluent: CH_3CN/H_2O (10:90); yield: 94%; yellow solid; m.p. 178–181 °C (isopropanol/diethyl ether). $[\alpha]_D = -29.2$ ($c = 0.31$, MeOH); ^{1}H NMR (500 MHz, D_2O): $\delta = 8.10$ (d, $^3J(H,H) = 7.8$, 1H, 3H-NPS), 7.48–7.03 (m, 6H, Ar), 6.60 (d, $^3J(H,H) = 7.8$, 1H, 6H-NPS), 4.17 (t, $^3J(H,H) = 7.3$, 1H, 3-H), 3.75 (m, 1H, 8-H), 3.66 (m, 1H, 4-H), 3.08 (m, 2H, 3-CH_2), 2.69 (m, 2H, 3′-H), 2.57 (m, 1H, 7-H), 2.02 (m, 1H, 7-H), 1.29 ppm (m, 4H, 1′-H, 2′-H); ^{13}C NMR (100 MHz, D_2O): $\delta = 173.00$ (2-CO), 171.25 (5-CO), 156.70 (C=N), 144.27–112.03 (14C, Ar), 70.66 (6-C), 58.11 (3-C), 49.02 (8-C), 41.04 (3′-C), 34.43 (1′-C), 29.59 (3-CH_2), 29.05 (7-C), 21.70 ppm (2′-C); ESI-MS: *m/z*: 522.3 $[M+1]^+$; elemental analysis calcd (%) for $C_{25}H_{27}N_7O_4S{\cdot}HCl$: C 53.81, H 5.06, N 17.57; found C 53.75, H 5.26, N 17.84.

*Isomer (3S,6S) (**17b**):* From **13b**; yield: 99%; yellow solid; m.p. 170–173 °C (isopropanol/diethyl ether). $[\alpha]_D = -14.2$ ($c = 0.23$, MeOH); ^{1}H NMR (300 MHz, D_2O): $\delta = 8.08$ (d, $^3J(H,H) = 8.0$, 1H, 3H-NPS), 7.55–7.02 (m, 6H, Ar), 6.47 (d, $^3J(H,H) = 7.8$, 1H, 6H-NPS), 4.36 (m, 1H, 3-H), 3.73 (m, 2H, 8-H), 3.32 (dd, $^3J(H,H) = 5.3$, 15.1, 1H, 3-CH_2), 3.04 (dd, $^3J(H,H) = 5.3$, 15.1, 1H, 3-CH_2), 2.85 (m, 2H, 3′-H), 2.41 (m, 1H, 7-H), 2.04 (m, 1H, 7-H), 1.32 ppm (m, 4H, 1′-H and 2′-H). ^{13}C NMR (100 MHz, D_2O): $\delta = 172.15$ (2-CO), 171.00 (5-CO), 156.85 (C=N), 137.61–112.02 (14C, Ar), 71.64 (6-C), 53.07 (3-C), 49.00 (8-C), 40.80 (3′-C), 32.24 (1′-C), 27.99 (3-CH_2), 24.24 (7-C), 22.10 ppm (2′-C); ESI-MS: *m/z*: 522.3 $[M+1]^+$; elemental analysis calcd (%) for $C_{25}H_{27}N_7O_4S{\cdot}HCl$: C 53.81, H 5.06, N 17.57; found C 53.83, H 5.18, N 17.53.

General procedure for the synthesis of tetrahydro-β-carboline derivatives: *N*-Boc-L-Lys(Fmoc)-OH (0.534 g, 1.14 mmol) was coupled to a HMBA resin (0.19 g, 0.19 mmol) in anhydrous DMF (6 mL), by means of diisopropylcarbodiimide (DIPCDI; 0.176 mL, 1.14 mmol) and catalytic 4-dimethylaminopyridine (10%), with orbital stirring overnight. The resin was washed repeatedly with DMF and DCM (5×0.5 min). Then, the Boc group was removed by stirring the mixture for 2 h at room temperature with TFA/EDT/TA/phenol/H_2O/TIPS (68.5:10:10:5:3.5:1; 10 mL). After the washings, a second coupling was performed with *N*-Boc-L-Trp-OH (0.173 g, 0.57 mmol), HOBt (0.077 g, 0.57 mmol), and DIPCDI (0.088 mL, 0.57 mmol). Following the removal of the new Boc group by the above procedure, treatment with benzaldehyde or *o*-nitrobenzaldehyde (5.7 mmol) in TMOF (0.3 mL) at room temperature for 5 h was performed. This treatment was repeated once. After the corresponding washings, 25% TFA in DCM (3 mL) was added, and the mixture was stirred for 2 h at room temperature. Removal of the Fmoc group was performed with a mixture of DBU/HOBt/DMF (0.059 mL:0.05 g:5 mL). Cleavage of the product was achieved by heating the resin at 50 °C with MeOH/TEA (9:1, 3 mL). After being gently stirred overnight, the mixture was allowed to cool to room temperature and filtered, and the filtrate, after the addition of 2 drops of concentrated HCl, was evaporated to dryness and lyophilized from acetonitrile-water to yield the desired products as hydrochloride salts. Final product purification was performed on reversed-phase SPE cartridges (Discovery DSC-18LT) with the solvent system specified in each case.

*(1S,R,3S,1′S)-3-[*N*-(5′-amino-1′-methoxycarbonyl)pentyl]-1-phenyl-1,2,3,4-tetrahydro-β-carboline (**32**):* Eluent: gradient from 100% H_2O to 100% CH_3CN; yield: 33%; white solid; ^{1}H NMR (300 MHz, CD_3OD): $\delta = 7.71$–6.89 (m, 9H, Ar), 5.22 and 5.11 (2s, 1H, 1-H), 4.10 (m, 1H, 1′-H), 3.63 (m, 1H, 3-H), 3.60 and 3.58 (2s, 3H, OMe), 3.35 (m, 1H, 4-H), 3.24–2.94 (m, 2H, 5′-H), 2.81–2.68 (m, 1H, 4-H), 1.58–1.15 ppm (m, 6H, 2′-, 3′-, 4′-H); ESI-MS: *m/z*: 435.3 $[M+1]^+$.

*(1S,R,3S,1′S)-3-[*N*-(5′-amino-1′-methoxycarbonyl)pentyl]-1-(o-nitro)-phenyl-1,2,3,4-tetrahydro-β-carboline (**33**):* Eluent: gradient from 100% H_2O to 100% MeOH; yield: 48%; white solid. ^{1}H NMR (300 MHz, CD_3OD): $\delta = 7.96$–6.98 (m, 8H, Ar), 5.94 and 5.77 (2s, 1H, 1-H), 4.13 (m, 1H, 1′-H), 3.77 (m, 1H, 3-H), 3.70 and 3.67 (2s, 3H, OMe), 3.40–3.10 (m, 3H, 4-H and 5′-H), 2.96–2.88 (m, 1H, 4-H), 1.70–1.40 ppm (m, 6H, 2′-, 3′-,d 4′-H); ESI-MS: *m/z*: 480.2 $[M+1]^+$.

Recombinant rat TRPV1 and human NMDAR channel expression in Xenopus *oocytes and channel blockade*: All the procedures have been described in detail elsewhere.[29,30] Briefly, capped cRNA for TRPV1 (kindly provided by Dr. David Julius), and the NR1 and NR2A subunits of the NMDA receptor was synthesized from linearized cDNA by using the mMESSAGE mMACHINE from AMBION (Texas). cRNA (0.2 mg mL^{-1}) was microinjected ($V = 50$ nL) into defolliculated oocytes (Stage V and VI) as described. Oocytes were functionally assayed 2–4 days after cRNA injection. Whole-cell currents from rat TRPV1-injected oocytes were recorded in standard Ringer solution (10 mM HEPES, pH 7.4, 115 mM NaCl, 2.8 mM KCl, 2.8 mM $BaCl_2$) with a two-microelectrode voltage-clamp amplifier at 20 °C. TRPV1 channels were activated by application of 10 μM capsaicin in the absence or presence of compounds (10 μM) at a holding potential (V_h) of −60 mV. Whole-cell currents from oocytes injected with NR1/NR2A (1:3, *w/w*) subunits were recorded in standard Ringer solution upon activation with L-glutamate (100 μM) supplemented with glycine (10 μM) at $V_h = -60$ mV. Responses were normalized with respect to that evoked in the absence of channel blockers. Data are given as mean ± SEM, with *n* (number of oocytes) ≥5.

Use of animals: All experiments were approved by the Institutional Animal and Ethical Committee of the University Miguel Hernández, and were in accordance with the guidelines of the European Economic Community (86/609/EEG), the National Institutes of Health, and the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

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