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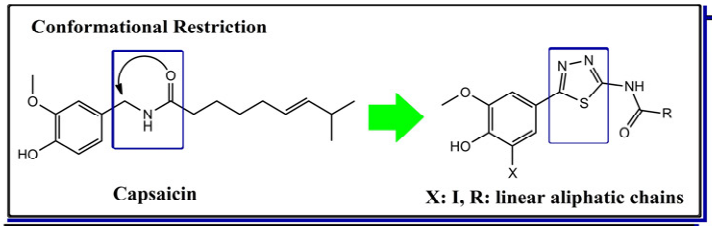

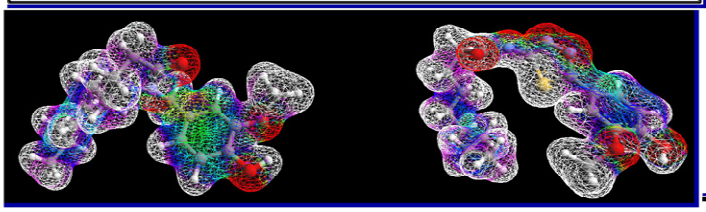

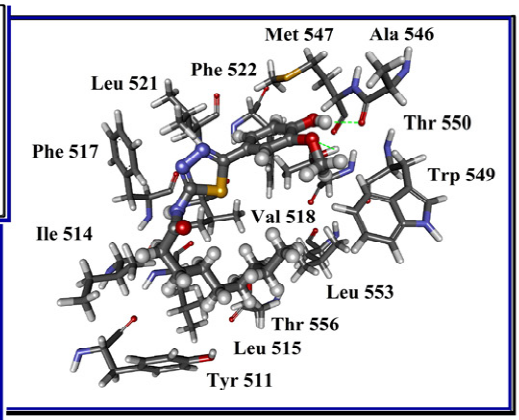

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Design and synthesis of conformationally restricted capsaicin analogues based in the 1, 3, 4-thiadiazole heterocycle reveal a novel family of transient receptor potential vanilloid 1 (TRPV1) antagonists

Carolyne Lespay Rebolledo[a], Pamela Sotelo Hitshfeld[b], Sebastián Brauchi[b] and Miguel Zárraga O.[a,*]

[a]*Department of Organic Chemistry, Faculty of Chemical Sciences, University of Concepcion, Edmundo Larenas 160C, Concepción, 1430000, Chile.*
[b]*Institute of Physiology, Faculty of Medicine, University Austral Of Chile, Campus Isla Teja 511-0566,Valdivia, 5090000, Chile.*

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ABSTRACT

4-hydroxy-3-methoxybenzaldehyde was used as starting material to obtain a number of 1, 3, 4-thiadiazole alkylamide derivatives. The pharmacological properties of these conformationally restricted capsaicin analogues were evaluated on HEK-293T cells transiently expressing TRPV1 receptor. By means of a high-throughput calcium imaging assay we find that 1, 3, 4-thiadiazoles (compounds **8-15**) act as potent antagonists of the capsaicin receptor, inhibiting both, the capsaicin- and temperature- dependent activation. Docking studies suggested a different binding orientation on the vanilloid binding site when compared with capsaicin analogues, such as 5-iodononivamide. Overall, our studies suggest that 1, 3, 4 thiadiazoles interact with capsaicin's binding region of the receptor, although using a different set of interactions within the vanilloid binding pocket.

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* Corresponding author. Tel.: +56-41-2203921; fax: +56-41-2245974; e-mail: mzarraga@udec.cl

1. Introduction

Transient receptor potential vanilloid 1 (TRPV1) proteins are non-selective, ligand-gated cation channels with permeability to calcium ions. They are well described as polymodal nociceptors allowing for the integration of multiple stimulus during the activation process. These include capsaicin, pH, PIP2, T°> 43, and voltage (1, 2, 3). TRPV1 receptors have a direct role in the physiology and physiopathology of the pain responses showing a strong correlation with their tissue expression, which includes peripheral terminals as well as specific brain areas related with the nociceptive process (4). Thereby, TRPV1 receptors represent an important pharmacological target, which already have provided a broad diversity of drugs engineered for the treatment of inflammatory chronic and neuropathic pain (5, 6).

Capsaicin was the first TRPV1 agonist described, even though its effects, such as burning pain, increment of the intracellular calcium and depolarization of DRG sensory neurons, and potential analgesic effects, were known long before to the identification of the receptor (7, 8). Structurally, capsaicin has been an important lead for drug development targeting TRPV1 receptors, leading to the synthesis and identification of important analogues with well described modifications (9-15). This has allowed the identification of the minimal structural requirements essential to modulate TRPV1 activity. According to this, both capsaicin and capsaicin-like compounds can be divided into three pharmacophoric structural features (Figure 1). These correspond to a region A with capacity of forming hydrogen bonds (9); a polar region B (10), and a lipophilic region C (11). Modifications performed on these regions have allowed the identification of a great diversity of compounds with antagonist activity (9-22). Within the modifications described so far, stand out the antagonist effect induced by iodine atom in capsaicin analogues, such as 5-iodononivamide or 6-iodononivamide (25). Likewise, modifications in the region B such as the substitution of the amide group by the urea or thiourea group notably increased the agonist or antagonist activity in both capsaicin analogues as in non-capsaicin like compounds (10, 16).

In order to increase the antagonist potency, several optimization strategies have been incorporated in TRPV1 compounds, in which the conformational restriction of linker groups has shown to be a useful way for this purpose. The conformational restriction has been incorporated mainly in the linker group located between regions B and C, through of the substitution by polar heterocyclic groups, such as pyrimidine (17), oxazol (18), thiazol (19) among other groups, achieving potent compounds known as conformationally-restricted TRPV1 antagonist. However, the linker group between regions A and B corresponding to methylene or amino groups, has been conserved in these kind of antagonists because the absence or the substitution by non-flexible groups, such as carbonyl group has shown to cause lost or decreasing of the activity on TRPV1 receptors (17-22). Although it is true, that many compounds with restriction conformational have been reported as potent antagonist TRPV1 and several modifications have been studied for expand the three pharmacophoric areas mentioned before, the changes on the linker group between regions A and B through conformational restriction with heterocyclic groups is little frequent.

Thereby, in this work have been proposed the design of conformationally-restricted capsaicin analogues by replacing the amide group of capsaicin with the 1, 3, 4-thiadiazole heterocycle, followed by the incorporation of an amide group as linker group between regions B and C (**Figure 1**). This isosteric replacement has allowed evaluating both the isosteric efficiency of this heterocycle and the effect of conformational restriction between regions A and B for these capsaicin analogues, which have not been evaluated before through of the substitution by a heterocyclic group. Thus, from 4-hydroxy-3-methoxybenzaldehyde as starting material, were carried out several steps of synthesis, which allowed obtaining 1, 3, 4-thiadiazole alkylamides (**8-15**) as novel conformationally-restricted capsaicin analogues. These analogues included both iodinated derivatives on the fifth position of vanillyl group and non-iodinated derivatives, in order to evaluate the effect induced by iodine atom on this kind of derivatives, due to the differences of agonist effect and antagonist effect, observed between compounds non-iodinated and iodinated, respectively on TRPV1 receptors (17, 14). So, as a consequence of the high polarity and the conformational restriction induced by 1, 3, 4-thiadiazole heterocycle, together with the effect of the amide group as linker group were obtained a novel family of potent TRPV1 antagonists.

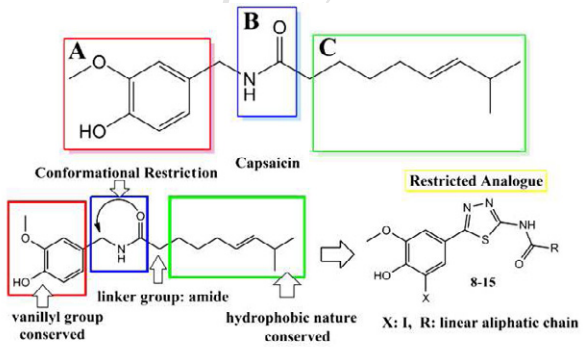


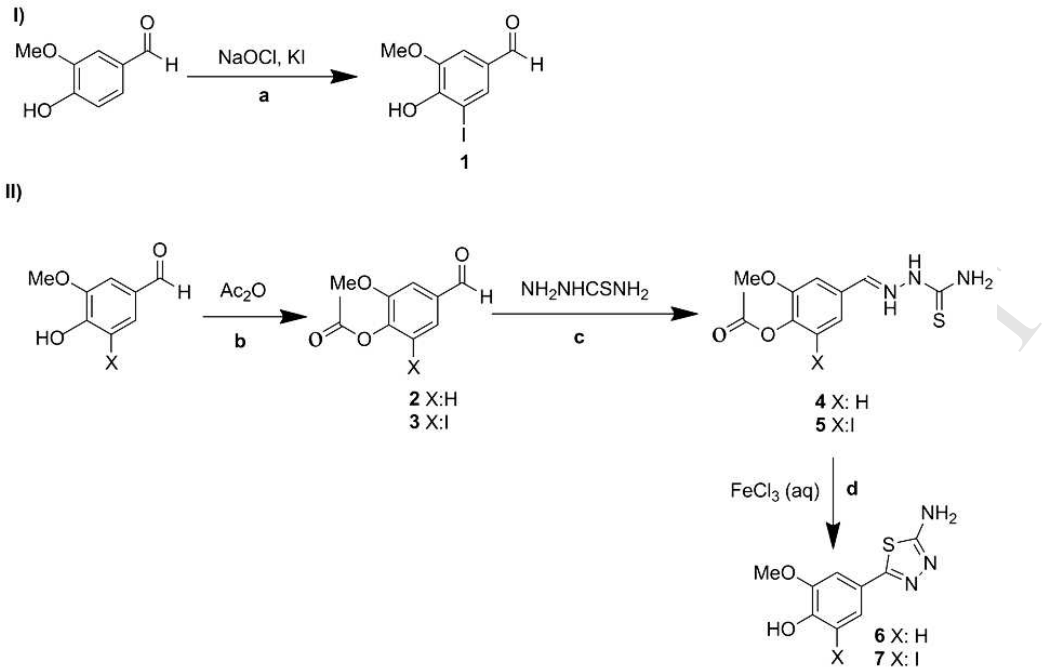
Figure 1. Pharmacophoric features for capsaicin and design of conformationally restricted capsaicin analogues.

2. Results and Discussion

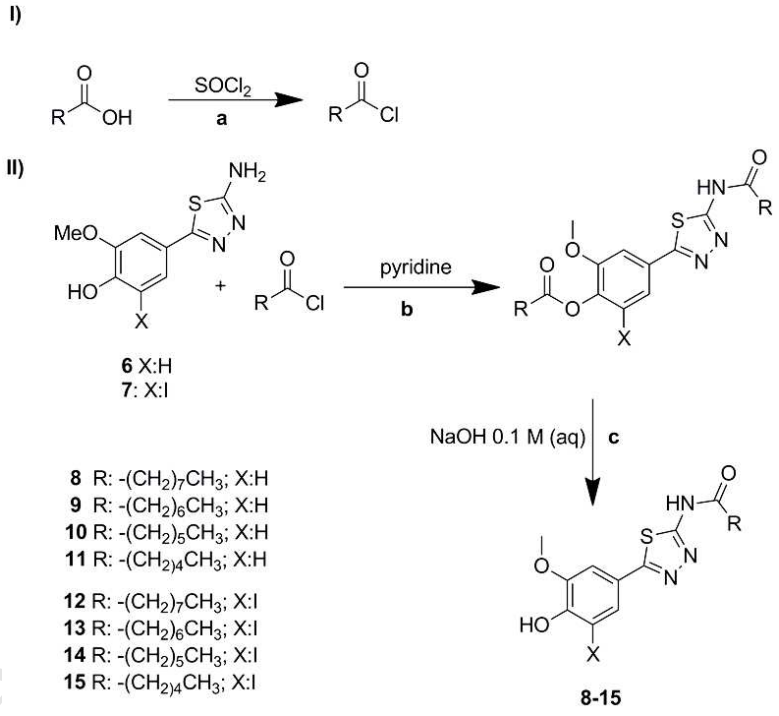
2.1. Chemistry

The 1, 3, 4-thiadiazole alkylamide derivatives (**8-15**) were synthetized from 4-hydroxy-3-methoxybenzaldehyde in three steps. Previously, 4-hydroxy-3-methoxybenzaldehyde was iodinated (**scheme 1-I**) through *in situ* oxidation of potassium iodide with commercial hypochlorite (4.7%). This iodination method allowed obtaining the monoiodinated product (**1**), with a yield of 72%.

According to **scheme 1-II** the first step consisted in the synthesis of heterocycle 1, 3, 4-thiadiazole through oxidative cyclization of the thiosemicarbazones **4** and **5** with an aqueous solution of ferric chloride. Thus, 2-amino-1, 3, 4-thiadiazole **6** and **7** were obtained with good yields that corresponded to 60% and 45% respectively. The next step, involved the formation of amides between 2-amino-1, 3, 4-thiadiazole **6** or **7** and acyl derivatives coming from linear aliphatic carboxylic acids with chains of different longitude. According to **scheme 2**, the carboxylic acids were converted to acyl chlorides with thionyl chloride. Later, *in situ* and at room temperature the 2-amino-1, 3, 4-thiadiazole **6** or **7** was added into the reaction medium followed by the addition of pyridine. The esterified amide derivatives were obtained by this method, therefore the last step of synthesis consisted in an alkaline hydrolysis reaction with NaOH 0.1 M under reflux at 60°C approximately for achieve the formation of a medium of reaction homogenous. This method led to obtaining



Scheme 1. Reaction Conditions: (a) methanol, water-ice bath, agitation for 1 hour (b) room temperature, H_2SO_4 (c) agitation for 1 hour, methanol, glacial acetic acid, pH 4-5, reflux for 2 hours (d) methanol, reflux for 1-2 hours.



Scheme 2. Reaction Conditions: (a) Reflux at 80º C for 16 hours, (b) Agitation for 3 hours at room temperature and (c) Methanol for 3 hours at 60°C.

the 1, 3, 4-thiadiazole alkylamide derivatives **8-15**, which were purified by chromatography and crystallization, achieving satisfactory yields of synthesis.

2.2. Biological Evaluation

All the 1, 3, 4-thiadiazole derivatives obtained were tested in transiently transfected HEK-293T cells expressing rat TRPV1 (rTRPV1). The biological activity of the derivatives was evaluated on the activation of the rTRPV1 receptor by

temperature and capsaicin. TRPV1 activation was measured by means of a high-throughput calcium influx assay; following the fluorescence of Fluo-4 AM loaded cells in a real time PCR thermocycler (23). This method allowed us to follow multiple conditions simultaneously during a temperature ramp as well as to maintain controlled temperatures.

First, the response to temperature changes for control conditions was evaluated from 22°C to 56 °C. These results are shown in **Figure 2a** in which the temperature-induced calcium influx was normalized with respect to the fluorescence obtained at 50°C for rTRPV1. The temperature activation curve for rTRPV1 receptors rose around 40ºC and it saturated at 54º C. The calcium influx induced by increasing temperature was modest or not observed in the cultures previously incubated with reported TRPV1 blockers, such as BCTC, lanthanum, or ruthenium red (6). In contrast, the incubation in presence of 30 nM capsaicin increased the total response (**Supporting information, Figure 1**), as expected according to the allosteric potentiation reported for TRPV1 channels (Latorre et al. 2009). The effect of 1, 3, 4-thiadiazole derivatives (**8**-**15**) on the activity of rTRPV1 was therefore evaluated at 50°C. As seen in **Figure 2b,** the cells incubated in the presence of thiadiazole derivatives displayed a severely diminished calcium influx signal when compared with control conditions. Furthermore, was observed that cells incubated in the presence of thiadiazole derivatives inhibited the calcium influx evoked by 30 nM of capsaicin (**Figure 2c**). According to these results, a low micromolar range (1µM) of the thiadiazole derivatives was enough to block both capsaicin- and temperature- dependent activation of rTRPV1 receptors (**Table 1**).

Using the same approach, dose-response curves were built and the half maximal inhibitory concentration (IC_{50}) for previous-

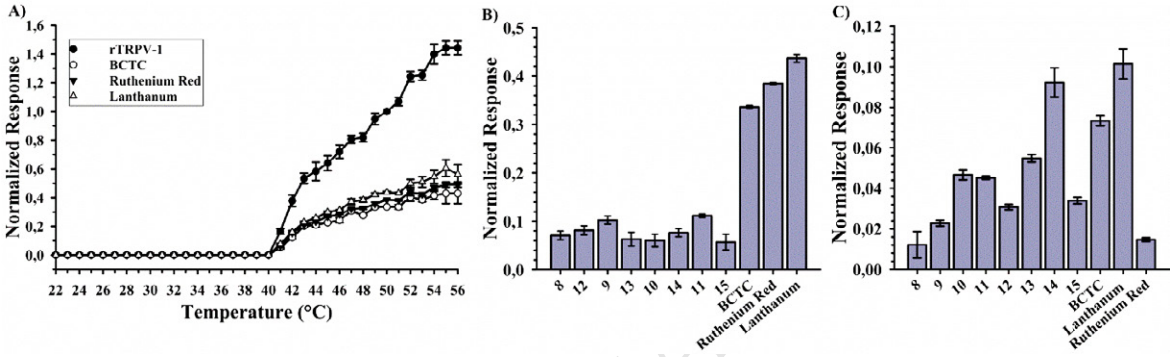


Figure 2. Activity of 1, 3, 4-thiadiazole derivatives on rat TRPV1 receptors. (**A**) Calcium imaging recordings performed on HEK-293T cell overexpressing rTRPV1. The temperature ramp induced a significant raise in the $[Ca^{2+}]_i$ curve over 40°C. The response after incubations with the TRPV1 blockers Lanthanum (100 µM), Ruthenium Red (10 µM) and BCTC (1.0 µM) and in absence of them (rTRPV-1) is shown. The changes in fluorescence were estimated by the following correction (F-F0)/F0, and further normalized with respect to the normal (i.e. untreated transfected cells) response at 50°C (black circles). Data represent mean ± s. d. (n=8). (**B**) Bar plot for the response elicited by incubations with 1.0 µM of thiadiazoles and compared with the antagonistic activity of BCTC (1.0 µM), Lanthanum (100 mM) and Red Ruthenium (10 µM). The relative fluorescence was normalized with respect to the normal TRPV1 response at 50°C and was represented as mean ± s. d. for two assays performed independently (n=3). (**C**) Bar plot for the inhibitory effect of the thiadiazole derivatives (1.0 µM) with respect to the response observed after capsaicin stimulation. The response was normalized to the $[Ca^{2+}]_i$ signal induced by 30 nM capsaicin at 25 °C. The responses correspond to the mean ± s. d. (n=10). All data analyzed were statistically different ***P < 0.05 (one-way ANOVA).

Table 1. Percentage of inhibition for 1, 3, 4-thiadiazole derivatives evaluated at 1.0 µM on rat TRPV1 receptors

Compound	% Inhibition[a] (Temperature)	% Inhibition[a] (Capsaicin)	IC_{50} (nM)[a] (Capsaicin)	IC_{50} (nM)[a] (Temperature)
8	92.92 ± 0.88	98.75 ± 0.65	0.18 ± 0.04	0.05 ± 0.01
9	89.78 ± 0.83	97.72 ± 0.14	0.15 ± 0.04	-
10	93.95 ±1.31	95.33 ± 0.25	0.77 ± 0.02	1.26 ± 0.01
11	88.85 ± 0.35	95.47 ± 0.07	2.61 ± 0.05	-
12	91.86 ± 0.93	96.92 ± 0.13	0.34 ± 0.04	0.15 ± 0.05
13	93.68 ± 1.42	94.52 ± 0.18	0.33 ± 0.03	1.09 ± 0.49
14	92.36 ± 0.83	90.78 ± 0.72	6.07 ± 0.01	-
15	94.29 ± 1.64	96. 60 ± 0.16	1.47 ± 0.03	0.29 ± 0.03
BCTC	66.38 ± 0.32	92.66 ± 0.25	4.86 ± 0.12	2.12 ± 0.35

[a] Values were obtained in two independent experiments and expressed as mean ± s. d.

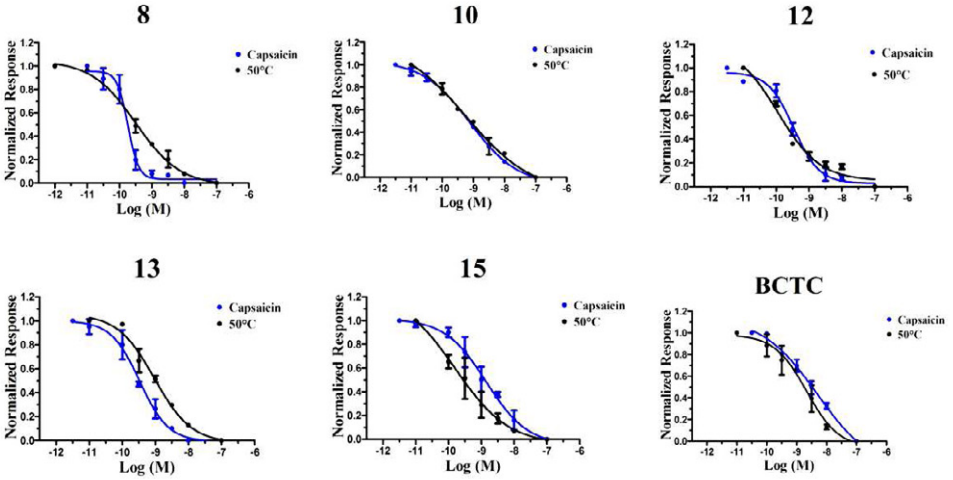


Figure 3. Dose-Response Curves obtained for 1, 3, 4-thiadiazole derivatives through capsaicin and temperature-induced activation on rat TRPV1. The fluorescence values of temperature activation mode were obtained at 50°C to build the curves. The relative fluorescence was calculated for each concentration and normalized with respect to the maxima fluorescence. Values are expressed as mean ± s. d. (n=3). Solid traces represent a fit of the data to the Hill equation.

ly tested derivatives was calculated (**Figure 3 and Table 1**). The potency determined by dose-response curves confirmed the low nanomolar order of magnitude necessary for antagonist activity of 1, 3, 4-thiadiazole derivatives. BCTC was used as control because its general use as a more specific TRP channels inhibitor (24). In the performed experiments, the IC_{50} for BCTC was determined to be 4.8 nM and 2.1 nM on capsaicin, and temperature activation respectively. This is in good agreement with previously reported active concentrations (~5nM; 23). Compounds **8** and **9** were the most potent among all the evaluated compounds (IC_{50}= 40pM; **Table 1**), showing higher affinity when compared with other antagonists such as iodo-nonivamide and iodo-resiniferatoxin (25-27).

In order to evaluate whether 1, 3, 4-thiadiazole derivatives share capsaicin's mechanism of action, we further evaluated their activity in the avian TRPV1 receptors (chicken clone; cTRPV1) which are insensitive to capsaicin. Although capsaicin unresponsive, cTRPV1 receptors are activated by heat or protons, consistent with their physiological role as nociceptors (28).

According to our calcium imaging approach, the activation of the cTRPV1 receptor induced by temperature was absent in presence of lanthanum, ruthenium red, and all the thiadiazole derivatives tested (**Figure 4b**). This time, the effect of BCTC was modest in comparison with the thiadiazole derivatives (**Table 2**). This may imply that the natural substitutions present in cTRPV1 clone are important not only for capsaicin-dependent activation but also for BCTC-dependent inhibition. Interestingly, thiadiazole derivatives displayed a lesser but still potent inhibition of the cTRPV1 temperature-activation when compared to the response obtained with the rTRPV1 receptor (**Table 2)**. These results indicated that although the natural structural variations present in cTRPV1 are not necessary for the molecular mechanism underlying the effect of thiadiazole derivatives, they are important in the modulation of the inhibitory effect.

Notably, both families of thiadiazole derivatives tested (i.e. iodinated and non-iodinated) presented the same trend of antagonistic activity, suggesting that the 1, 3, 4-thiadiazole heterocycle is responsible for the differences observed in the potency of the compounds when compared themselves or to other capsaicin analogues reported before (15, 25). Furthermore, the antagonistic effect shown by the derivatives with the non-iodinated vanillyl group was in opposition to the effect reported for other capsaicin analogues containing amide, thiourea, or the 1, 2, 3-triazole group as "B region", all of them elicited a strong agonistic effect (10, 15).

In general terms, the level of inhibition of capsaicin-induced activation of rTRPV1 indicate that the non-iodinated derivatives are better antagonists in comparison with the iodinated derivatives. Our analysis also underscores the compounds with longest aliphatic chains (**8**, **9**, **12** and **13)** as the most potent. On the other hand, the inhibitory responses observed for the case of temperature-induced activation of rTRPV1 showed that derivatives **8, 12** and **15**, having the longest and the shortest linear aliphatic chains were the most potent. Interestingly, when the same temperature activation assay was performed on the capsaicin-insensitive chicken TRPV1 receptor, the derivatives **11** and **15** with the shortest aliphatic chains showed greater potency and in general the iodinated derivatives showed slightly a better potency than non-iodinated derivatives (**Table 2**).

3. SAR analysis, docking and electrostatic potential

Docking studies have shown that vanillyl group of all vanilloid compounds (e.g. capsaicin) interacts with the same aminoacids in the vanilloid binding site (29), and mutagenesis studies have showed that Y511, S512 and T550 residues are associated with the sensibility to vanilloid compounds (28, 30).

According to the results presented here, the presence of capsaicin altered the interaction of the thiadiazole derivatives within the receptor (**Figure 2 and Table 1**). Additionally, residues that alter the pathway for capsaicin-dependent activation also, alter (but not impaired) the effect of the synthesized derivatives. Therefore, our results suggest to us that to elicit its antagonistic activity, the thiadiazole derivatives require a pathway of activation that share elements with the one of capsaicin. These interactions not necessarily have to do with bin-

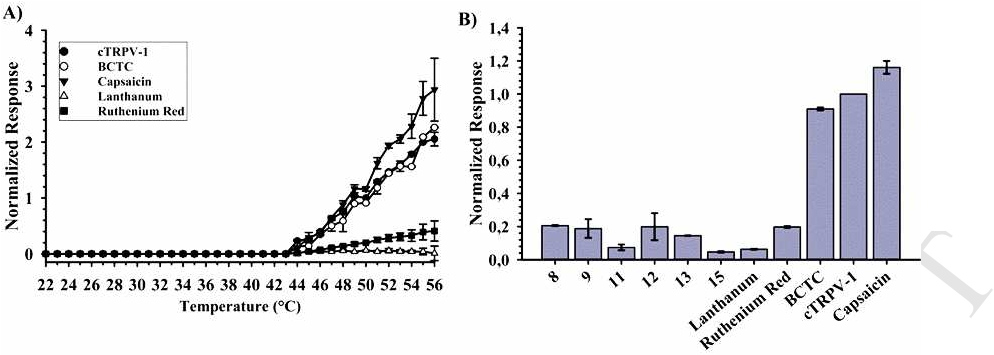


Figure 4. Temperature-induced activation in chicken TRPV1 for **A**) conditions control corresponding to cells incubated in presence of lanthanum (100 mM), ruthenium red (10 µM), BCTC (1.0 µM), capsaicin (30 nM) and in absence of them (cTRPV-1). **B)** 1, 3, 4-thiadiazole derivatives evaluated at 1.0 µM. The relative fluorescence was normalized with respect to the response of untreated transfected cells at 50°C. The relative fluorescence measurements were expressed as mean ± s. d. for two assays performed independently (n=3). Data obtained for the antagonist effect are statistically different ***P < 0.05 (one-way ANOVA) with the exception of capsaicin and BCTC.

Table 2. Antagonist activity of 1, 3, 4-thiadiazoles evaluated at 1.0 µM on chicken TRPV1 receptors

Compound	% Inhibition[a]
8	79.38 ± 0.30
9	81.18 ± 5.62
11	92.56 ± 1.72
12	80.07 ± 8.13
13	85.43 ± 0.15
15	95.29 ± 0.41
BCTC	8.97 ± 0.89

[a] Values were obtained in two independent experiments and expressed as mean ± s. d.

ding properties, since they may also include the allosteric communication between the agonist-sensing domain and the pore.

In order to evaluate these structural features of thiadiazole derivatives that are responsible for their higher activity with respect to their non-restricted analogs, and to explore into the mechanism of protein-ligand interaction, we performed docking studies on the vanilloid binding site of a rTRPV1 (Gavva 2004). The docking results for capsaicin (**Figure 5a**) indicated that the vanillyl group interacts with Tyr 511 by means of the formation of hydrogen bonds, whereas the alkyl group is oriented towards a hydrophobic pocket formed by Val 518, Met 547, Leu 521, Phe 517, Phe 522 and Thr 550. This docked pose is in agreement with the mutagenesis studies reporting that Tyr 511 and Thr 550 are associated with the sensibility of TRPV1 receptors to vanilloids (28, 30). The binding orientation adopted by 5-iodononivamide was similar to the one observed for capsaicin on the vanilloid binding site (**Figure 5b**), and involved the same set of residues. In contrast, the docking results for 1, 3, 4-thiadiazole derivatives (**Figure 5** and **Table 3**) showed not only a different binding orientation into the vanilloid binding site but also a different set of interactions. According to this, the vanillyl group interacts with Thr 550 through the formation of hydrogen bonds and the alkyl group orients toward a hydrophobic pocket formed by Ile 514, Leu 515, Thr 556, Leu 553, and Trp 549 whereas, the thiadiazole heterocycle and the amide group are oriented near of the residues of Phe 517, Leu 521, Val 518 and Tyr 511.

When we cross correlate these results obtained by molecular docking with the inhibitory effect of thiadiazole derivatives on capsaicin-dependent rTRPV1 activation, we can observe that the most potent compounds are characterized by an interaction between Thr 550 and the vanillyl group of the ligand through hydrogen bond formation. This is accompanied by polar interactions between the thiadiazole group and polar residues of the receptor. On the other hand, the less potent compounds interact by hydrogen bond formation between Tyr 511 and the amide group of thiadiazole derivatives (**Figure 5d**). When we analyze the same correlation for the case of the inhibition of the temperature-induced activation, the docking results indicated that the vanillyl group of the most potent derivatives interacts with Thr 550, together with an apparent important contribution from the polar interactions established by the thiadiazole and amide groups (**Table 3**). Therefore, when we compared compounds **8**, **12**, and **15** our results suggest that Thr 550 promotes a greater antagonistic potency when compared with Tyr 511 on capsaicin-induced responses, while polar interactions between thiadiazole and amide groups promotes greater antagonistic potency on the temperature-induced response.

The differences between capsaicin and thiadiazole derivatives are extensive to the active conformation of the compounds once on the binding pocket. On **figure 6** we can observe that agonists and antagonists adopt different three dimensional configurations. We analyzed then the electrostatic potential for the bioactive conformation of thiadiazole derivatives (**Figure 6).** Our analysis reveals that thiadiazole derivatives possess an electrostatic potential pattern resembling that of capsaicin and 5-iodononivamide. According to the pharmacophoric division mentioned initially for capsaicin, the regions with the highest electron density were the oxygen of the carbonyl group and the nitrogen atoms of the thiadiazole group in the region B, and the oxygen atoms of the hydroxyl and methoxyl groups located in the region A. Conversely, the lowest electron density was observed in the aromatic ring of the vanillyl group (region A). Moreover, an unequal distribution of electron density was observed on the region C formed by the alkyl group. The latter might mediate Van der Waals or hydrophobic interactions, which would explain the

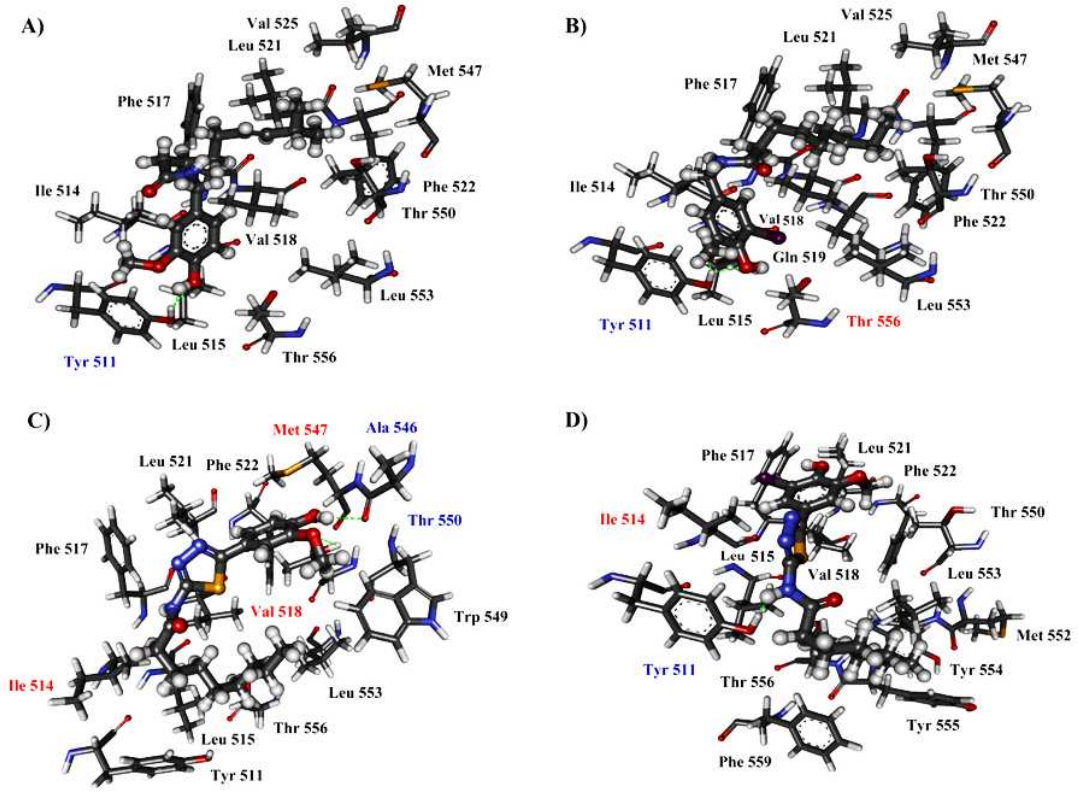


Figure 5. Docking solutions into the vanilloid binding site of TRPV1 receptor for **(A)** Capsaicin, **(B)** 5-iodononivamide, **(C)** thiadiazole **8** and **(D)** thiadiazole **13.** The interactions by means of hydrogen bonds are shown in green, whereas the residues involved the formation of hydrogen bonds are shown in blue, the residues involved in the polar interactions are shown in red and the residues involved in the hydrophobic interactions are shown in black.

Table 3. Summary of docking results for lowest energy poses of capsaicin, 5-iodononvamide, BCTC and 1, 3, 4-thiadiazole derivatives. The residues involved in the interaction on vanilloid binding site and the nature of interaction are shown. Blue color indicates the residues forming hydrogen bond, red color indicates the residues involved in the polar interaction and black color indicates the hydrophobic or Van der Waals interactions.

Compound	Docking Residues	E pose (Kcal/mol)
Capsaicin	**Tyr 511**, Ile 514, Leu 515, Phe 517, Val 518, Leu 521, Phe 522, Val 525, Met 547, Thr 550, Leu 553, Thr 556	-8.14
5-iodononivamide	**Tyr 511**, Ile 514, Leu 515, Phe 517, Val 518, Gln 519, Leu 521, Phe 522, Val 524, Met 547, Thr 550, Leu 553, Thr 556	-8.33
BCTC	**Tyr 511**, Leu 515, Val 518, Leu 521, Phe 522, Val 525, Phe 543, Met 547, Thr 550, Leu 553, Tyr 554, Tyr 555, Phe 559	-8.52
8	Tyr 511, **Ile 514**, Leu 515, Phe 517, **Val 518**, Leu 521, Phe 522, **Met 547**, **Ala 546**, Trp 549, **Thr 550**, Leu 553, Thr 556	-6.82
12	Tyr 511, Ile 514, Leu 515, Val 518, **Ala 546**, **Trp 549**, **Thr 550**, **Asn 551**, **Met 552**, **Leu 553**, Tyr 555, Thr 556, Phe 559	-6.76
9	Val 518, Leu 521, Phe 522, Val 525, Met 547, Trp 549, **Thr 550**, Leu 553, Tyr 555, Thr 556, Phe 559	-6.57
13	**Tyr 511**, **Ile 514**, Leu 515, Phe 517, Val 518, Leu 521, Phe 522, Thr 550, Leu 553, Met 552, Tyr 554, Tyr555, Thr 556, Phe 559	-7.49
10	**Tyr 511**, Ile 514, Leu 515, Val 518, Leu 521, Trp 549, Thr 550, Met 552, Leu 553, Met 552, Tyr 555, Thr 556, Phe 559	-7.01
14	**Tyr 511**, Ile 514, Leu 515, Phe 517, Val 518, Leu 521, Trp 549, Thr 550, Met 552, Leu 553, Met 552, Tyr555, Thr 556, Phe 559	-7.42
11	**Tyr 511**, Ile 514, Leu 515, Phe 517, Val 518, Leu 521, Thr 550, Met 552, Leu 553, Tyr555, Thr 556, Phe 559	-5.90
15	**Tyr 511**, Leu 515, Val 518, Phe 522, Trp 549, **Thr 550**, Asn 551, Leu 553, **Tyr554**, Tyr 555, **Thr 556**, Arg 557, Phe 559	-8.55

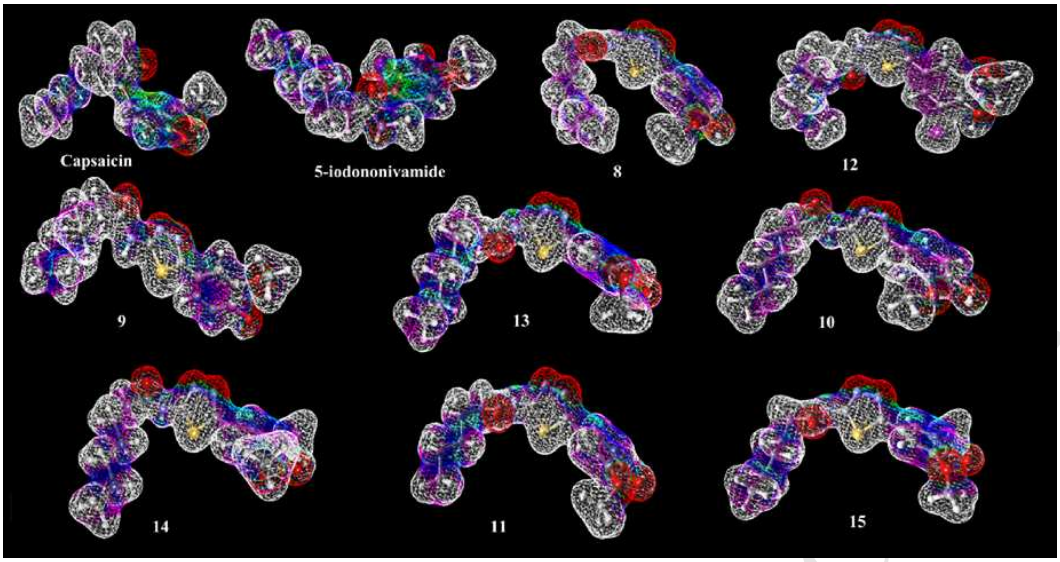


Figure 6. Map of electrostatic potential for docked poses of capsaicin, 5-iodononivamide and 1, 3, 4-thiadiazole derivatives. Red color represent zones with high electron density, Blue color represent zones with low electron density and White color represent zones with neutral electron density.

greater antagonistic potency obtained when longer alkyl groups were introduced.

The results obtained from our *in-silico* studies, specially the similarities observed on the electrostatic potential, suggest a common binding region for capsaicin and thiadiazole derivatives into the receptor, however, having both a different set of interactions with the receptor. These differences may be determined by the structural restrictions intrinsic to the designed antagonists together with the orientation of the compounds inside the binding pocket.

The fact that BCTC showed a reduced activity on cTRPV1 when compared with the inhibition caused in rTRPV1 well correlate with our structural studies. The docked conformation of BCTC inside the vanilloid binding site share similarities with capsaicin rather than to thiadiazole derivatives (**Table 3**), causing then a modest inhibitory effect on channel activity. We speculate that capsaicin and BCTC are able to bind to cTRPV1, however, the chemical energy of binding is not transduced to the pore domain, probably due to the presence of a different set of allosteric interactions, allowing for thiadiazole inhibition but impairing capsaicin-dependent activation.

4. Conclusion

The conformationally restricted capsaicin analogues 1, 3, 4-thiadiazole derivatives showed a potent antagonistic activity (low nanomolar) on capsaicin- and temperature- activated TRPV1 channels. The modification from agonist to antagonist activity, seems to be associated with conformational effects and an increased polarity on the region B of the analogues. Furthermore, our results indicated that the iodinated thiadiazole derivatives are approximately 100 times more potent antagonists than other iodinated capsaicin analogues reported in the past (14, 15). According to our structural studies this greater antagonist potency observed for thiadiazole derivatives was possible due to a different binding orientation into the vanilloid binding site, different to the one observed for other capsaicin derivatives, such as 5-iodononivamide.

5. Experimental Section

5.1 Chemistry

Unless otherwise noted all materials and solvents were obtained from Merck and were used without previous purification. The reactions involved moisture sensitive reagents were carried out under presence of $CaCl_2$ anhydride. The reactions were followed by means of thin layer chromatography and were revealed by UV light, sulfuric/vanillin spray and Folin reactive. Preparative column chromatography was performed using 60G-F254 silica gel (MERCK). The melting points were determined on a Bûchi oil-heated melting point apparatus and are uncorrected; the ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance-400 MHz (Avance 400, Bruker, Germany, 2002). The FT-IR spectrums were recorded with a Nicolet Nexus spectrometer (Nexus, Nicolet Instrument. Inc, Madison WI 53711, USA) and the elemental analysis was performed on Elemental Analyzer CE Instrument (EA 1108, Fisons, Italy, 1995).

5.1.1 Synthetic Procedures

5.1.1.1. *4-Hydroxy-3-iodo-5-methoxybenzaldehyde* (**1**). In a 100 ml Erlenmeyer flask was added 4-hydroxy-3-methoxybenzaldehyde (66 mmol, 10 g) and potassium iodide (66 mmol, 9.73 g) in 20 ml of methanol. The reaction mixture was cooled under an ice-water bath for 15 minutes and after was added drop to drop, using an addition funnel and under stirring, 74 ml of sodium hypochlorite commercial (66 mmol, 75 ml) and then the reaction mixture was stirred for 1 hour, maintaining the ice-water bath. After was added 20 ml of an aqueous solution of sodium thiosulphate (10% p/v) and stirred for 15 minutes at room temperature and then acidified with hydrochloric acid (37%) until pH 3-4, the precipitate formed was filtered and washed with cooled water. The crude product was crystallized from isopropyl alcohol and **1** was obtained as light yellow crystals.

5.1.1.2. *4-Formyl-2-methoxyphenyl acetate* (**2**). In a 100 ml Erlenmeyer flask was added 4-hydroxy-3-methoxybenzaldehyde (66 mmol, 10 g) and an excess of acetic anhydride (27 mmol, 30 ml) and then under stirring at room temperature was added 0.3 ml

of sulphuric acid concentrated. The reaction mixture was maintained in stirring continued for 4 hours and then slowly was added cold water until to form a solid, which was filtered and washed with abundant water. The crude product was crystallized from methanol to give **2** as light orange-pink crystals.

5.1.1.3. *4-Formyl-2-iodo-6-methoxyphenylacetate* (**3**). Similar procedure for synthesis of **2** was followed. It was used 4-hydroxy-3-iodo-5-methoxybenzaldehyde (14.40 mmol, 4 g) and acetic anhydride (212 mmol, 20 ml). The product **3** was obtained as light brown crystals.

5.1.1.4. *2-Methoxyphenyl acetate thiosemicarbazone* (**4**). In a 250 ml round bottom flask a mixture of thiosemicarbazide (3.73 g, 41 mmol) and **2** (8 g, 41 mmol) in 50 ml of methanol was prepared and acidified until pH 4-5 with acetic acid glacial and refluxed for 6 hours under stirring. The reaction mixture was cooled in an ice-water bath and the precipitate formed was filtrated and crystallized from methanol to give **4** as light yellow crystals.

5.1.1.5. *2-Iodo-6-methoxyphenylacetate thiosemicarbazone* (**5**). Similar procedure for synthesis of **4** was followed, were used thiosemicarbazide (12 mmol, 1.09 g) and **3** (12 mmol, 4 g). The product **5** was obtained as light yellow crystals.

5.1.1.6. *2- Amino- 5 - (4-hydroxy-3-methoxyphenyl) - 1, 3, 4-thiadiazole* (**6**). In a 250 ml round bottom flask was added **4** (19.5 mmol, 5g), 20 ml of ferric chloride aqueous solution (59 mmol, 9.48g) and 30 ml methanol. The reaction mixture was refluxed under stirring for 1-2 hours and filtered in hot on activated carbon and then an aqueous solution of citric acid (11.52 g, 60 mmol) and sodium citrate (6.42 g, 30 mmol) was added and refluxed for 1 hour under stirring. After the solution was neutralized with 10% aqueous ammonia to pH 4-5 and cooled in an ice- water bath. The precipitate was washed several times with cooled water and then was crystallized with a mixture of ethanol/methanol 1:1 to give **6** as a bright yellow powder.

5.1.1.7. *2-Amino-5-(4-hydroxy-3-iodo-5-methoxyphenyl)-1, 3, 4-thiadiazole* (**7**). Similar procedure for synthesis of **6** was followed. It was mixed **5** (10.60 mmol, 4g) and a ferric chloride aqueous solution (32 mmol, 10 ml, 5.2g,). Finally, the product was crystallized with a mixture of water/methanol 1:1 to give **7** as a light brown powder.

5.1.1.8. *N-[5-(4-hydroxy-3-methoxyphenyl)-1, 3, 4- thiadiazol-2-yl)] nonamide* (**8**). In a 250 ml round bottom flask dried previously, was added 1 ml of nonanoic acid (11 mmol) and 0.6 ml of thionyl chloride (8.30 mmol), the reaction mixture was stirred and refluxed for 16 hours at 80°C . Later, the reaction mixture was cooled at room temperature and under vigorous stirring was rapidly added **6** (0.89 mmol, 200 mg) to reaction medium, followed of 2 ml of pyridine. The mixture reaction was stirred at room temperature for 2 hours and then partitioned between an aqueous acid solution and dichloromethane; the organic layer was extracted and washed with a saturated aqueous solution of sodium chloride. After the concentrated organic layer was hydrolyzed in presence 2 ml of a solution sodium hydroxide 0.1 M in 10 ml of methanol and was refluxed for 3 hours under stirring. The hydrolysis mixture was acidified until pH 4 with hydrochloric acid 37%, a white precipitate was formed and then filtered followed of the purification by column chromatography using 20% ethyl acetate/hexane, and finally the product obtained was crystallized in 1:4 water / methanol to give **8** as white crystals.

5.1.1.9. *N-[5-(4-hydroxy-3-iodo-5-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] nonamide* (**12**). The product was obtained by similar procedure for synthesis of **8**. It was used 1ml of nonanoic acid (11 mmol) and 0.6 ml of thionyl chloride (8.30 mmol). After was added 200 mg of **7** (0.89 mmol) and **12** was obtained as a brown powder.

5.1.1.10. *N-[5-(4-hydroxy-3-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] octanamide* (**9**). The product was obtained by similar procedure for synthesis of **8**. It was used 1ml of octanoic acid (13 mmol) and 0.8 ml of thionyl chloride (11 mmol). And then 200 mg (0.89 mmol) of **6** was added, obtaining **9** as white crystals in a 55.27% of yield.

5.1.1.11. *N-[5-(4-hydroxy-3-iodo-5-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] octanamide* (**13**). Similar procedure for synthesis of **8** was followed. It was used 1 ml of octanoic acid (13 mmol) and 0.8 ml of thionyl chloride (11 mmol). And then 200 mg (0.57 mmol) of **7** was added, obtaining **13** as a brown powder.

5.1.1.12. *N-[5-(4-hydroxy-3-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] heptanamide* (**10**). The product was obtained by similar procedure for synthesis of **8**. It was used 1 ml of heptanoic acid (14 mmol) and 0.9 ml of thionyl chloride (12 mmol). And then 200 mg (0.89 mmol) of **6** was added, obtaining **10** as white crystals.

5.1.1.13. *N-[5-(4-hydroxy-3-iodo-5-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] heptanamide* (**14**). The synthetic procedure was similar to described for **8**. It was used 1 ml (14 mmol) of heptanoic acid and 0.9 ml of thionyl chloride (12 mmol). And then 200 mg (0.57 mmol) of **7** was added, obtaining **14** as a brown powder.

5.1.1.14. *N-[5-(4-hydroxy-3-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] hexanamide* (**11**). The product was obtained by similar procedure for synthesis of **8**. It was used 1 ml of hexanoic acid (16 mmol) and 0.9 ml of thionyl chloride (12 mmol). And then 200 mg (0.89 mmol) of **6** was added, obtaining **11** as white crystals.

5.1.1.15. *N-[5-(4-hydroxy-3-iodo-5-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] hexanamide* (**15**). The synthetic procedure was similar to described for **8**. It was used 1 ml (16 mmol) of hexanoic acid and 0.9 ml of thionyl chloride (12 mmol). And then 200 mg (0.57 mmol) of **7** was added, obtaining **15** as a brown powder.

5.1.2. Characterization of Compounds

5.1.2.1. *4-Hydroxy-3-iodo-5-methoxybenzaldehyde* (**1**). Yield: 75%; mp: 182°C; TLC: Rf = 0.6 (dichloromethane). IR (KBr) ν cm^{-1}: 1415.48, 1455.92, 1491.49, 1578.21, 1665.52, 2842.86, 3145.56.

5.1.2.2. *4-Formyl-2-methoxyphenyl acetate* (**2**). Yield: 71%; mp: 66°C; TLC: Rf =: 0.8 (70% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 1029.62, 1210.29, 1279.61, 1428.30, 1468.36, 1509.57, 1596.57, 1684.91, 1756.07, 2968.94, 2846.29, 3015.06.

5.1.2.3. *4-Formyl-2-iodo-6-methoxyphenylacetate* (**3**). Yield: 99%; mp: 62°C; TLC: Rf = 0.7 (60% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 1203.78, 1275.01, 1418.37, 1461.59, 1579.20, 1685.51, 1768.15, 2966.65, 3051.19.

5.1.2.4. *2-Methoxyphenyl acetate thiosemicarbazone* (**4**). Yield: 89%; mp: 119°C; TLC: Rf = 0.6 (70% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 1029.58, 1214.36, 1294.31, 1368.13, 1464.26, 1509.53, 1538.31, 1595.55, 1762.03, 3014.01, 3160.41, 3263.58, 3395.89.

5.1.2.5. *2-Iodo-6-methoxyphenylacetate thiosemicarbazone* (**5**). Yield: 85%; mp: 184°C; TLC: Rf = 0.63 (80% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 1279.48, 1409.29, 1460.59, 1514.49, 1587.62, 1763.25, 2830.59, 2972.53, 3151.73, 3262.22.

5.1.2.6. *2- Amino- 5 - (4-hydroxy-3-methoxyphenyl) - 1, 3, 4-thiadiazole* (**6**). Yield: 60%; mp: 263°C; TLC: Rf = 0.03 (70% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 1282.25, 1370.74, 1423.82, 1511.31, 1580.33, 1642.43, 2784.57, 3094.62, 3214.19, 3287.48. ^{1}H NMR (400 MHz, DMSO) δ ppm: 3.86 (s, 3H), 4.25 (s, 2H), 6.96-6.94 (d, J=8.2 Hz, 1H), 7.19-7.17 (dd, J= 8.2 Hz, 1.9 Hz, 1H), 7.30-7.29 (d, J= 1.8 Hz, 1H), 9.20 (s, 1H, OH). ^{13}C NMR (101 MHz, DMSO) δ ppm: 168.24, 156.07, 150.05, 148.17, 120.76, 119.82, 115.95, 109.51, 55.77.

5.1.2.7. *2-Amino- 5 - (4-hydroxy-3-iodo-5-methoxyphenyl) - 1, 3, 4-thiadiazole* (**7**). Yield: 45%; mp: 281°C; TLC: Rf = 0.23 (80% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 1413.95, 1487.43, 1586.70, 1637.74, 3293.55; ^{1}H NMR (400 MHz, DMSO) δ ppm: 3.81 (s, 3H), 5.69 (s, 1H), 7.273- 7.268 (d, J= 2.17 Hz, 1H), 7.282 (s, 2H), 7.469-7.464 (d, J= 1.9 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ ppm: 56.10, 84.57, 109.10, 124.04, 128.14, 147.17, 147.83, 155.03, 168.01.

5.1.2.8**.** *N-[5-(4-hydroxy-3-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] nonamide* (**8**). Yield: 57%; mp: 142°C; TLC: Rf = 0.28 (60% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 3538.20, 3398, 3155.60, 2920.21-2730.72, 1680.21, 1573.98. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.86-0.83 ppm (t, J= 6.8 Hz,3H), 1.50-1.24 (m, 10H), 1.86-1.79 (m, 2H), 2.78-2.76 (t, 7.7 Hz, 2H), 3.96 (s, 3H), 5.9 (s, OH), 7.01-6.99 (d, J= 8.2 Hz, 1H), 7.43-7.41 (dd, J=8.2 Hz, 1.9 Hz, 1H), 7.52 (d, J= 1.7 Hz, 1H), 12.56 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm: 172.45, 163.40, 159.93, 148.56, 147.39, 123.24, 121.78, 115.40, 109.43, 56.52, 36.93, 32.26, 29.80, 29.72, 29.63, 25.93, 23.11, 14.52. Molecular Formula: $C_{18}H_{25}N_3O_3S$. Anal. Calcd: C (59.48), H (6.93), N (11.56), O (13.21), S (8.82). Found: C (59.55), H (7.04), N (10.96), O (13.92), S (8.53).

5.1.2.9. *N-[5-(4-hydroxy-3-iodo-5-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] nonamide* (**12**). Yield: 22.85%; mp: 123°C; TLC: Rf = 0.31 (60% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 3536.22, 3334.22, 3161.33, 2924.58-2853.71, 1694.22. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.88-0.82 (dd , J= 14.1 Hz, 8,0 Hz,3H), 1.46-1.24 (m, 10H), 1.83-1.80 (m, 2H), 2.78-2.74 (t, 7.0 Hz, 2H), 3.98 (s, 3H), 7.44 (s, 1H), 7.85 (s, 1H), 9.77 (s, 1H), 12.74 (s, 1H).^{13}C NMR (101 MHz, $CDCl_3$) δ ppm: 172.47, 161.75, 160.29, 148.51, 146.70, 130.36, 124.71, 109.44, 56.91, 36.91, 32.26, 29.78, 29.71, 29.62, 25.86, 23.11, 14.53. Molecular Formula: $C_{18}H_{24}IN_3O_3S$. Anal. Calcd: C (44.18), H (4.94), N (8.59), O (9.81), S (6.55), I (25.93). Found: C (44.21), H (4.56), N (8.37), O (8.18), S (5.33), I (29.35).

5.1.2.10. *N-[5-(4-hydroxy-3-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] octanamide* (**9**). Yield: 55.27%; mp: 139°C; TLC: Rf = 0.27 (60% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 3308. 01, 3154.41, 3154.41, 2946.86-2861.81, 1672.79. ^{1}H NMR (400 MHz, DMSO) δ ppm: 0.85-0.81 (t, J=6.9 Hz, 3H), 1.25-1.21 (m, 8H), 1.61-1.55 (m, 2H), 2.47-2.44 (t, J= 6.8Hz, 2H), 3.83 (s, 3H), 6.9-6.87 (m, 1H), 7.30-7.28 (dd, J= 8.2 Hz, 2.1 Hz, 1H), 7.44 (d, J= 2.0 Hz, 1H), 9.71 (s, 1H), 12.50 (s, 1H).^{13}C NMR (101 MHz, DMSO) δ ppm: 171.49, 162.00, 157.46, 149.17, 148.10, 121.48, 120.64, 115.95, 109.92, 55.68, 34.87, 31.13, 28.49, 28.39, 24.65, 22.08, 13.96. Molecular Formula: $C_{17}H_{23}N_3O_3S$. Anal. Calcd: C (58.43), H (6.63), N (12.02), O (13.74), S (9.18). Found: C (58.45), H (5.97), N (14.64), O (11.70), S (8.96).

5.1.2.11. *N-[5-(4-hydroxy-3-iodo-5-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] octanamide* (**13**). Yield: 33.46%; mp: 158°C; TLC: Rf = 0.28 (60% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 3386.16, 3386.16, 3158.92, 2929.56-2864.87, 1695.60. ^{1}H NMR (400 MHz, DMSO) δ ppm: 0.87-0.84 (t, J= 6.7 Hz, 1H), 1.28-1.24 (m, 8H), 1.63-1.59 (m, 2H), 2.20-2.16 (t, J= 7.4Hz, 2H), 3.36 (s, 3H),7.48-7.47 (d, J= 1.9 Hz, 1H), 7.78-7.77 (d, J= 1.9 Hz, 1H), 10.04 (s, 1H), 12.57 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ ppm: 172.88, 166.65, 158.75, 147.60, 141.94, 137.50, 132.94, 128.57, 74.39, 56.54, 31.45, 28.90, 28.79, 28.70, 24.96, 22.41, 14.30. Molecular Formula: $C_{17}H_{22}IN_3O_3S$. Anal. Calcd: C (42.95), H (4.66), N (8.84), O (10.10), S (6.75), I (26.70). Found: C (43.03), H (4.52), N (8.91), O (9.53), S (6.53), I (27.48).

5.1.2.12. *N-[5-(4-hydroxy-3-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] heptanamide* (**10**). Yield: 55.48%; mp: 224°C; TLC: Rf = 0.26 (60% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 3536. 01, 3212.54, 3091.83, 2930.79-2775.56, 1676.99. ^{1}H NMR (400 MHz, DMSO) δ ppm: 0.83-0.87 (dd, J= 8.9Hz, 4.8 Hz, 3H), 1.32-1.23 (m, 6H), 1.63-1.56 (m, 1H), 2.49-2.46 (t, J=7.9 Hz, 2H), 3.85 (s, 3H), 6.91-6.89 (d, J=8.2 Hz, 1H), 7.20-7.17 (dd, J=8.2 Hz, 2.1Hz), 7.46 (d, J=2.0 Hz), 12.51 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ ppm: 171.51, 168.42, 162.05, 157.49, 156.17, 150.02, 149.20, 148.19, 148.13, 121.51, 120.87, 120.68, 119.88, 115.99, 115.95, 109.94, 109.52, 55.80, 55.71, 34.91, 30.99, 28.23, 24.64, 22.01, 13.96. Molecular Formula: $C_{16}H_{21}N_3O_3S$. Anal. Calcd: C (57.29), H (6.31), N (12.53), O (14.31), S (9.56). Found: C (57.52), H (6.27), N (12.31), O (13.87), S (10.03).

5.1.2.13. *N-[5-(4-hydroxy-3-iodo-5-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] heptanamide* (**14**). Yield: 29.55%; mp: 192°C; TLC: Rf = 0.27 (60% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 3400.52, 3400.52, 3163.52, 2929.09-2863.98, 1696.07. ^{1}H NMR (400 MHz, DMSO) δ ppm: 0.86-0.82 (t, J=6.87Hz, 3H), 1.29-1.23 (m, 6H), 1.63-1.55 (m, 2H), 2.49-2.45 (t, J=8.06Hz),3.33 (s, 3H), 7.46 (s, 1H), 7.76 (s, 1H), 10.01 (s, 1H), 12.55 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ ppm: 171.56, 160.48, 157.91, 148.60, 147.34, 128.73, 123.32, 109.93, 84.89, 56.31, 56.22, 34.89, 30.95, 28.19, 24.60, 21.97, 13.95. Molecular Formula: $C_{16}H_{21}IN_3O_3S$. Anal. Calcd: C (41.66), H (4.37), N (9.11), O (10.40), S (6.95), I (27.51). Found: C (41.88), H (4.23), N (8.99), O (11.70), S (5.17), I (28.03).

5.1.2.14. *N-[5-(4-hydroxy-3-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] hexanamide* (**11**). Yield: 41.11%; mp: 234°C; TLC: Rf = 0.24 (60% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 3402.67, 3402.67, 3162.49, 2997.19-2742.07, 1679.61. ^{1}H NMR (400 MHz, DMSO) δ ppm: 0.89-0.85 (t, J=6.8 Hz, 3H), 1.35-1.23 (m, 6H), 1.65-1.58 (m, 2H), (t, J=, 2H), 2.49-2.46 (t, J=7.5 Hz, 2H), 3.86 (s, 3H), 6.89-6.87 (d, J= 8.2Hz, 1H), 7.33-7.30 (dd, J= 8.2 Hz, 1.9Hz, 1H), 7.47-7.46 (d, J= 1.8Hz), 9.68 (s, 1H), 12.52 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ ppm: 171.80, 170.06, 159.61, 159.03, 151.67, 141.90, 130.46, 127.92, 110.92, 93.61, 56.50, 34.65, 33.03, 26.55, 21.69, 13.67. Molecular Formula: $C_{15}H_{19}N_3O_3S$. Anal. Calcd: C (56.06), H (5.96), N (13.07), O (14.93), S (9.98). Found: C (56.39), H (6.04), N (13.02), O (15.39), S (9.16).

5.1.2.15. *N-[5-(4-hydroxy-3-iodo-5-methoxyphenyl)-1, 3, 4-thiadiazol-2-yl)] hexanamide* (**15**). Yield: 22.66%; mp: 205°C; TLC: Rf = 0.24 (60% ethyl acetate/ether petroleum). IR (KBr) ν cm^{-1}: 3404.63, 3404.63, 3164.74, 2927.67-2735.99, 1699.04. ^{1}H NMR (400 MHz, DMSO) δ ppm: 0.88-0.85 (t, J= 6.8Hz, 3H), 1.32-1.25 (m, 4H), 1.65-1.58 (m, 2H), 2.49-2.46 (t, J=7.4Hz, 2H), 3.91 (s, 3H), 7.48-7.47 (d, J= 1.7 Hz, 1H), 7.78-7.77 (d, J= 1.8 Hz, 1H), 10.02 (s, 1H), 12.57 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ ppm: 171.54, 160.47, 157.91, 148.60, 147.91, 147.34,

147.26, 128.74, 128.30, 124.12, 123.34, 109.91, 109.20, 84.87, 84.66, 56.31, 56.23, 34.86, 30.74, 24.34, 21.86, 13.85. Molecular Formula: $C_{15}H_{18}IN_3O_3S$. Anal. Calcd: C (40.28), H (4.06), N (9.39), O (10.73), S (7.17), I (28.37). Found: C (40.61), H (3.98), N (7.89), O (11.83), S (6.16), I (29.53).

5.2 Biological Evaluation

5.2.1. Compound preparation and delivery

The compounds were prepared as a stock solution of 10.000X in DMSO and after for assay were diluted to a concentration of 1 µM in 99 % of Ringer buffer (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Glucose, 15 mM de HEPES 7.35 pH, 292 mOSM). Solutions of capsaicin (Sigma), BCTC (ENZO life Science), Lanthanum chloride (Sigma) and Ruthenium red (Alexis biochemicals) were used in a final concentration of 30 nM, 1 µM, 100 mM and 10 µM, respectively.

5.2.2. HighThroughput calcium imaging assay: TRPV1 activation assay by capsaicin

Fluorescence measurements were carried out in real time PCR thermocycler (StepOne plus, Applied Biosystem, USA), setted in relative-quantification mode. The measurements were carried out at 25°C in 13 cycles and registered each 10 seconds, using a filter for fluoresceine (483-533 nM). Fluorescence recordings were exported processed for analysis. Each measurement was background subtracted considering non-capsaicin incubated HEK-293T as base line. The relative fluorescence (F-F0/F0) was calculated for every curve. The response was then normalized with respect to the response of capsaicin-incubated cells. Data is expressed as mean value ± standard deviation, from two independent experiments (N=10).

5.2.3. Highthrouput calcium imaging assay: TRPV1 activation assay by temperature

Fluorescence measurements were carried out in real time PCR thermocycler (StepOne plus, Applied Biosystem, USA), setted in melting-curve mode. The temperature ramp (1°C / 35 seconds) from 22°C up to 56°C. Controls for background were taken at 25°C. The measurements were carried out using a filter for fluoresceine (483ex-533em). Fluorescence recordings were exported processed for analysis. Each measurement was background subtracted considering the signal from non-incubated HEK-293T at 25°C as base line. The relative fluorescence (F-F0/F0) was calculated for every curve. The response was then normalized with respect to the response obtained for transfected cells exposed at 50 °C of temperature. Normalized response to temperature was expressed as mean ± standard deviation, from two independent experiments run (N=3).

5.2.4. Dose-response Curve

Dose-response curves were as described for capsaicin and temperature activation. Transfected HEK-293T cells were loaded with Fluo-4AM and then incubated in the presence of different concentrations of each compound corresponding to 0, 1 µM; 0, 01 µM; 0, 003 µM; 0,001 µM; 0, 0003 µM, 0, 0001 µM y 0, 00001 µM. Semilogaritmic plots for the normalized fluorescence were made and then the data was fitted to a sigmoid curve of inhibition (GraphPad software, California, USA). Values of half maxima inhibitory concentration (IC_{50}) were extracted and expressed as mean ± standard deviation, from two independent experiments run (N=3).

5.2.5. Statistical Analysis

Data is expressed as mean ± standard deviation. Significant differences among groups were assessed using a one-way ANOVA test. Statistical significance was defined as $P < 0.05$. Analysis was calculated using GraphPad Prism 5 software (GraphPad software, California, USA).

5.3. Computational Methods

5.3.1. Docking

Docking computations were carried out using Arguslab 4.0.1 (Planaria Software LLC, Seattle, WA) with AScore scoring functions to find the lowest energy binding modes. The flexibility of ligands was accounted for calculates of docking. The rat TRPV1 model was used and the vanilloid binding site was defined around of Tyr 510, Met 547, Trp 549 and Thr 550, obtaining a box size of 14.51 x 10 x 24 A°. The docking experiments were carried out with the following parameters: Doking Engine: ArgusDock, Calculation Type: Dock and Number poses: 150.

5.3.2. Electrostatic Potential

The electrostatic potential were calculated using Arguslab 4.0.1 (Planaria Software LLC, Seattle, WA). First the energy of docked poses was calculated by semi-empirical PM3 method at RHF level of theory. The run type was set at SCF level with maximum 200 iterations with a convergence of 10^{-10} Kcal/mol. After the grids generated were used for obtain a map the electrostatic potential onto a surface of the electron density.

6. Acknowledgements

We thank Dr. AM Zárraga Olavarría for her technical support. The cTRPV1 clone was kindly provided by Dr. David Julius and the rat TRPV1 model was kindly provided by Dr. Narender R. Gavva. This work was supported by DIUC 210.023.046-1.0 (University of Concepcion) and FONDECYT grant 1110906 (SB). Brauchi Laboratory is part of CISNe-UACh.

7. Abbreviations

DRG: dorsal root ganglion; rTRPV1: rat TRPV-1; cTRPV1: chicken TRPV1; TLC: thin layer chromatography; Rf: retention factor.

8. Associated content

Supporting Information. Temperature ramp for capsaicin on rTRPV1, details for cell culture, transfection methods and docking molecular for 1, 3, 4-thiadiazole derivatives.

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Highlights

Several 1, 3, 4-thiadiazole alkylamides have been synthetized as conformationally restricted capsaicin analogues.

All compounds were evaluated on rat and chicken TRPV1 receptor.

1, 3, 4-thiadiazole derivatives showed to act as potent antagonist of TRPV1 receptor for capsaicin and temperature activation mode.

Supporting Information

Design and synthesis of conformationally restricted capsaicin analogues based in the 1, 3, 4-thiadiazole heterocycle reveal a novel family of transient receptor potential vanilloid 1 (TRPV1) antagonists

Carolyne Lespay Rebolledo[a], Pamela Sotelo Hitshfeld[b], Sebastián Brauchi[b] and Miguel Zárraga O.[a,*]

[a]*Department of Organic Chemistry, Faculty of Chemical Sciences, University of Concepcion, Edmundo Larenas 160C, Concepción, 1430000, Chile.*
[b]*Institute of Physiology, Faculty of Medicine, University Austral Of Chile, Campus Isla Teja 511-0566,Valdivia, 5090000, Chile.*

* Corresponding author. Tel.: +56-41-2203921; fax: +56-41-2245974; e-mail: mzarraga@udec.cl

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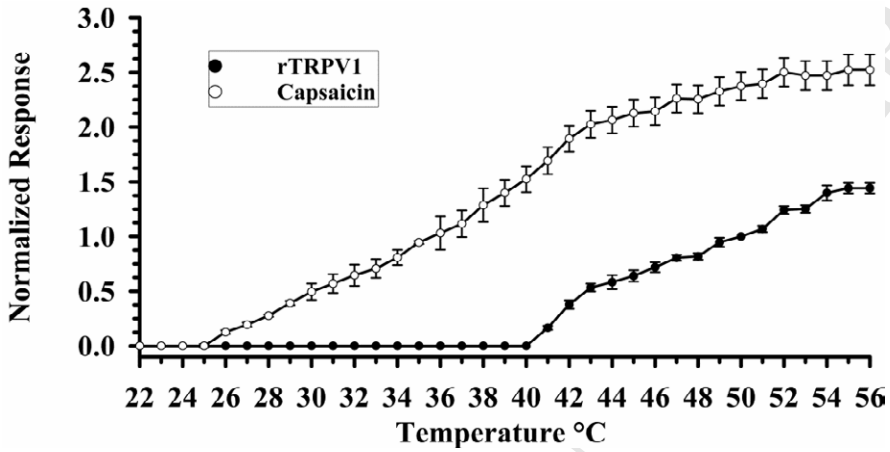
∗ Corresponding author. Tel.: +56-41-2203921; fax: +56-41-2245974; e-mail: mzarraga@udec.cl

Figure 1. Temperature-induced activation on rat TRPV1 receptors in transfected HEK-293T cells. The temperature increment response curve is obtained from cells incubated in presence and absence (rTRPV1) of 30 nM of capsaicin. Relative fluorescence normalised represent the fluorescence mean ± s. d. (n=3). Data obtained for the agonist effect of capsaicin are statistically different with respect to rTRPV1 ***P < 0.05 (One-way ANOVA test).



Biological procedures

Cell Culture.

HEK-293T cells was cultured in a DMEM medium, supplemented with 5% of fetal bovin serum (HyClone), 50 U/ml penicillin (HyClone), 50mg/ml streptomycin (HyClone) and 2mM L-glutamin (GIBCO). The cultures were supporting to 37°C temperature, in an ambient of 5% CO_2 and 80% de relative humidity.

DNA Transfection

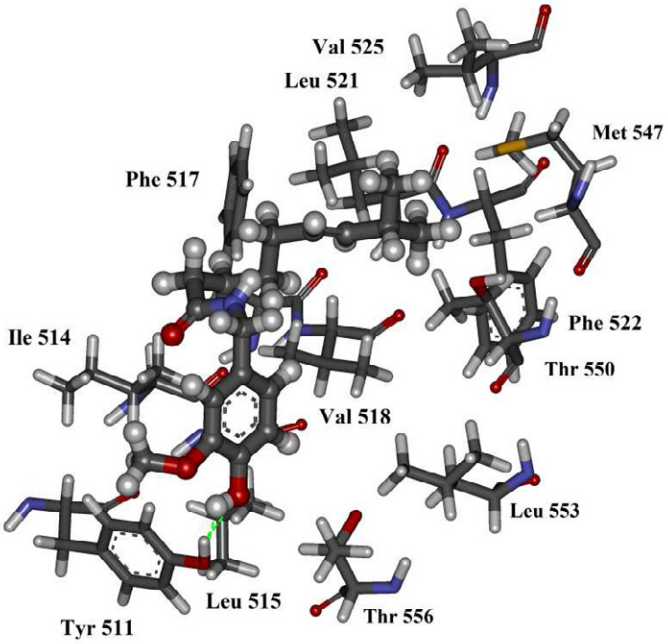
HEK-293T cells were transfected with pcDNA3 TRPV-1 of rat using Lipofectamine 2000 reagent (Invitrogen). In a tube was added 97 µL Optimem (Gibco) and 3 µL de Lipofectamine 2000, in a second tube was added 1 µg of DNA and a volume of Optimen until complete 100 µL. Both were incubate for 10 minutes. After the content of both tubes was mixed and incubated for another additional 15 minutes additional. The mixture was added to RT-PCR plaques of 60 mm with a confluence of 60% of cells and work with them 48hrs post transfection. Furthermore, HEK-293T cells were transfected with pcDNA3 TRPV1 of chicken using Mirus Transfection Reagent (Mirus) according with manufacture instructions. The mixture was added to plaques of 60 mm with a confluence of 60% of cells and work with them 48 hrs post transfection.

Loading cells with Fluo-4AM

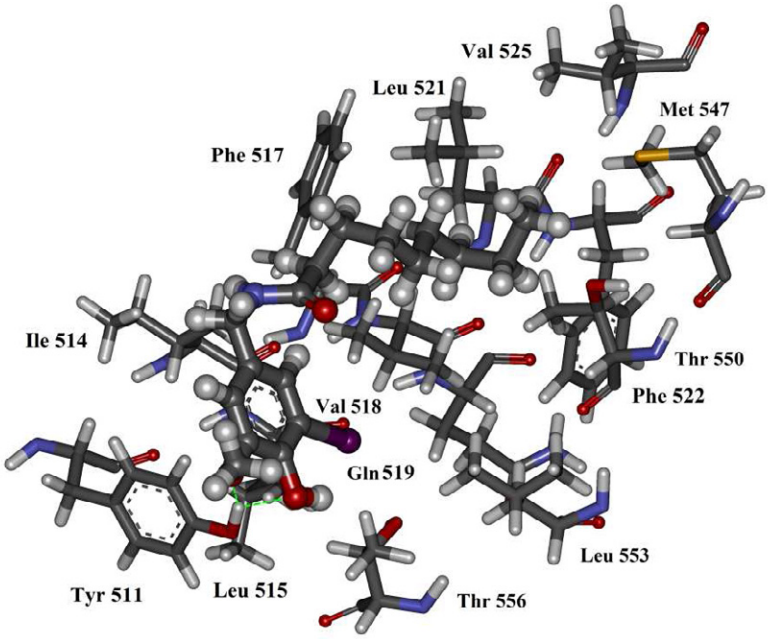
After 48 hours of transfection, cells were treated with 200 µL of trypsin, it incubates for 5 minutes at room temperature, and then cells were soft pipetted and to transferred to a falcon tube of 15 mL and then were centrifuged to a speed of at 6000 rpm for 10 minutes. After this cells were resuspended in 1 ml Ringer buffer (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Glucose, 15 mM de HEPES 7.35 pH, 292 mOSM) , and then 1 µl Fluo-4AM (Invitrogen) 1000X was added and incubated for 30 minutes at 37ºC. Later cells were centrifuged and washed 3 times with PBS ringer buffer 1X, pH 7.34 for remove excess of fluorescent probe. Finally cell pellet was resuspended in 1.5 ml Ringer buffer.

Docking Molecular into TRPV1 receptor

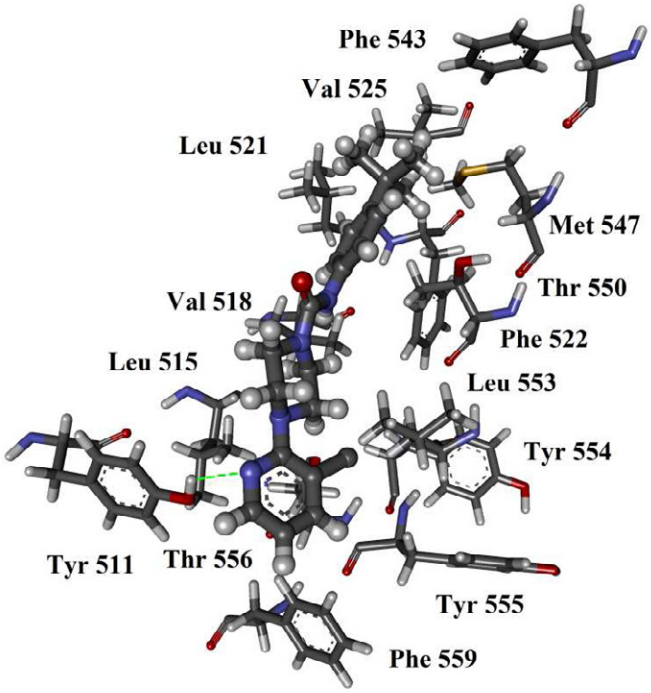
a) docked pose for capsaicin



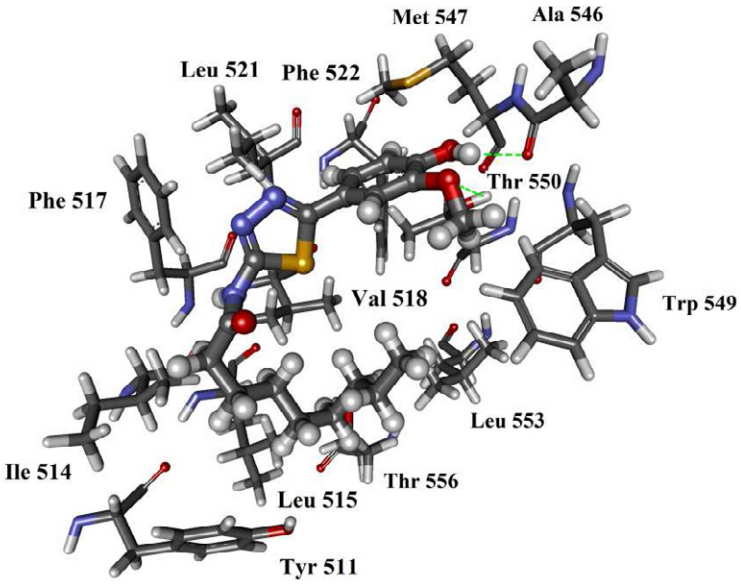
b) docked pose for 5-iodononivamide



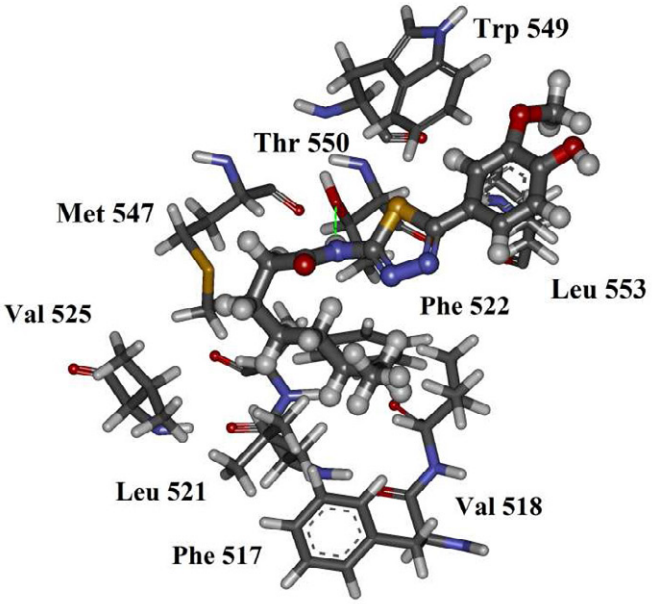
c) docked pose for BCTC



d) docked pose for 1, 3, 4-thiadiazole derivative 8



e) docked pose for 1, 3, 4-thiadiazole derivative 9



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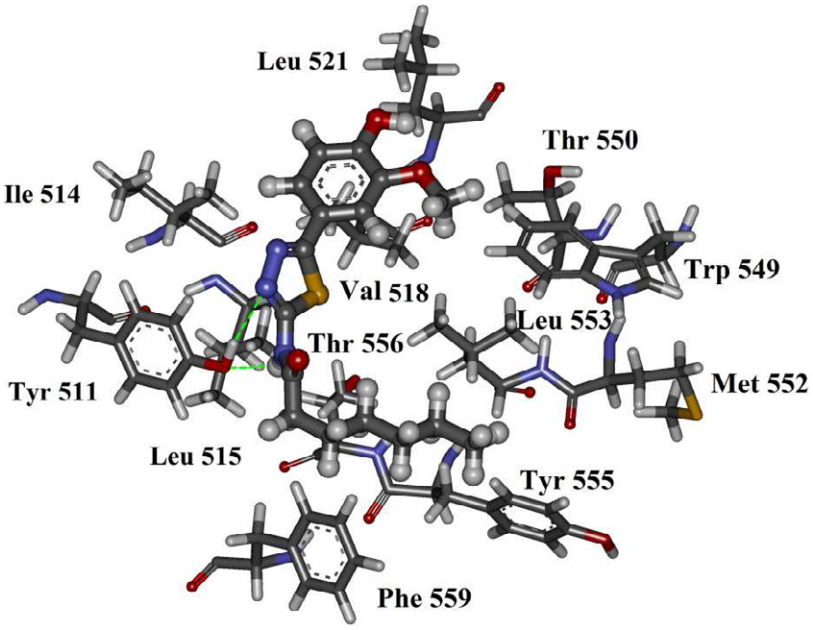
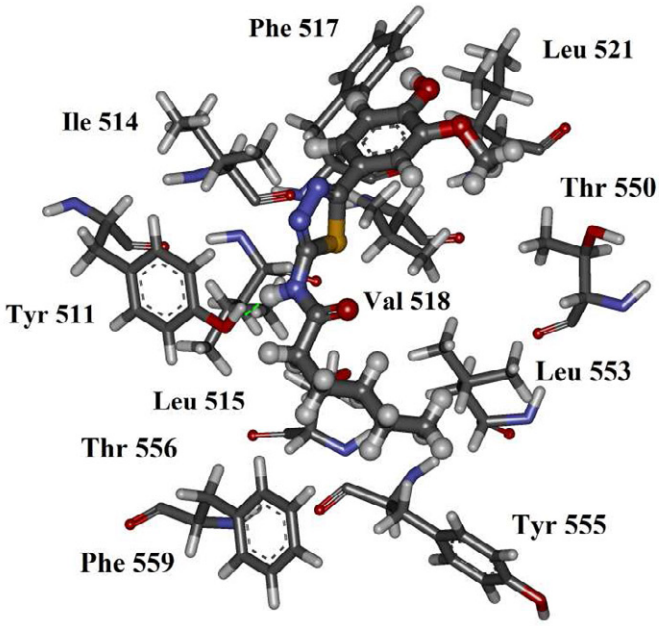


g) docked pose for 1, 3, 4-thiadiazole derivative 11



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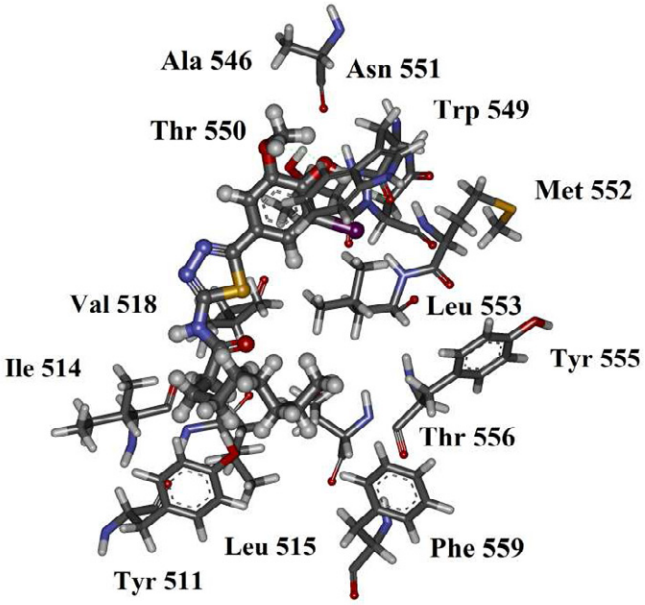

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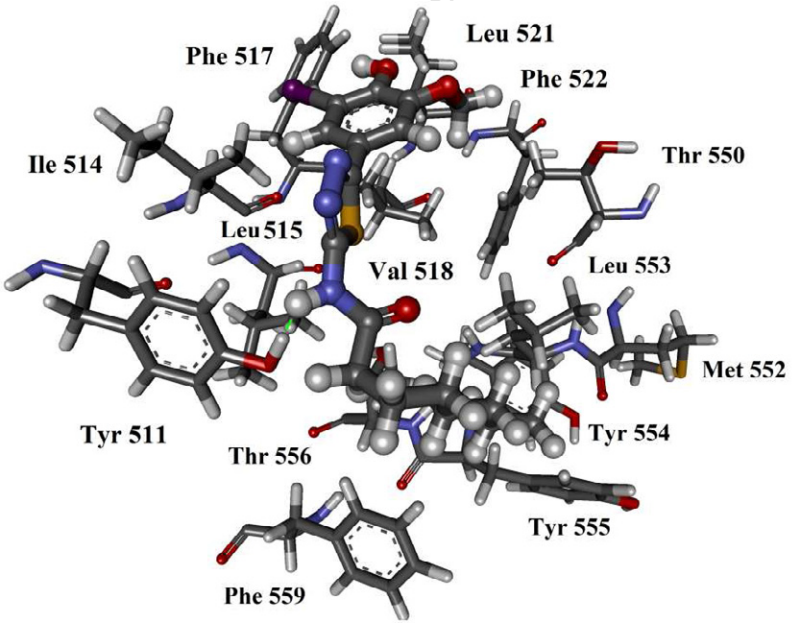
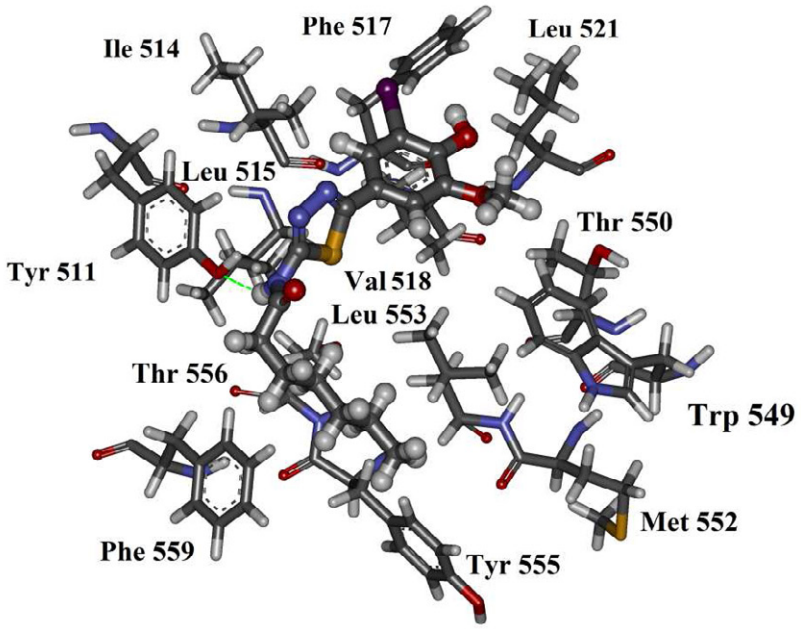
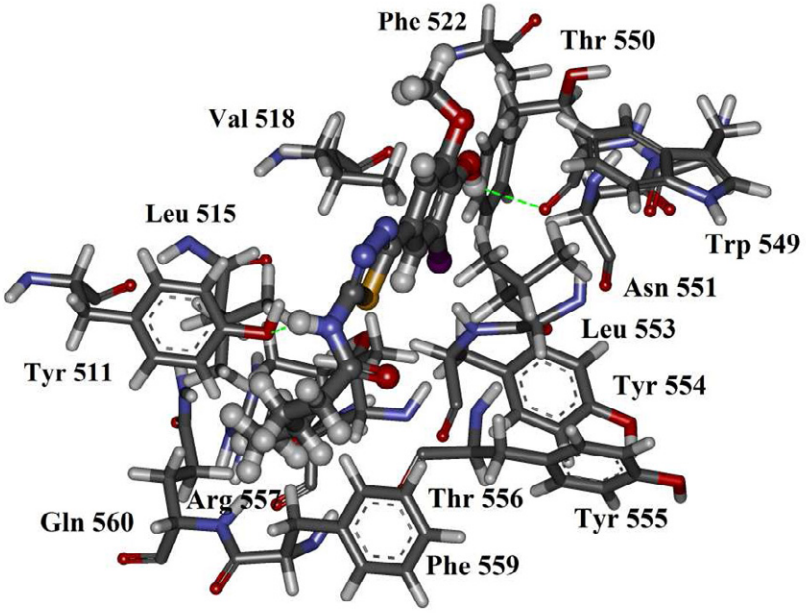


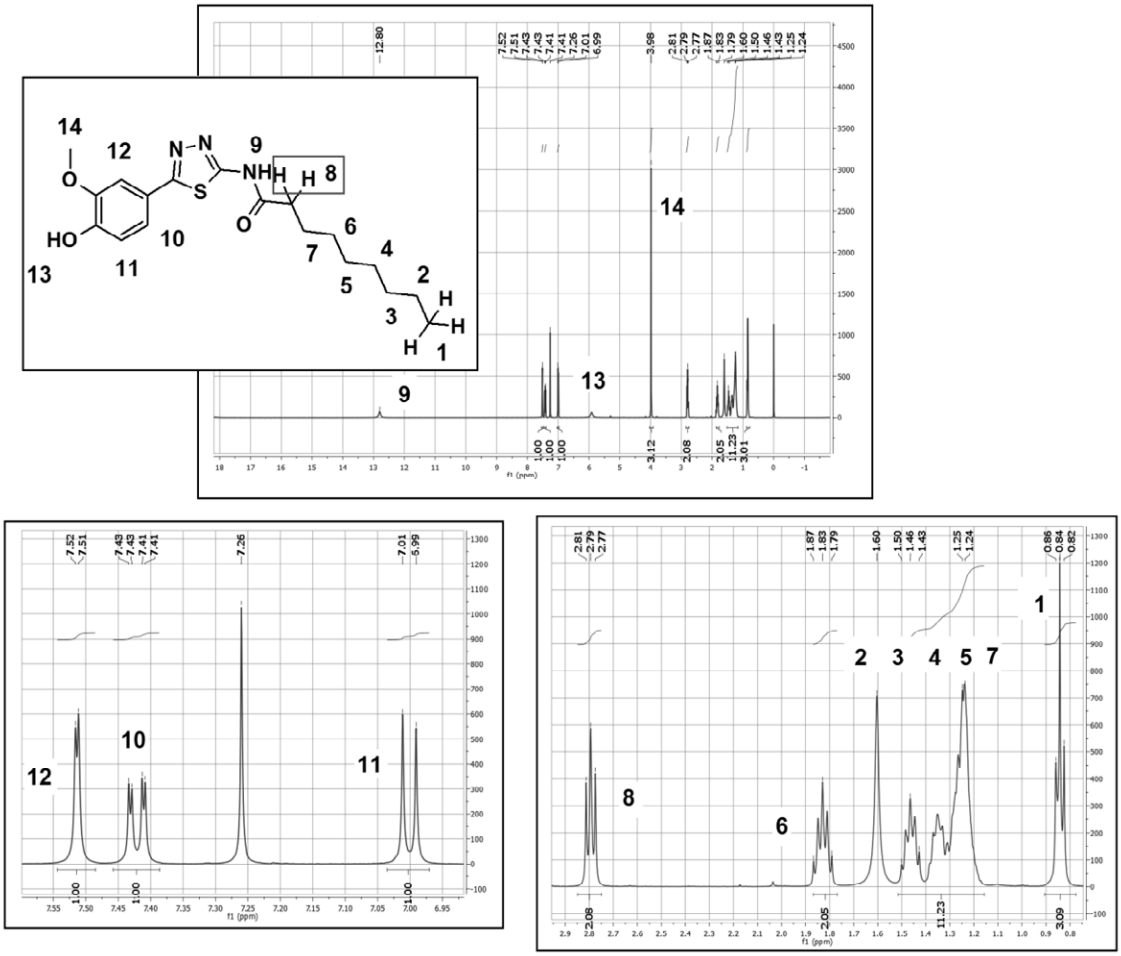
j) docked pose for 1, 3, 4-thiadiazole derivative 14



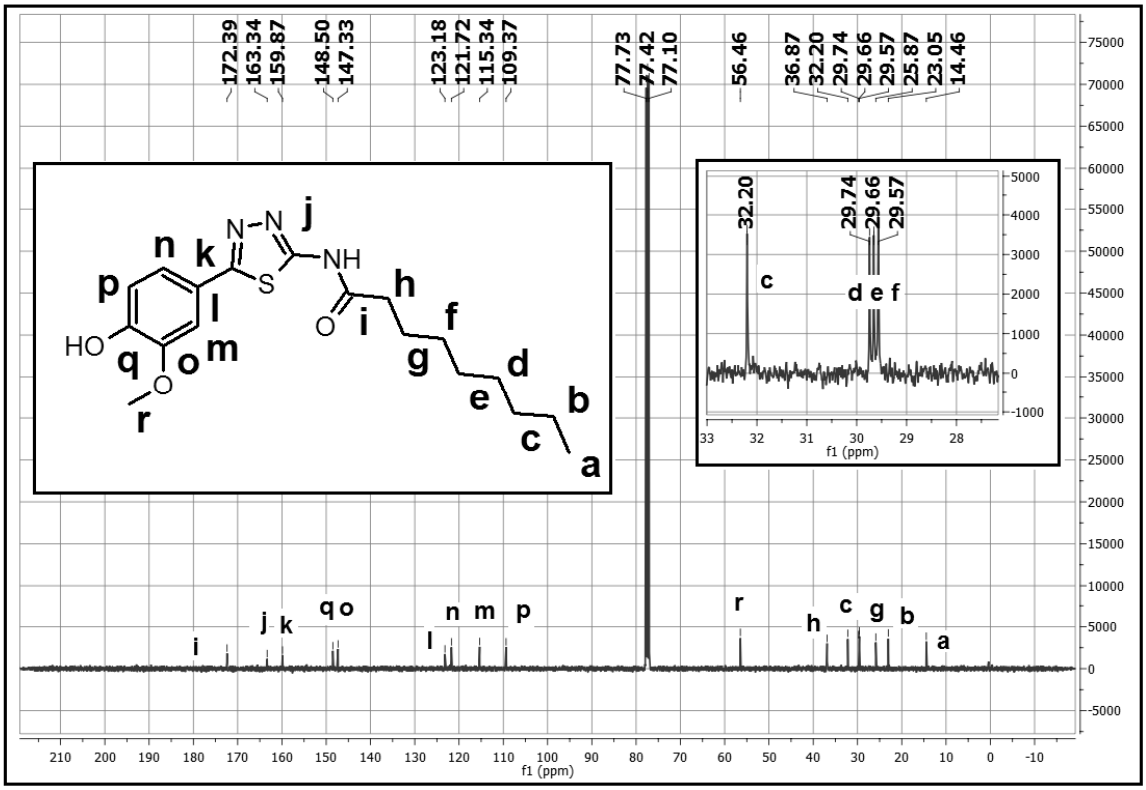
k) docked pose for 1, 3, 4-thiadiazole derivative 15



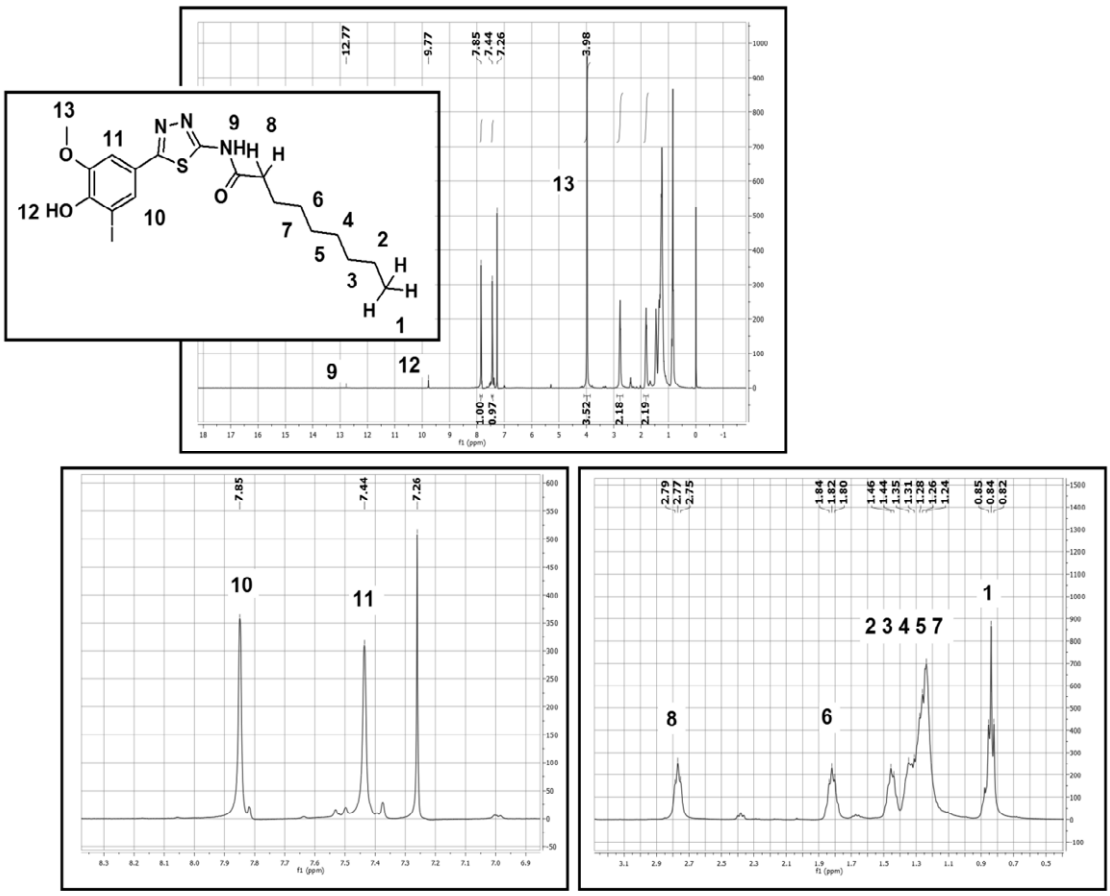
H^1-NMR Spectrum of compound 8



C^{13}-NMR spectrum of compound 8



H[1]-NMR Spectrum of compound 12



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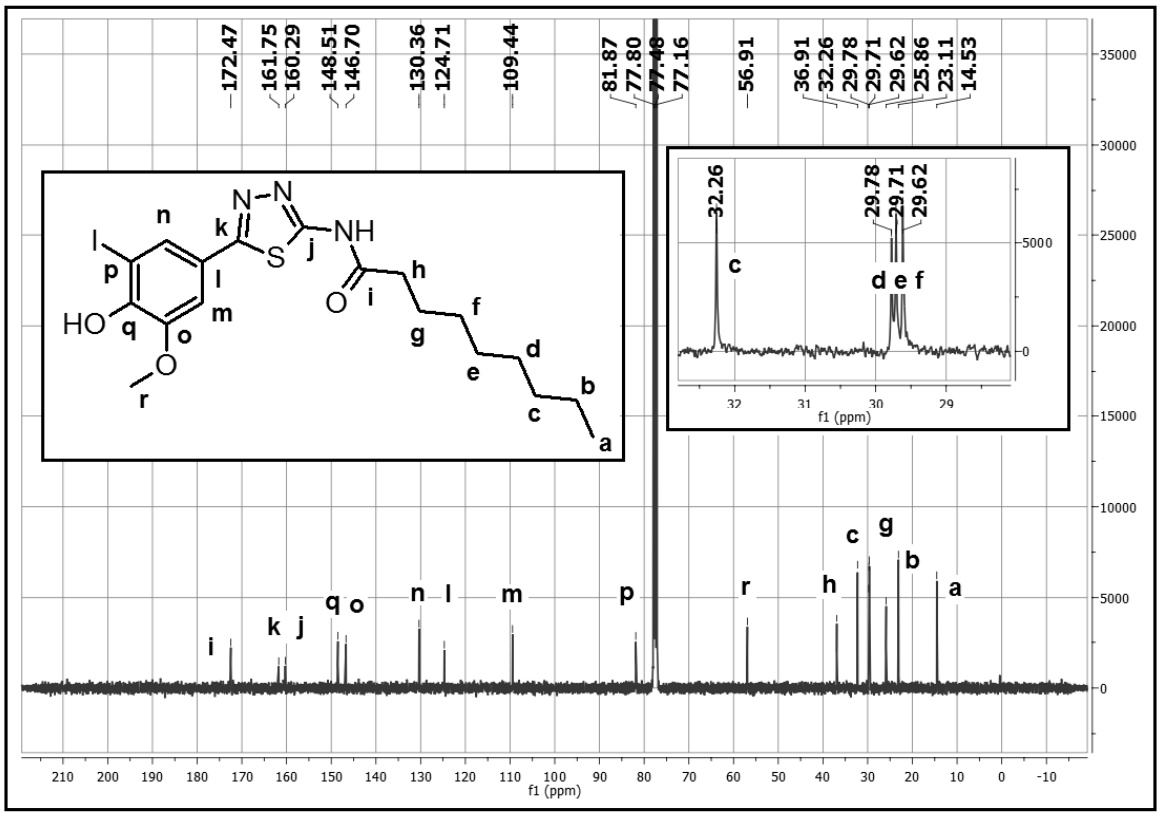

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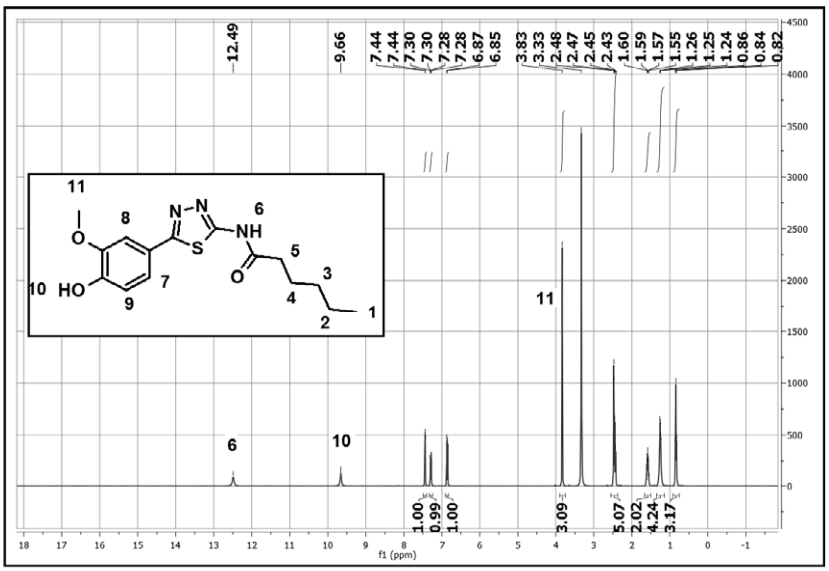
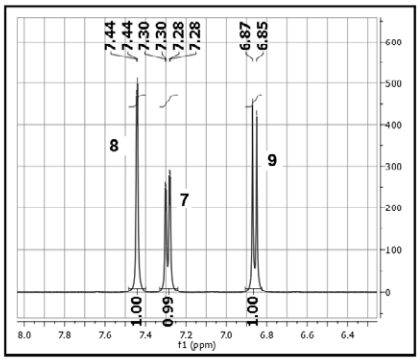
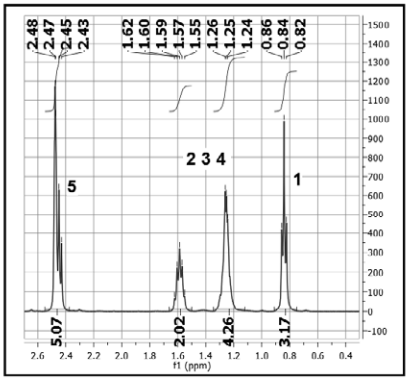
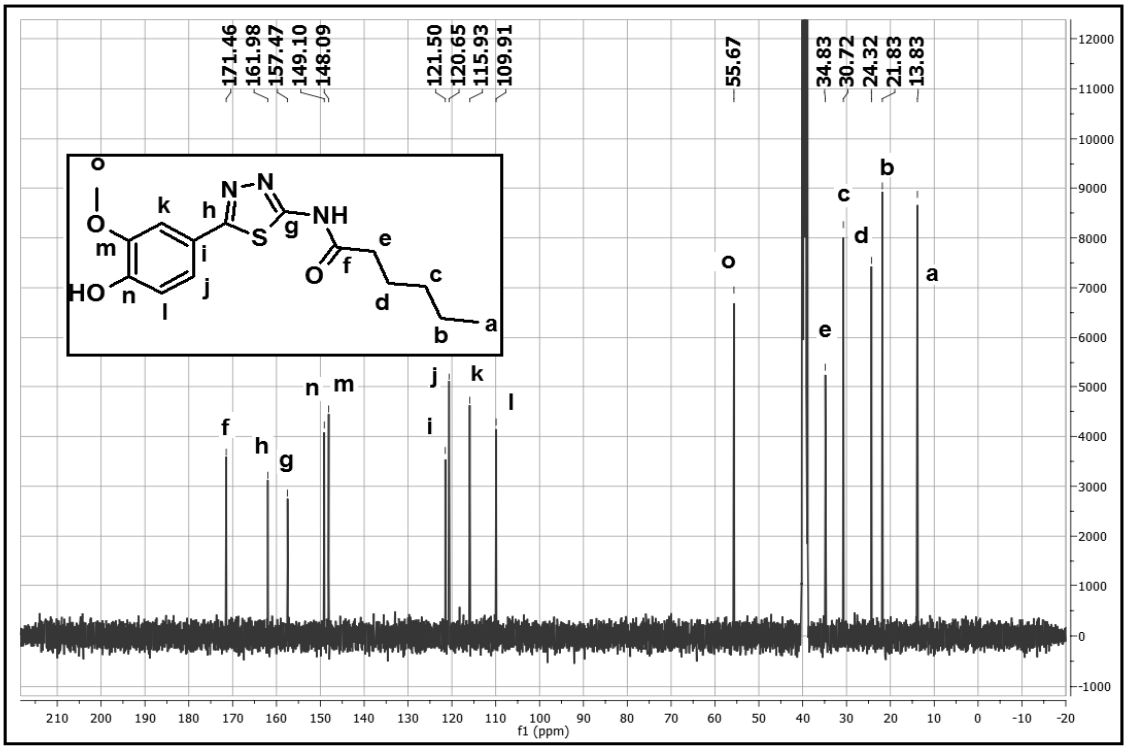
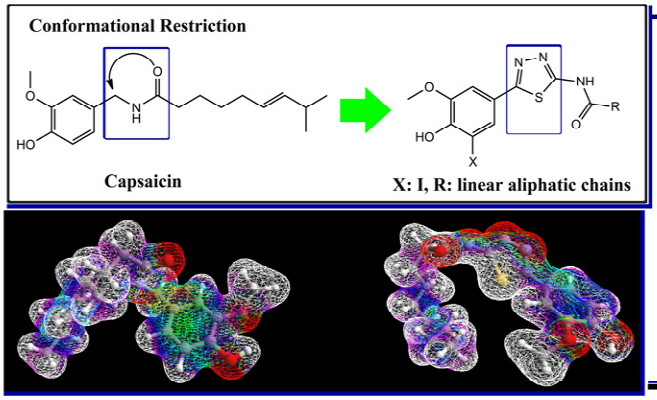


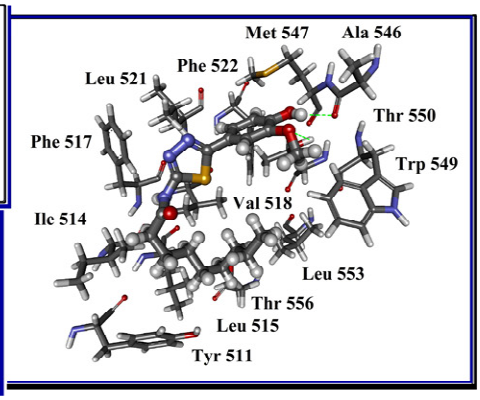




C^{13}-NMR Spectrum of compound 11







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