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ARTICLE

# New transient receptor potential TRPV1, TRPM8 and TRPA1 channel antagonists from a single linear β,γ-diamino ester scaffold

Received 00th January 20xx,

Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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A high throughput screening campaign identified nine β,γ–diamino ester derivatives as TRP modulators. A discrete library of new derivatives (23 components) was prepared in one-pot two step reductive amination reaction, and evaluated for their ability to block the agonist-induced calcium influx in cells expressing human TRPV1, TRPM8 and TRPA1 channels. Selective antagonists for each channel, as well as dual TRPV1/TRPM8 and TRPM8/TRPA1 ligands, were obtained after subtle modification of this linear scaffold. SAR studies revealed the preferred substituents for the selective blockade of the three TRP channels under study. The most potent TRPV1 antagonists displayed submicromolar $IC_{50}$ values.

## Introduction

Membrane transient receptor potential (TRP) proteins are voltage- and ligand-gated ion channels with a variety of biological functions and apparently implicated in diverse pathological conditions. According to sequence similarities, mammalian TRP channels comprise six subfamilies with low sequence identity among them (as low as 20%), named TRPA (ankyrin), TRPC (canonical), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin), and TRPV (vanilloid).[1]

A few members of the TRP family (TRPV1-4, TRPM8 and TRPA1), belonging to three different subfamilies, are the so-called thermoTRP channels, which participate in the detection of temperature changes and also integrate different noxious stimuli.[2] Thus, TRPV1 is a non-selective $Ca^{2+}$ channel activated by noxious temperatures (>43ºC), acidic pH and vanilloid compounds. TRPV1 expression is overregulated under acute inflammatory states[3-5] and in chronic pain conditions,[6] and its activity is potentiated by proalgesic mediators after inflammation and tissue injury.[7, 8] TRPM8 channels have a physiological role in detecting low temperature (10-33 °C),[9] and are also over-expressed in sensory neurons after nerve injury or inflammation,[10] as well as involved in cold allodynia and hyperalgesia[11]. TRPA1 is also a non-selective $Ca^{2+}$ channel activated by multiple stimuli, including harmful cold temperatures, acids, and numerous chemical pollutants.[12-15] TRPA1 receptors are coexpressed with TRPV1 channels in C-fiber sensory neurons,[16] and seems to have a crucial role in neuronal and non-neuronal neuropathic pain [17-19].

Since patients with inflammatory or neuropathic pain suffer from hypersensitivity to mechanical, thermal and/or chemical stimuli, an approach to develop successful analgesic therapies may be to target TRPV1, TRPM8 and TRPA1 nociceptors.[20] However, despite the big number of compounds currently available in this field, their poor specificity and side effects justify the need for new compounds.[21] A few representative modulators of these three types of TRP channels are depicted in Chart 1. Knowledge about the molecular requirements that makes a particular family of compounds to bind to one or more of these channels is still scarce and, in general, the described compounds for every TRP channel are structurally very different among them.[21]

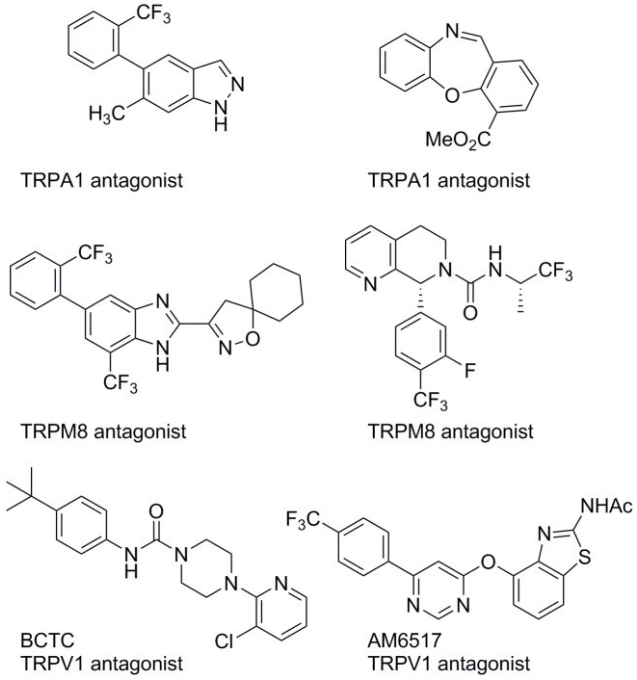


Chart 1. Representative TRPA1, TRPM8 and TRPV1 antagonists

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Electronic Supplementary Information (ESI) available: [Protocol for the evaluation of compound **4b** in the CFA-induced paw inflammation model and obtained results]. See DOI: 10.1039/x0xx00000x

Within our programs of innovative chemical libraries we have recently described a series of β,γ–diamino ester derivatives **1-4** as valuable building block intermediates to heterocyclic compounds (Chart 2).[22] Following our interest in TRP modulators,[23-26] some of these derivatives were evaluated in our HTS screening platform for the search of new TRP channel ligands. Within them, we found either selective antagonists for TRPV1 and TRPM8 or dual TRPM8/TRPA1 blockers, although they are very closely related compounds. To explore further the interest of these β,γ–diamino ester derivatives as TRP ligands, and to look deeply into the structural particularities behind the TRP channel type preferences, in this paper we describe the results of the biological evaluation of compounds **1**-**4**, along with the preparation and HTS screening of new derivatives within this series.

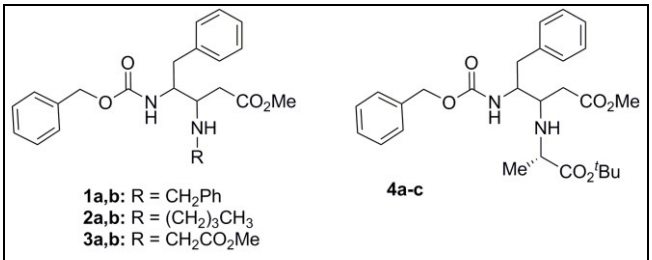


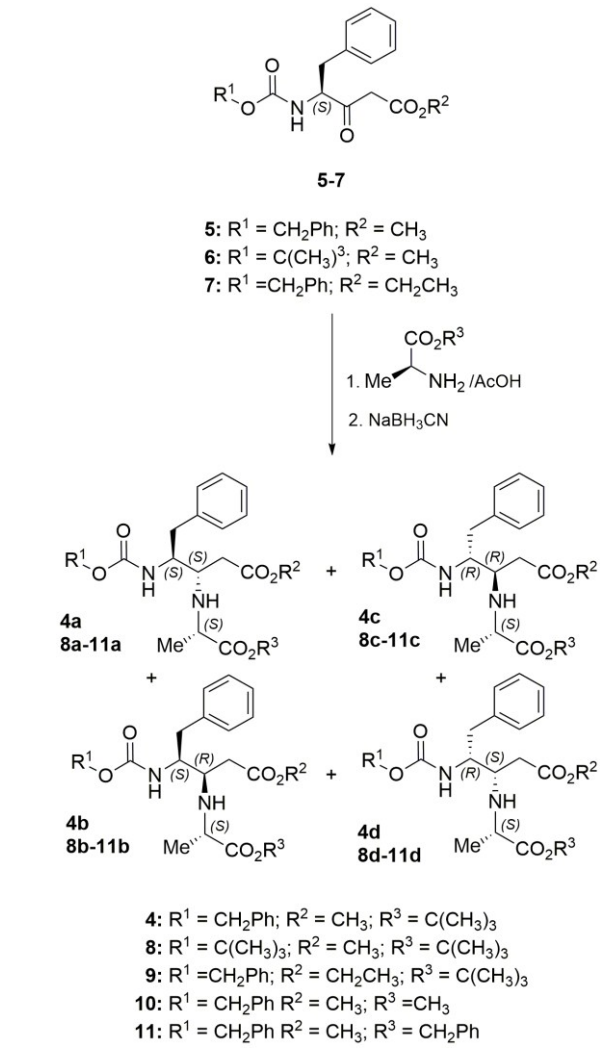

Chart 2. β,γ–Diamino ester derivatives previously synthesized by us

## Results and discussion

### Chemistry

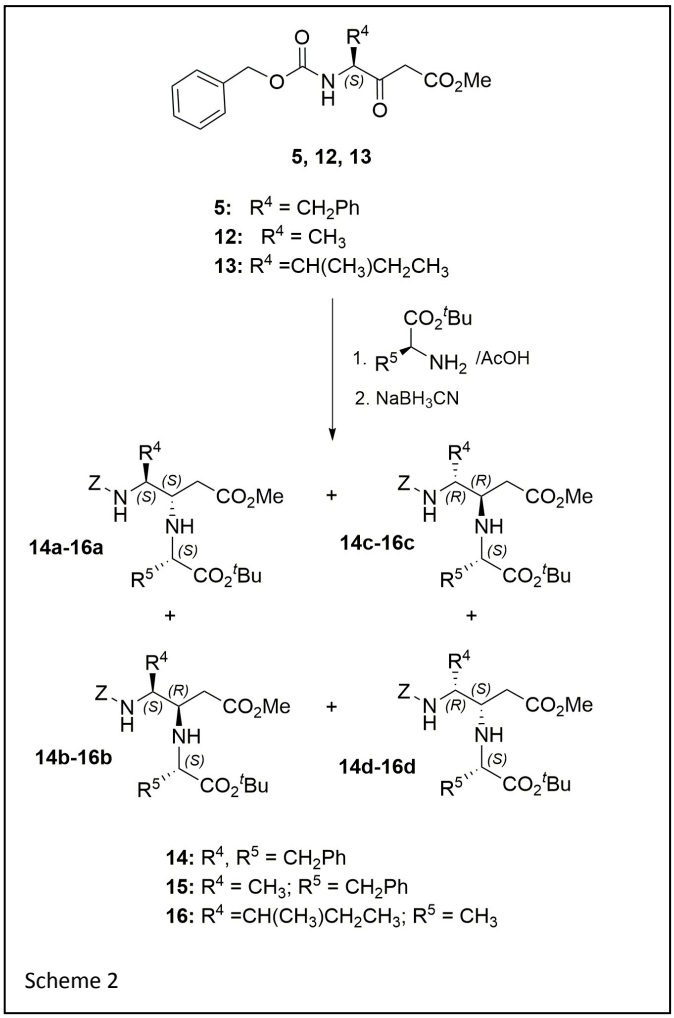
To understand the role of the benzyloxycarbonyl (Z) and ester groups in **4**, we first aim to prepare the Boc analogue **8** and some ester variations, **9**-**11**. Highly functionalized β,γ–diamino esters **8**-**11**, derived from Phe and Ala, were prepared following our previously described method for analogues **4a-c**.[22] Briefly, the reaction of β–ketoesters **5-7** with the corresponding Ala-derived α–amino esters in the presence of AcOH led to imine intermediates, which were then reduced with $NaBH_3CN$. This two-step reductive amination procedure afforded compounds **4**, **8**-**11** as mixtures of diastereoisomers that, in most cases, can be separated by column chromatography. As described, three isomers had been isolated for compound **4** (**a**-**c**), and their configurations had indirectly been determined through transformation to pyrrolidinone derivatives.[22] Similarly, the formation of three major stereoisomers was observed in the case of compound **8**-**10**, where only traces of a fourth diastereosiomer could be detected by HPLC-MS (not isolated). However, for the Ala-OBn derivative **11**, all four possible diastereoisomers were formed, although isomers **11c** and **11d** could not be separated in pure form. In each case the configurational assignment was performed by comparison of NMR and HPLC data with those of **4a**-**c** (Scheme 1).

The loss of the stereochemical integrity of the initial amino acid-derived β–ketoester, leading to diastereoisomers **c** and **d**, could be explained through the existence of imine-enamine tautomerism, as previously determined by reduction with $NaBD_3CN$.[22]

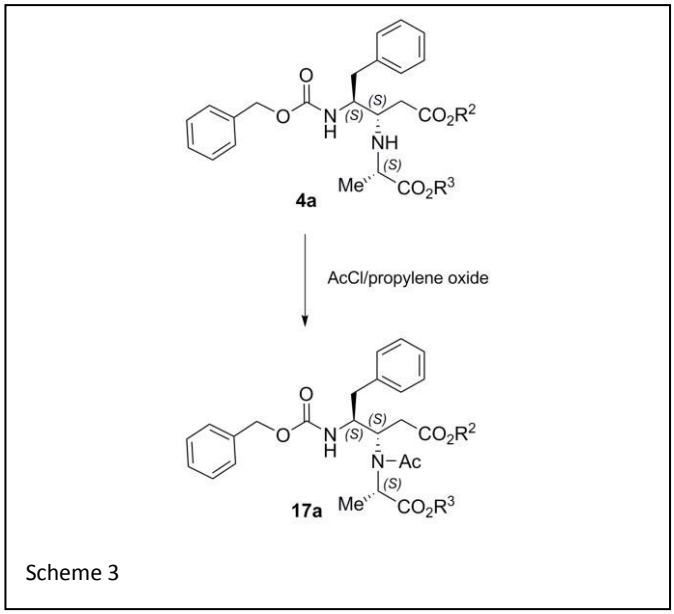



Scheme 1

To set light on the importance of the starting amino acids side chains, we then prepared compounds **14-16**, in which the initial Ala-OtBu derivative was changed by Phe-OtBu, and the Z-Phe-derived ketoester was substituted by those resulting from Z-Ala and Z-Ile. Phe-Phe-derived compound **14** was obtained as a 23:44:23:10 mixture of **a**-**d** diastereoisomers, with **14c**-**14d** isolated as an inseparable mix. However, three isomers were detected for the Ala-Phe analogue **15**, although only isomers **15a** and **15b** were separated in pure form. Four main isomers were obtained in the case of compound **16**, from which isomers **a**, **b** and **d** could be totally separated. As the Ile residue has an additional stereogenic center, other minor isomers were detected by HPLC and in certain NMR fractions (not isolated). As indicated above, the configuration assignment was tentatively done by comparison with isomers **4a**-**c**.




Scheme 2




Scheme 3

Finally, to explore the importance of the 3-NH group, we prepare the corresponding acetil derivative **17a** by treatment of compound **4a** with acetyl chloride in the presence of propylene oxide as HCl scavenger.

**Biological Evaluation**

All compounds were assayed on TRPV1, TRPM8 and TRPA1 channels, stably expressed in the appropriate cell lines (see experimental for details). The agonist-induced intracellular $Ca^{2+}$ signals were measured by microfluorography, using a fluorescence plate reader, in the absence and in the presence of test compounds. Capsaicin (TRPV1), menthol (TRPM8) and allylisothyocyanate (TRPA1) were used as the respective agonists. The obtained results were compared toward those of AMTB (TRPM8 antagonist) and ruthenium red (TRPV1, TRPA1 unspecific antagonist). The obtained results are recorded in Tables 1 and 2.

As shown in Table 1, N-benzyl and N-n-butyl derivatives **1a,b** and **2a,b** showed an interesting TRPM8 blockade activity (up to 91 and 60 % inhibition of the menthol-induced channel activation at 50 and 5 µM, respectively). In addition, at the indicated concentrations, they are quite selective against TRPV1 (significant blockade was observed only at the high concentration) and especially against TRPA1. Interestingly, Gly analogues **3a,b** at the lower concentration (5 µM) decreased TRPM8 activity but were able to nicely block TRPA1 channels. It seems that the incorporation of a polar, H-accepting ester moiety favors TRPA1 recognition, while more hydrophobic residues (Bn, Bu) are preferred at the TRPM8. Interestingly, Ala-derived compounds **4a-c** behave as potent and selective TRPV1 antagonists. Thus, a minor modification of compound **3**, by the incorporation of a small Me group in **4**, gave to a shift in the selectivity, suggesting that this group could occupy a cavity in TRPV1 channels that is not present/accesible in TRPM8 and TRPA1. In general, each distereoisomer of the same compound displayed very similar activities and selectivities. It is interesting to note that the initial ketoester **1** (5 µM) did not show any significant antagonist activity on the channels under study (data not shown).

Modifications at the different parts of molecule **4** also provided us with valuable structural information. Thus, the benzyloxycarbonyl group in **4a,c** might be interchanged by a *tert*-butoxycarbonyl moiety in derivatives **8a,c** without apparent loss of TRPV1 antagonist activity, and similar selectivities. However, removal of the urethane moiety and cyclization to the corresponding pyrrolidinone derivatives led to inactive compounds in all studied TRP channels (data not shown), telling us on the importance of a hydrophobic substituent on the Phe nitrogen of **4** and **8**.

When the $R^2$ substituent in **4** (Me) is changed by an ethyl group in **9**, all diastereoisomers were able to maintain the potent TRPV1

antagonist properties, but an increase in the TRPM8 blocking activity was also detected, specially at the higher concentration. Therefore, Et-derivatives **9** can be considered as dual TRPV1/TRPM8 antagonists.

Concerning the $R^3$ substituent, different results were obtained when the *tert*-butyl group was replaced by its methyl (**10**) or benzyl (**11**) counterparts. While Me derivatives **10a-c** were mainly inactive in the three TRP channel assayed, Bn analogues **11a-c** lost the TRPV1 antagonist activity, although they showed a significant ability to inhibit the $Ca^{2+}$ entry through TRPM8 and TRPA1 channels, upon activation with their respective agonists. A similar dual TRPM8/TRPA1 antagonist activity was observed in the case of compounds **14a,c,** in which a Phe-OtBu residue was incorporated instead of the Ala-OtBu in **4**. The fact that Phe-OtBu and Ala-OBn derived compounds **11** and **14** showed similar activity/selectivity profile could associate the presence of an aromatic ring by this part of the molecule with a preference for TRPM8 and TRPA1 channels.

The role of the $R^4$ benzyl group is not negligible, since compounds with the reverse sequence **15a-b** were only able to maintain certain TRPV1 and TRPM8 antagonist activity at high concentrations, and completely loss it for TRPA1 channels. The importance of the phenyl group of this benzyl moiety was corroborated by the lack of activity of the Ile analogue **16b** at 5 μM concentration, compared to **4b**.

Finally, the decreased TRPV1 antagonist activity of the acetyl derivative **17a**, compared to its free NH analogue **4a**, suggests a possible direct participation of the NH group either in a saline bridge or in an H-bond formation.

As mentioned previously, no big differences in activity were found among diastereoisomers of the same compound, suggesting that the pocket within the studied TRP channels responsible for the interaction with this family of compounds is quite big, allowing different spatial arrangements to accommodate.

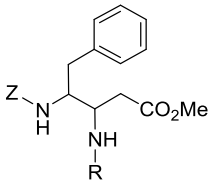
To further validate the TRP-blocking activity for the apparently most potent and selective TRP antagonists, the corresponding dose-response curves were obtained for selected compounds. Table 3 sumarizes the $IC_{50}$ values of N-benzyl derivatives **1a-b** for TRPM8 receptors, Gly-analogue **3a** for TRPA1, and Ala-derived compounds **4a-c** and **8a-c** against TRPV1 channels. TRPM8 selective antagonists **1a** and **1b** show similar micromolar $IC_{50}$ values, regardless of the configuration at C3. A related value was obtained for the TRPA1 antagonists **3a**. Diastereoisomeric Phe-Ala diamino esters **4** and **8** display micromolar or submicromolar $IC_{50}$ values for TRPV1, quite similar among them, with compound **4b** showing the highest potenty. Although the configuration does not play a crucial role in the antagonist activity, confirming previous results, the 3*R*,4*S*,1'*S* diasteroisomers seems to give to slightly higher potencies.

In *in vivo* experiments, compound **4b** produced some elevations of PWL in the plantar test, at a 2 mg/Kg dose ip (see ESI, Fig S1). Although not statistically significant, it seems that this compound could decrease the thermal hyperalgesia induced in mice by CFA injection. However, no positive signs of activity were observed in the mechanical von Frey test (mechanical hypersensitivity) at this dose. Unfortunately, we could not evaluate the effect at higher doses due to low solubility issues.

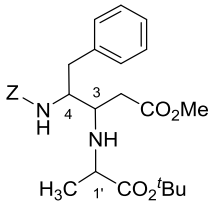
## Conclusions

In summary, medicinal chemistry efforts initiated from a HTS of synthetic intermediates resulted in the discovery of new selective hits for TRPV1, TRPM8 and TRPA1 blockade, just by incorporating minor changes in the structure of a single β,γ-diaminoester linear scaffold. Compounds were prepared by a two-steps reductive amination process that afforded separable mixtures of diastereoisomers. Starting with Phe-derived β–ketoesters, the SAR demonstrated that the addition of simple 3-NHBn or 3-NHBu substituents resulted in TRPM8 antagonists. Compounds including a second amino acid residue indicated that 3-Gly-OMe-containing derivatives are selective TRPA1 antagonists (**3**), while the corresponding 3-Ala-OtBu analogues are potent and selective TRPV1 blockers (**4**). An increase in the hydrophobicity around the $R^2$ substituent enhanced the ability to block the TRPM8 activation, while keeping the TRPV1 antagonists activity, thus resulting in dual TRPV1/TRPM8 blockers. At $R^3$, a *tert*-butyl ester is clearly preferred for the vanilloid channel while Bn analogues are better for theinhibition of the agonist-activation of both TRPM8 and TRPA1 channels. Dual TRPM8/TRPA1 blockers were also found when the 3-Ala-OtBu moiety was substituted for the more hydrophobic 3-Phe-OBn residue. Concerning the stereochemistry, no strict requirements were observed, with all *SSS*, *RSS* and *SSR* configurations allowed (slightly better results for the *RSS* diastereoisomers). Although further pharmacological characterization of compounds within this series is limited by their low solubility, and probably poor pharmacokinetics, due in part to the high number of rotable bonds, this work allowed the identification of substituents and amino acid residues that led to selective modulators of the indicated three types of TRP channels. Attaching these particular combinations of substituent on more rigid scaffolds that allowed different 3D-arrangements could serve to discover new potent and selective TRP blockers with improved PK profile. Efforts in this direction are in progress in our labs.

Table 1. Activities of compounds **1**-**4** against TRPV1, TRPM8 and TRPA1 (Expressed as % Blockade at the indicated concentrations)




**1,2,3**

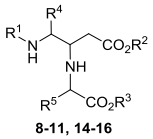



**4**

| Compd. | Config. 3,4 or 3,4,1' | R | Concentration (μM) | TRPV1[a] | TRPM8[b] | TRPA1[c] |
|---|---|---|---|---|---|---|
| **1a** | *S,S* | $CH_2Ph$ | 50<br>5 | 31,50 ±16,63<br>29,48 ± 6,46 | 89,08 ± 5,40<br>57,54 ± 1,67 | NA |
| **1b** | *R,S* | $CH_2Ph$ | 50<br>5 | 65,05 ± 2,72<br>14,60 ±24,26 | 91,89 ± 4,07<br>60,66 ± 5,34 | NA |
| **2a** | *S,S* | $(CH_2)_3CH_3$ | 50<br>5 | 89,68 ±11,88<br>21,11 ± 8,00 | 74,93 ± 3,11<br>52,98 ± 6,46 | NA |
| **2b** | *R,S* | $(CH_2)_3CH_3$ | 50<br>5 | 54,95 ± 3,51<br>15,14 ± 7,65 | 83,91 ± 4,42<br>56,79 ± 6,11 | NA |
| **3a** | *S,S* | $CH_2CO_2Me$ | 50<br>5 | 57,38 ± 3,59<br>22,00 ± 3,51 | 84,36 ± 5,41<br>37,83 ±11,03 | 80,14 ± 14,67<br>70,34 ± 13,25 |
| **3b** | *R,S* | $CH_2CO_2Me$ | 50<br>5 | 84,22 ± 9,08<br>24,46 ±15,73 | 79,51 ± 4,71<br>36,96 ± 14,30 | 60,86 ± 10,71<br>66,87 ± 17,04 |
| **4a** | *S,S,S* | – | 50<br>5 | 99,19 ± 12,10<br>73,50 ± 9,62 | NA | NA |
| **4b** | *R,S,S* | – | 50<br>5 | 109,28 ± 6,54<br>72,25 ±11,10 | 62,29 ± 10,46<br>-15,13 ± 19,57 | NA |
| **4c** | *R,R,S* | – | 50<br>5 | 106,76 ± 9,82<br>49,91 ± 22,57 | 57,45 ± 22,47<br>-8,20 ± 17,97 | NA |
| **Ruthenium red** | | – | 10 | 100% | | 100% |
| **AMTB** | | – | 10 | | 100% | |

[a] Blockade of Ca2+ entry trough TRPV1 channel by peptides (Capsaicin, 10 μM, was used as the agonist). b Blockade of $Ca^{2+}$ entry trough TRPM8 channel by peptides (Menthol, 100 μM, was used as the agonist). c Blockade of Ca2+ entry trough TRPA1 channel by peptides (allyl isothyocyanate, 500 M, was used as the agonist). c Compounds were assayed at 50 μM (up) and 5μM (down) concentrations. NA: blockade lower than 25% at the higher concentration assayed.

Table 2. Activities of compounds **8**-**11** and **14**-**17** against TRPV1, TRPM8 and TRPA1
(Expressed as % Blockade at 50 and 5 μM concentrations)




**8-11, 14-16**

| Compd. | Config. 3,4,1' | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | TRPV1[a] | TRPM8[b] | TRPA1[c] |
|---|---|---|---|---|---|---|---|---|---|
| **8a** | *S,S,S* | **Boc** | Me | $^t$Bu | Bn | Me | 106,76±9,82<br>52,12±12,39 | 88,55±13,60<br>14,64±8,42 | NA |
| **8b** | *R,S,S* | **Boc** | Me | $^t$Bu | Bn | Me | 103,46±8,27<br>62,00±10,03 | 33,14±21,05<br>9,56±2,95 | NA |
| **8c** | *R,R,S* | **Boc** | Me | $^t$Bu | Bn | Me | 89.97±2.62<br>49.45±4,86 | 38,96±13,62<br>8,34±11,67 | NA |
| **9a** | *S,S,S* | Z | **Et** | $^t$Bu | Bn | Me | 88,30±8,70<br>74,58±5,24 | 85,72±5,01<br>70,77±5,49 | NA |
| **9b** | *S,R,S* | Z | **Et** | $^t$Bu | Bn | Me | 95,83±5,81<br>72,35±17,12 | 87,90±6,25<br>39,78±7,97 | NA |
| **9c** | *R,R,S* | Z | **Et** | $^t$Bu | Bn | Me | 92,95±12,14<br>50,49±16,58 | 96,97±6,91<br>40,51±8,85 | NA |
| **10a** | *S,S,S* | Z | Me | **Me** | Bn | Me | 75,53±1,24<br>23,74±15,03 | 57,83±5,92<br>13,48±7,33 | NA |
| **10b** | *R,S,S* | Z | Me | **Me** | Bn | Me | 28,41±12,41<br>9,65±11,52 | 51,83±8,30<br>16,14±16,27 | NA |
| **10c** | *R,R,S* | Z | Me | **Me** | Bn | Me | NA | 46,54±7,18<br>19,92±7,52 | NA |
| **11a** | *S,S,S* | Z | Me | **Bn** | Bn | Me | NA | 96,89±2,67<br>69,79±6,29 | 93,46±9,16<br>67,07±7,47 |
| **11b** | *R,S,S* | Z | Me | **Bn** | Bn | Me | 42,49±6,83<br>17,92±1,74 | 96,68±3,01<br>43,10±8,24 | 90,61±15,78<br>67,84±15,94 |
| **11c,d** | *R,R,S*<br>*S,R,S* | Z | Me | **Bn** | Bn | Me | 63,56±5,18<br>24,24±7,06 | 95,91±4,66<br>65,65±3,47 | 90,05±18,55<br>71,53±4,30 |
| **14a** | *S,S,S* | Z | Me | $^t$Bu | Bn | **Bn** | 67,05±9,43<br>15,16±12,92 | 102,85±3,00<br>73,47±7,41 | 57,07±18,64<br>44,20±16,94 |
| **14b** | *R,S,S* | Z | Me | $^t$Bu | Bn | **Bn** | 90,77±2,17<br>45,92±14,74 | 86,91±3,09<br>47,09±5,84 | 78,14±9,53<br>60,18±11,21 |
| **14c,d** | *R,R,S*<br>*S,R,S* | Z | Me | $^t$Bu | Bn | **Bn** | 85,37±5,86<br>42,17±8,30 | 92,96±4,22<br>40,50±13,24 | 81,99±9,40<br>54,25±11,60 |
| **15a** | *S,S,S* | Z | Me | $^t$Bu | **Me** | **Bn** | 54,46±8,92<br>17,90±9,01 | 86,74±5,26<br>34,81±5,85 | NA |
| **15b** | *R,S,S* | Z | Me | $^t$Bu | **Me** | **Bn** | 91,09±3,78<br>31,40±4,84 | 86,35±6,97<br>29,10±22,35 | NA |
| **16b** | *R,S,S* | Z | Me | $^t$Bu | **s-Bu** | Me | 61,88±11,62<br>6,46±3,77 | 84,92±13,64<br>27,32±8,42 | NA |
| **17a** | *S,S,S* | Z | Me | $^t$Bu | Bn | Me | 114,22±9,16<br>50,16±11,85 | 72,98±14,78<br>-23,67±10,83 | NA |

[a] Blockade of Ca2+ entry trough TRPV1 channel by peptides (Capsaicin, 10 μM, was used as the agonist). b Blockade of $Ca^{2+}$ entry trough TRPM8 channel by peptides (Menthol, 100 μM, was used as the agonist). c Blockade of Ca2+ entry trough TRPA1 channel by peptides (allyl isothyocyanate, 500 M, was used as the agonist). c Compounds were assayed at 50 μM (up) and 5μM (down) concentrations. NA: blockade lower than 25% at the higher concentration assayed.

Table 3. Potency of selected compounds for TRP channels

| Compd. | Configuration 3,4 or 3,4,1' | Channel | $IC_{50}$ (μM) |
|---|---|---|---|
| **1a** | *S,S* | TRPM8 | 3,64±0,34 |
| **1b** | *R,S* | TRPM8 | 2,75±0,18 |
| **3a** | *S,S* | TRPA1 | 1,40±0,70 |
| **4a** | *S,S,S* | TRPV1 | 0.62 ± 0.32 |
| **4b** | *R,S,S* | TRPV1 | 0.30 ± 0.33 |
| **4c** | *S,S,R* | TRPV1 | 1.75 ± 0.50 |
| **8a** | *S,S,S* | TRPV1 | 1.13 ± 1.80 |
| **8b** | *R,S,S* | TRPV1 | 0.47 ± 0.80 |
| **8c** | *S,S,R* | TRPV1 | 3.17 ± 0.76 |

## Experimental

*General methods*. All reagents were of commercial quality. Solvents were dried and purified by standard methods. $^1$H NMR spectra were recorded at 300 or 400 MHz in $CDCl_3$. $^{13}$C NMR spectra were registered at 75 MHz. Electrospray mass spectra (positive mode) were also recorded. Analytical TLC was performed on aluminium sheets with a 0.2 mm layer of silica gel F254. Silica gel 60 (230-400 mesh) was used for column chromatography. Silica gel SPE cartridges (Supelco) were also used for compound purification. Analytical HPLC was performed on a Novapak $C_{18}$ (3.9 × 150 mm, 0.004mm), Deltapak $C_{18}$ (3.9 × 150 mm, 0.004mm) or on a Sunfire $C_{18}$ (3.9 x 150 mm, 4μM) column, with a flow rate of 1mL/min, using a tuneable UV detector set at 214nm. Mixtures of MeCN (solvent A) and 0.05% TFA in $H_2O$ (solvent B) were used in the mobile phase. The solvent mixtures are specified in each case. Electrospray mass spectra (positive mode) were also recorded. Compounds **1a,b-4a-c** were prepared as described.[21] β–Ketoesters **5-7**, **12** and **13** were prepared by standard procedures, and their analytical and spectroscopic data are coincident with those described.[27-30]

**Methyl (4*S*)-*tert*-butoxycarbonylamino-(3*S*)-[(1'*S*-*tert*-butoxy-carbonyl)eth-1'-yl]amino-5-phenylpentanoate (8a)**
(EtOAc:hexane, 1:8). Yield: 19 % (syrup). $t_R$ = 13.04 min (5 to 100% A in 15 min). $^1$H NMR ($CDCl_3$) δ: 7.29-7.18 (m, 5H, Ar), 4.81 (d, 1H, *J* = 9.2, 4-NH), 3.88 (m, 1H, H-4), 3.63 (s, 3H, $OCH_3$), 3.28 (q, 1H, *J* = 6.9, H-1'), 2.95 (m, 1H, H-3), 2.80 (d, 2H, *J* = 7.4, H-5), 2.49 (dd, 1H, *J* =15.5, 6.2, H-2), 2.42 (dd, 1H, *J* =15.5, 7.2, H-2), 1.68 (bs, 1H, 3-NH), 1.44 (s, 9H, C$H_3$ $^t$Bu, Boc), 1.34 (s, 9H, C$H_3$ $^t$Bu), 1.24 (d, 3H, *J* = 6.9, H-2'). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 175.4, 172.2 (CO), 155.5 (NCO), 138.3 (C Ar), 129.2, 128.3, 126.2 (CH Ar), 81.1, 79.9 (C $^t$Bu), 57.0 (C1'), 54.9 (C4, C3), 51.6 ($OCH_3$), 39.2 (C5), 38.1 (C2), 28.3, 27.9 ($CH_3$ $^t$Bu), 20.20 (C2'). MS: 451.6 $[M+1]^+$. Anal. Cald. for $C_{24}H_{38}N_2O_6$: C, 63.98; H, 8.50; N, 6.22. Found: C, 63.75; H, 8.44; N, 6.04.

**Methyl (4*S*)-*tert*-butoxycarbonylamino-(3*R*)-[(1'*S*-*tert*-butoxy-carbonyl)eth-1'-yl]amino-5-phenylpentanoate (8b)**
(EtOAc:hexane, 1:8). Yield: 29 % (syrup). $t_R$ = 12.42 min (5 to 100% A in 15 min). $^1$H NMR ($CDCl_3$) δ: 7.31-7.19 (m, 5H, Ar), 4.98 (d, 1H, *J* = 8.5, 4-NH), 3.89 (m, 1H, H-4), 3.65 (s, 3H, $OCH_3$), 3.29 (q, 1H, *J* = 6.8, H-1'), 3.11 (dt, 1H, *J*=6.2, 2.8, H-3), 2.89 (dd, 1H, *J* = 13.6, 6.1, H-5), 2.78 (dd, 1H, *J* = 13.6, 7.7, H-5), 2.49 (dd, 1H, *J* =15.6, 6.3, H-2), 2.39 (dd, 1H, *J* =15.6, 6.5, H-2), 1.69 (bs, 1H, 3-NH), 1.47 (s, 9H, C$H_3$ $^t$Bu), 1.37 (s, 9H, C$H_3$ $^t$Bu), 1.25 (d, 3H, *J* = 6.8, H-2'). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 174.9, 172.2 (CO), 155.6 (NCO), 138.1 (C Ar), 129.3, 128.3, 126.2 (CH Ar), 81.1, 79.0 (C $^t$Bu), 55.1 (C1'), 54.9 (C4), 54.5 (C3), 51.6 ($OCH_3$), 38.5 (C5), 37.5 (C2), 28.3, 27.9 ($CH_3$ $^t$Bu), 19.8 (C2'). MS: 451.6 $[M+1]^+$. Anal. Cal. for $C_{24}H_{38}N_2O_6$: C, 63.98; H, 8.50; N, 6.22. Found: C, 63.59; H, 8.61; N, 6.07.

**Methyl (4*R*)-*tert*-butoxycarbonylamino-(3*R*)-[(1'*S*-*tert*-butoxycarbonyl)eth-1'-yl]amino-5-phenylpentanoate (8c)**
(EtOAc:hexane, 1:8). Yield: 20 % (syrup). $t_R$ = 12.21 min (5 to 100% A in 15 min). $^1$H NMR ($CDCl_3$) δ: 7.22-7.09 (m, 5H, Ar), 4.81 (m, 1H, 4-NH), 3.85 (m, 1H, H-4), 3.61 (s, 3H, O$CH_3$), 3.25 (q, 1H, *J* = 6.9, H-1'), 3.04 (dt, 1H, *J*=7.2, 5.7, H-3), 2.86 (dd, 1H, *J* = 13.6, 5.2, H-5), 2.66 (m, 1H, H-5), 2.50 (dd, 1H, *J* =15.2, 5.7, H-2), 2.40 (dd, 1H, *J* =15.2, 7.2, H-2), 1.69 (bs, 1H, 3-NH), 1.38 (s, 9H, C$H_3$ $^t$Bu), 1.24 (s, 9H, C$H_3$ $^t$Bu), 1.14 (d, 3H, *J* = 6.8, H-2'). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 174.9, 172.6 (CO), 155.5 (NCO), 138.2 (C Ar), 129.2, 128.3, 126.3 (CH Ar), 80.9, 79.0 (C $^t$Bu), 56.7 (C3), 55.2 (C1'), 54.5 (C4), 51.7 ($OCH_3$), 37.4 (C5), 37.0 (C2), 28.2, 27.9 ($CH_3$ $^t$Bu), 19.6 (C2'). MS: 451.6 $[M+1]^+$. Anal. Cal. for $C_{24}H_{38}N_2O_6$: C, 63.98; H, 8.50; N, 6.22. Found: C, 63.62; H, 8.70; N, 5.98.

**Ethyl (4*S*)-benzyloxycarbonylamino-(3*S*)-[(1'*S*-*tert*-butoxy-carbonyl)eth-1'-yl]amino-5-phenylpentanoate (9a)**
(EtOAc:hexane, 1:8). Yield: 16 % (syrup). $t_R$ = 14.03 min (5 to 100% A in 15 min). $^1$H NMR ($CDCl_3$) δ: 7.32-7.19 (m, 10H, Ar), 5.17 (d, 1H, *J* = 9.3, 4-NH), 5.05, 4.98 (d, 1H, J = 12.1, $OCH_2$ Z), 4.07 (m, 2H, $OCH_2$), 3.97 (m, 1H, H-4), 3.31 (q, 1H, *J* = 6.8, H-1'), 2.98 (t, 1H, *J* = 6.1, H-3), 2.84 (d, 2H, *J* = 7.4, H-5), 2.46 (dd, 1H, *J* =15.1, 7.0, H-2), 2.44 (dd, 1H, *J* =15.1, 5.5, H-2), 1.69 (bs, 1H, 3-NH), 1.44 (s, 9H, $CH_3$ $^t$Bu), 1.25 (d, 3H, *J* = 7.0, H-2'), 1.20 (t, 3H, *J* = 7.1, C$H_3$ OEt). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 175.2, 171.5 (CO), 156.1 (NCO), 138.1, 136.0 (C Ar), 129.1, 128.4, 128.3, 127.9, 126.3 (CH Ar), 81.1 (C $^t$Bu), 66.4 ($OCH_2$ Z), 60.5 ($OCH_2$), 57.1 (C1'), 55.5 (C4), 54.7 (C3), 39.1 (C5), 38.3 (C2), 27.9 ($CH_3$ $^t$Bu), 19.9 (C2'), 14.0 (C$H_3$ OEt). MS: 499.6 $[M+1]^+$.
Anal. Calcd. for $C_{28}H_{38}N_2O_6$: C, 67.45; H, 7.68; N, 5.62. Found: C, 67.21; H, 7.98; N, 5.49.

**Ethyl (4*S*)-benzyloxycarbonylamino-(3*R*)-[(1'*S*-*tert*-butoxy-carbonyl)eth-1'-yl]amino-5-phenylpentanoate (9b)**
(EtOAc:hexane, 1:8). Yield: 31 % (syrup). $t_R$ = 13.22 min (5 to 100% A in 15 min). $^1$H NMR ($CDCl_3$) δ: 7.32-7.17 (m, 10H, Ar), 5.36 (d, 1H, *J* = 8.6, 4-NH), 5.07, 5.00 (d, 1H, *J* = 12.3, O$CH_2$ Z), 4.01 (m, 2H, O$CH_2$ OEt), 3.95 (m, 1H, H-4), 3.28 (q, 1H, *J* = 6.9, H-1'), 3.12 (dt, 1H, *J* = 6.2, 2.9, H-3), 2.93 (dd, 1H, *J* = 14.0, 5.7, H-5), 2.81 (dd, 1H, *J* = 14.0, 7.5, H-5), 2.96 (dd, 1H, *J* =15.6, 6.0, H-2), 2.81 (dd, 1H, *J* =15.6, 6.3, H-2), 1.71 (bs, 1H, 3-NH), 1.45 (s, 9H, C$H_3$ $^t$Bu), 1.23 (d, 3H, *J* = 6.9, H-2'), 1.20 (t, 3H, *J* = 6.9, C$H_3$ OEt). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 174.9, 171.6 (CO), 156.1 (NCO), 137.8, 136.6 (C Ar), 129.3, 129.2, 128.4, 128.3, 127.9, 127.9, 126.3 (CH Ar), 81.2 (C $^t$Bu), 66.4 ($OCH_2$ Z), 60.5 ($OCH_2$), 55.5 (C4), 55.0 (C1'), 54.5 (C3), 39.3 (C5), 37.6 (C2), 27.9 ($CH_3$ $^t$Bu), 19.7 (C2'), 14.1 (C$H_3$ OEt). MS: 499.6 $[M+1]^+$. Anal.

Calcd. for $C_{28}H_{38}N_2O_6$: C, 67.45; H, 7.68; N, 5.62. Found: C, 67.33; H, 7.96; N, 5.23.

**Ethyl (4*R*)-benzyloxycarbonylamino-(3*R*)-[(1'*S*-*tert*-butoxy-carbonyl)eth-1'-yl]amino-5-phenylpentanoate (9c)**

(EtOAc:hexane, 1:8). Yield: 16 % (syrup). $t_R$ = 13.36 min (5 to 100% A in 15 min). $^1$H NMR (300 MHz, $CDCl_3$) δ: 7.32-7.19 (m, 10H, Ar), 5.40 (bd, 1H, *J* = 8.2, 4-NH), 5.03, 4.96 (d, 1H, *J* = 12.6, OC$H_2$ Z), 4.13 (q, 2H, *J* = 7.1, OC$H_2$ OEt), 4.00 (m, 1H, H-4), 3.33 (q, 1H, *J* = 6.9, H-1'), 3.13 (q, 1H, *J* = 6.5, H-3), 2.93 (dd, 1H, *J* = 13.8, 5.3, H-5), 2.73 (dd, 1H, *J* = 13.8, 8.6, H-5), 2.50 (dd, 1H, *J* =15.2, 5.9, H-2), 2.42 (dd, 1H, *J* =15.2, 7.1 H-2), 1.67 (bs, 1H, 3-NH), 1.42 (s, 9H, C$H_3$ $^t$Bu), 1.24 (d, 3H, *J* = 6.9, H-2'), 1.21 (t, 3H, *J* = 6.9, C$H_3$ OEt). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 174.9, 172.1 (CO), 156.1 (NCO), 138.0, 136.7 (C Ar), 129.2, 128.4, 128.3, 127.8, 127.7, 126.4 (CH Ar), 81.0 (C $^t$Bu), 66.3 (OCH$_2$ Z), 60.6 (OCH$_2$ OEt), 56.8 (C3), 55.4 (C4, C1'), 37.3 (C2, C5), 27.9 (CH$_3$ $^t$Bu), 19.6 (C2'), 14.1 (*C*H$_3$ OEt). MS: 499.6 $[M+1]^+$. Anal. Calcd. for $C_{28}H_{38}N_2O_6$: C, 67.45; H, 7.68; N, 5.62. Found: C, 67.36; H, 7.77; N, 5.35.

**Methyl (4*S*)-benzyloxycarbonylamino-(3*S*)-[(1'*S*-methoxy-carbonyl)eth-1'-yl]amino-5-phenylpentanoate (10a)**

($CH_2Cl_2$:ether:hexane, 1:1:2). Yield: 16 % (syrup). $t_R$ = 8.89 min (20 to 100% A in 20 min). $^1$H NMR (400 MHz, $CDCl_3$) δ: 7.26-7.13 (m, 10H, Ar), 5.12 (m, 1H, 4-N*H*), 4.98, 4.89 (d, 1H, *J* = 12.3, OC$H_2$), 3.92 (m, 1H, H-4), 3.62 (s, 3H, OCH$_3$), 3.56 (s, 3H, OCH$_3$), 3.39 (m, 1H, H-1'), 2.99 (m, 1H, H-3), 2.78 (m, 2H, H-5), 2.45 (m, 2H, H-2), 1.56 (bs, 1H, 3-NH), 1.25 (d, 3H, *J* = 6.9, H-2'). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 175.8, 172.0 (CO), 156.1 (NCO), 137.8, 136.5 (C Ar), 129.1, 128.4, 127.9, 127.8, 126.4 (CH Ar), 66.5 (OCH$_2$), 56.6 (C4), 55.6 (C3), 55.4 (C1'), 52.0, 51.7 (OCH$_3$), 38.9 (C5), 37.8 (C2), 19.7 (C2'). MS: 443.6 $[M+1]^+$. Anal. Calcd. For $C_{24}H_{30}N_2O_6$: C, 65.14; H, 6.83; N, 6.33. Found: C, 65.01; H, 6.50; N, 6.39.

**Methyl (4*S*)-benzyloxycarbonylamino-(3*R*)-[(1'*S*-methoxy-carbonyl)eth-1'-yl]amino-5-phenylpentanoate (10b)**

($CH_2Cl_2$:ether:hexane, 1:1:2). Yield: 22 % (syrup). $t_R$ = 7.85 min (20 to 100% A in 20 min). $^1$H NMR (400 MHz, $CDCl_3$) δ: 7.28-7.13 (m, 10H, Ar), 5.24 (m, 1H, 4-NH), 4.98, 4.91 (d, 1H, *J* = 12.6, OCH$_2$), 3.91 (m, 1H, H-4), 3.63 (s, 3H, OCH$_3$), 3.55 (s, 3H, OCH$_3$), 3.40 (m, 1H, H-1'), 3.10 (m, 1H, H-3), 2.80 (m, 2H, H-5), 2.41 (m, 2H, H-2), 1.38 (bs, 1H, 3-NH), 1.22 (d, 3H, J = 6.9, H-2'). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 175.4, 172.1 (CO), 156.2 (NCO), 137.7, 136.5 (C Ar), 129.2, 128.4, 128.3, 127.9, 127.8, 126.4 (CH Ar), 66.5 (OCH$_2$), 55.5 (C4), 54.8 (C3), 54.6 (C1'), 52.0, 51.7 (OCH$_3$), 38.2 (C5), 37.2 (C2), 19.4 (C2'). MS: 443.6 $[M+1]^+$. Calcd. For $C_{24}H_{30}N_2O_6$: C, 65.14; H, 6.83; N, 6.33. Found: C, 64.88; H, 7.16; N, 6.00.

**Methyl (4*R*)-benzyloxycarbonylamino-(3*R*)-[(1'*S*-methoxy-carbonyl)eth-1'-yl]amino-5-phenylpentanoate (10c)**

($CH_2Cl_2$:ether:hexane, 1:1:2). Yield: 23 % (syrup). $t_R$ = 7.05 min (20 to 100% A in 20 min). $^1$H NMR (400 MHz, $CDCl_3$) δ: 7.32-7.18 (m, 10H, Ar), 5.25 (m, 1H, 4-NH), 5.00 (m, 2H, OCH$_2$), 3.99 (m, 1H, H-4)), 3.66 (s, 3H, OCH$_3$), 3.65 (s, 3H, OCH$_3$), 3.46 (m, 1H, H-1'), 3.14 (m, 1H, H-3), 2.93 (dd, 1H, *J* = 14.0, 5.2, H-5), 2.75 (m, 1H, H-5), 2.46 (m, 2H, H-2), 1.65 (bs, 1H, 3-NH), 1.25 (d, 3H, *J* = 6.9, H-2'). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 175.6, 172.4 (CO), 156.1 (NCO), 137.8, 136.5 (C Ar), 129.1, 128.4, 128.3, 127.9, 127.8, 126.5 (CH Ar), 66.5 (OCH$_2$), 56.7 (C3), 55.3 (C4), 54.8 (C1'), 51.9, 51.8 (OCH$_3$), 37.1 (C5), 36.9 (C2), 19.5 (C2'). MS: 443.6 $[M+1]^+$. Calcd. For $C_{24}H_{30}N_2O_6$: C, 65.14; H, 6.83; N, 6.33. Found: C, 64.95; H, 6.71; N, 6.05.

**Methyl (4*S*)-benzyloxycarbonylamino-(3*S*)-[(1'*S*-benzyloxy-carbonyl)eth-1'-yl]amino-5-phenylpentanoate (11a)**

($CH_2Cl_2$:ether:hexane, 1:1:3). Yield: 12 % (syrup). $t_R$ = 12.05 min (20 to 100% A in 20 min). $^1$H NMR (400 MHz, $CDCl_3$) δ: 7.39-7.19 (m, 15H, Ar), 5.19 (m, 1H, 4-NH), 5.17, 5.11 (d, 1H, J = 12.3, OCH$_2$), 5.06, 4.95 (d, 1H, J = 12.4, OCH$_2$), 3.99 (m, 1H, H-4), 3.59 (s, 3H, OCH$_3$), 3.51(m, 1H, H-1'), 3.08 (m, 1H, H-3), 2.85 (m, 2H, H-5), 2.50 (m, 2H, H-2), 2.05 (bs, 1H, 3-NH), 1.33 (d, 3H, *J* = 6.8, H-2'). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 175.3, 171.9 (CO), 156.1 (NCO), 137.8, 136.5, 135.5 (C Ar), 129.1, 128.6, 128.4, 128.3, 128.2, 127.9, 126.4 (CH Ar), 66.8, 66.5 (OCH$_2$), 56.8 (C1'), 55.5 (C4), 55.4 (C3), 51.7 (OCH$_3$), 38.9 (C5), 37.8 (C2), 19.7 (C2'). MS: 519.6 $[M+1]^+$. Anal. Calcd. for $C_{30}H_{34}N_2O_6$: C, 69.48; H, 6.61; N, 5.40. Found: C, 69.22; H, 6.62; N, 4.97.

**Methyl (4*S*)-benzyloxycarbonylamino-(3*R*)-[(1'*S*-benzyloxy-carbonyl)eth-1'-yl]amino-5-phenylpentanoate (11b)**

($CH_2Cl_2$:ether:hexane, 1:1:3). Yield: 19 % (syrup). $t_R$ = 11.60 min (20 to 100% A in 20 min). $^1$H NMR (400 MHz, $CDCl_3$) δ: 7.34-7.19 (m, 15H, Ar), 5.20 (m, 1H, 4-NH), 5.15 (m, 2H, OCH$_2$), 5.06, 4.99 (d, 1H, J = 12.3, OCH$_2$), 3.96 (m, 1H, H-4), 3.60 (s, 3H, OCH$_3$), 3.47 (m, 1H, H-1'), 3.15 (m, 1H, H-3), 2.76 (m, 2H, H-5), 2.45 (m, 2H, H-2), 1.67 (bs, 1H, 3-NH), 1.29 (d, 3H, J = 6.9, H-2'). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 174.9, 172.0 (*C*O), 156.1 (N*C*O), 137.7, 136.5, 135.5 (*C* Ar), 129.2, 128.6, 128.4, 128.3, 128.2, 127.9, 126.4 (*C*H Ar), 66.7, 66.5 (O*C*H$_2$Ph), 55.5 (*C4*), 54.8 (*C3*), 54.6 (*C1'*), 51.6 (O*C*H$_3$), 38.3 (*C*5), 37.3 (*C*2), 19.5 (*C*2'). MS: 519.6 $[M+1]^+$. Anal. Calcd. for $C_{30}H_{34}N_2O_6$: C, 69.48; H, 6.61; N, 5.40. Found: C, 69.31; H, 6.38; N, 5.48.

**Methyl (4*R*)-benzyloxycarbonylamino-(3*S,R*)-[(1'*S*-benzyloxy-carbonyl)eth-1'-yl]amino-5-phenylpentanoate (11c,d)**

($CH_2Cl_2$:ether:hexane, 1:1:3). Yield: 29 % (syrup). $t_R$ = 10.19 min (20 to 100% A in 20 min). Diastereosiomeric mixture **c**:**d** =1:1. $^1$H NMR (300 MHz, $CDCl_3$) δ: 7.35-7.15 (m, 15H, Ar), 5.24 (d, 1H, J = 8.0, 4-NH), 5.15, 5.09 (d, 1H, J = 12.3, OCH$_2$), 4.98 (m, 2H, OCH$_2$), 3.98 (m, 1H, H-4), 3.64, 3.62 (s, 3H, OCH$_3$), 3.51 (m, 1H, H-3'), 3.16, 3.10 (q, 1H, J = 6.0, H-1'), 2.92 (m, 1H, H-5), 2.72 (m, 1H, H-5), 2.48 (m, 2H, H-2), 1.60 (bs, 1H, 3-NH), 1.28, 1.27 (d, 3H, J = 7.0, H-2'). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 175.7, 175.4 (COO), 172.6, 172.4 (COO), 156.3, 156.1 (NCO), 138.1,138.0, 136.8, 136.7, 135.85, 135.8 (C Ar), 128.7, 128.6, 128.4, 128.3, 128.1, 127.9, 126.6 (CH Ar), 66.8, 66.6 (OCH$_2$), 57.0, 56.7 (C3), 55.0 (C4), 55.1, 55.0 (C1'), 51.1, 51.0 (OCH$_3$), 37.4, 37.2, 37.1 (C5, C2), 19.9, 19.6 (C2'). MS: 519.5 $[M+1]^+$.

**Methyl (4*S*)-benzyloxycarbonylamino-(3*S*)-[(1'*S*-*tert*butoxy-carbonyl-2'-phenyl)eth-1'-yl]amino-5-phenylpentanoate (14a)**

($CH_2Cl_2$:ether:hexane, 1:1:4). Yield: 15% (syrup). $t_R$ = 15.43 min (20 to 100% A in 20 min). $^1$H NMR ($CDCl_3$) δ: 7.33-6.96 (m, 15H, Ar), 5.03, 4.95 (d, 1H, *J* = 12.4 Hz, OC$H_2$), 4.90 (bd, 1H, *J* = 10.0 Hz, 4-NH), 3.81 (m, 1H, H-4), 3.61 (s, 3H, OCH$_3$), 3.42 (m, 1H, H-1'), 2.95 (m, 2H, H-3, H-5), 2.71 (dd, 1H, J = 8.6, 13.3 Hz, H-5), 2.46 (m, 4H, H-2, H-2'), 1.38 (s, 9H, CH$_3$ $^t$Bu). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 174.5, 171.7 (COO), 156.0 (NCO), 138.0, 136.6 (C Ar), 129.6, 129.1, 128.4, 128.3, 128.2, 127.9, 127.8, 126.6, 126.2 (CH Ar), 81.5 (C $^t$Bu), 66.4 (OCH$_2$), 64.0 (C1'), 55.7 (C4), 55.3 (C3), 51.6 (OCH$_3$), 40.7 (C5), 38.9 (C2'), 38.3 (C2), 27.9 (CH$_3$ $^t$Bu). MS: 561.3 $[M+1]^+$, 583.3 $[M+23]^+$. Anal. Calcd. for $C_{33}H_{40}N_2O_6$: C, 70.69; H, 7.19; N, 5.00. Found: C, 70.34; H, 7.02; N, 5.21.

**Methyl (4*S*)-benzyloxycarbonylamino-(3*R*)-[(1'*S*-*tert*-butoxy-carbonyl-2'-phenyl)eth-1'-yl]amino-5-phenylpentanoate (14b)**

($CH_2Cl_2$:ether:hexane, 1:1:4). Yield: 28% (syrup). $t_R$ = 13.48 min (20 to 100% A in 20 min). $^1$H NMR ($CDCl_3$) δ: 7.33-7.15 (m, 15H, Ar), 5.23 (bd, 1H, *J* = 7.2 Hz, 4-NH), 5.06, 5.00 (d, 1H, *J* = 12.3 Hz, $OCH_2$), 3.91 (m, 1H, H-4), 3.56 (s, 3H, $OCH_3$), 3.44 (m, 1H, H-1'), 3.07 (m, 1H, H-3), 2.85 (m, 4H, H-5, H-2'), 2.31 (m, 2H, H-2), 1.33 (s, 9H, $CH_3$ $^t$Bu). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 173.6, 171.9 (COO), 156.0 (NCO), 137.7, 137.1, 136.6 (C Ar), 129.4, 129.3, 128.5, 128.4, 128.3, 128.0, 127.9, 126.7, 126.4 (CH Ar), 81.6 (C $^t$Bu), 66.5 ($OCH_2$), 61.1 (C1'), 55.3 (C4), 54.5 (C3), 51.6 ($OCH_3$), 40.3, 38.4 (C5, C2'), 36.9 (C2), 27.9 ($CH_3$ $^t$Bu). MS: 561.3 $[M+1]^+$, 583.3 $[M+23]^+$. Anal. Calcd. for $C_{33}H_{40}N_2O_6$: C, 70.69; H, 7.19; N, 5.00. Found: C, 70.85; H, 7.15; N, 4.86.

**Methyl (4*R*)-benzyloxycarbonylamino-(3*S,R*)-[(1'*S*-*tert*-butoxy-carbonyl-2'-phenyl)eth-1'-yl]amino-5-phenylpentanoate (14c,d)**

($CH_2Cl_2$:ether:hexane, 1:1:4). Yield: 21% (syrup). $t_R$ = 13.48 min (20 to 100% A in 20 min). Diasteroisomeric mixture **c**:**d** = 2.5: 1. $^1$H NMR (300 MHz, $CDCl_3$) δ: 7.34-7.05 (m, 15H, Ar), 5.33 (bd, 1H, J = 10.6 Hz, 4-NH isomer **d**), 5.00 (s, 2H, $OCH_2$ **d**), 4.93 (s, 2H, $OCH_2$ isomer **c**), 4.59 (bd, 1H, J = 8.2 Hz, 4-NH isomer **c**), 3.97 (m, 1H, H-4 **d**), 3.83 (m, 1H, H-4 **c**), 3.63 (s, 3H, $OCH_3$ **c**), 3.56 (s, 3H, $OCH_3$ **d**), 3.47 (dd, 1H, J = 8.0, 6.0, H-2' **d**), 3.47 (dd, 1H, J = 8.5, 5.7, H-2' **c**), 3.08 (m, 1H, H-3 **d**), 2.97 (m, 1H, H-3 **d**), 2.96-2.64 (m, 2H, H-5, H-3'), 2.46 (m, 4H, H-2 **c**), 2.25 (m, 4H, H-2 **d**), 1.37 (s, 9H, $CH_3$ $^t$Bu **c**), 1.36 (s, 9H, $CH_3$ $^t$Bu **d**). MS: 561.2 $[M+1]^+$.

**Methyl (4*S*)-benzyloxycarbonylamino-(3*S*)-[(1'*S*-*tert*-butoxy-carbonyl-2'-phenyl)eth-1'-yl]aminopentanoate (15a)**

($CH_2Cl_2$:ether:hexane, 1:1:3). Yield: 6 % (syrup). $t_R$ = 13.97 min (20 to 100% A in 30 min). $^1$H NMR ($CDCl_3$) δ: 7.28-7.10 (m, 10H, Ar), 4.99 (m, 2H, $OCH_2$), 4.84 (m, 1H, 4-NH), 3.57 (m, 4H, H-1', $OCH_3$), 3.32 (m, 1H, H-4), 2.80 (m, 2H, H-3, H-2'), 2.67 (dd, 1H, *J*=13.4, 8.0, H-2'), 2.34 (m, 2H, H-2), 1.52 (bs, 1H, 3-NH), 1.30 (s, 9H, $CH_3$ tBu), 0.85 (d, 3H, *J* = 6.7, H-5). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 174.3, 172.1 (CO), 155.9 (NCO), 137.7, 136.6 (C Ar), 129.5, 128.5, 128.2, 128.0, 126.5 (CH Ar), 81.5 (C $^t$Bu), 66.5 ($OCH_2$), 63.7 (C4), 57.6 (C3), 51.7 ($OCH_3$), 49.9 (C1'), 40.5 (C2'), 37.9 (C2), 27.9 ($CH_3$ $^t$Bu), 18.5 (*C5*). MS: 485.6 $[M+1]^+$. Anal. Calcd. For $C_{27}H_{36}N_2O_6$: C, 66.92; H, 7.49; N, 5.78. Found: C, 66.73; H, 7.12; N, 5.32.

**Methyl (4*S*)-benzyloxycarbonylamino-(3*R*)-[(1'*S*-*tert*-butoxy-carbonyl-2'-phenyl)eth-1'-yl]aminopentanoate (15b)**

($CH_2Cl_2$:ether:hexane, 1:1:3). Yield: 12 % (syrup). $t_R$ = 13.37 min (20 to 100% A in 30 min). $^1$H NMR ($CDCl_3$) δ: 7.37-7.18 (m, 10H, Ar), 5.29 (bd, 1H, J = 7.9, 4-NH), 5.11 (m, 2H, O*CH*$_2$), 3.73 (m, 1H, *H-4), 3.60 (s, 3H, OCH*$_3$), 3.43 (m, 1H, H-1'), 2.98 (m, 1H, H-3), 2.85 (m, 2H, H-2'), 2.36 (dd, 1H, J = 15.6, 6.3, H-2), 2.27 (dd, 1H, J = 15.6, 6.1, H-2), 1.45 (s, 9H, CH3 $^t$Bu), 1.17 (d, 3H, *J* = 6.7, H-5). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 173.8, 172.5 (COO), 156.2 (NCO), 137.4, 136.8 (C Ar), 129.5, 128.6, 128.4, 128.1, 126.7 (CH Ar), 81.7 (C $^t$Bu), 66.7 ($OCH_2$), 61.0 (C1'), 57.1 (C3), 51.8 ($OCH_3$), 49.9 (C4), 40.3 (C2'), 36.7 (C2), 28.1 ($CH_3$ $^t$Bu), 18.3 (C5). MS: 485.2 $[M+1]^+$. Anal. Calcd. For $C_{27}H_{36}N_2O_6$: C, 66.92; H, 7.49; N, 5.78. Found: C, 66.58; H, 7.83; N, 5.40.

**Methyl (4*R*)-benzyloxycarbonylamino-(3*R*)-[(1'*S*-*tert*-butoxy-carbonyl-2'-phenyl)eth-1'-yl]aminopentanoate (15c)**

Data extracted from a 2:1 mixture of **15b,c**. ($CH_2Cl_2$:ether:hexane, 1:1:3). Yield: 42 % (syrup). $t_R$ = 13.24 min (20 to 100% A in 30 min). $^1$H NMR ($CDCl_3$) δ: 7.37-7.18 (m, 10H, Ar), 5.77 (bd, 1H, J = 8.1, 4-NH), 5.11 (m, 2H, $OCH_2$), 3.66 (m, 1H, H-4), 3.60 (s, 3H, $OCH_3$), 3.48 (m, 1H, H-1'), 2.98 (m, 2H, H-3, H-2'), 2.75 (dd, 1H, J = 13.5, 8.3, H-2'), 2.19 (m, 2H, H-2), 1.46 (s, 9H, $CH_3$ $^t$Bu), 1.05 (d, 3H, *J* = 6.7, H-5). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 174.1, 172.2 (COO), 156.0 (NCO), 137.4, 137.0 (C Ar), 129.4, 128.6, 128.5, 128.1, 126.9 (CH Ar), 81.7 (C $^t$Bu), 66.5 ($OCH_2$), 61.6 (C1'), 57.5 (C3), 51.9 ($OCH_3$), 49.6 (C4), 40.0 (C2'), 37.3 (C2), 28.1 ($CH_3$ tBu), 15.5 (C5). MS: 485.4 $[M+1]^+$.

**Methyl (4*S*)-benzyloxycarbonylamino-(3*S*)-[(1'*S*-*tert*-butoxy-carbonyl)eth-1'-yl]amino-(5*S*)-methylheptanoate (16a)**

(EtOAc:hexane, 1:7). Yield: 13 % (syrup). $t_R$ = 4.78 min (15 to 95% A in 5 min). $^1$H NMR ($CDCl_3$) δ: 7.36 (m, 5H, Ar), 5.20 (bd, 1H, *J* = 7.2 Hz, 4-NH), 5.12, 5.06 (d, 1H, *J* = 12.1 Hz, O*CH*$_2$), 3.66 (s, 3H, O*CH*$_3$), 3.25 (m, 1H, H-3, H-4, *H*-1'), 2.45 (m, 2H, H-2), 1.51 (m, 2H, H-5, H-6), 1.45 (s, 9H, $CH_3$ $^t$Bu), 1.19 (d, 3H, J = 6.9, H-2'), 1.08 (m, 1H, H-6), 0.96 (d, 3H, J = 6.7, 5-$CH_3$), 0.86 (t, 3H, J = 7.3, H-7). $^{13}$C NMR ($CDCl_3$) δ: 175.5, 172.3 (COO), 156.8 (NCO), 136.9 (C Ar), 128.6, 128.2, 127.9 (CH Ar), 81.3 (C $^t$Bu), 66.8 ($OCH_2$), 59.3(C4), 57.2 (C-1'), 53.3 (C3), 51.8 ($OCH_3$), 38.8 (C2), 37.1 (C5), 28.1 ($CH_3$ $^t$Bu), 26.7 (C6), 20.0 (C2'), 15.9 (5-$CH_3$), 11.2 (C7). MS: 451.4 $[M+1]^+$. Anal. Calc. for $C_{24}H_{38}N_2O_6$: C, 63.98; H, 8.50; N, 6.22. Found: C, 63.91; H, 8.58; N, 5.89.

**Methyl (4*S*)-benzyloxycarbonylamino-(3*R*)-[(1'*S*-*tert*-butoxy-carbonyl)eth-1'-yl]amino-(5*S*)-methylheptanoate (16b)**

(EtOAc:hexane, 1:7). Yield: 21 % (syrup). $t_R$ = 4.36 min (15 to 95% A in 5 min). $^1$H NMR ($CDCl_3$) δ: 7.34 (m, 5H, Ar), 5.27 (bs, 1H, 4-NH), 5.11 (s, 2H, $OCH_2$), 3.66 (s, 3H, $OCH_3$), 3.53 (m, 1H, H-4), 3.24 (m, 2H, H-3, H-1'), 2.43 (m, 2H, H-2), 1.56 (m, 1H, H-5), 1.45 (s, 9H, $CH_3$ $^t$Bu), 1.24 (m, 2H, H-6), 1.20 (d, 3H, J = 6.9, H-2'), 0.91 (t, 3H, J = 7.2, H-7), 0.88 (d, 3H, J = 6.5, 5-$CH_3$). $^{13}$C NMR ($CDCl_3$) δ: 175.2, 172.4 (COO), 157.1 (NCO), 136.9 (C Ar), 128.6, 128.1, 128.0 (CH Ar), 81.3 (C $^t$Bu), 66.7 ($OCH_2$), 58.0 (C4), 54.9 (C-1'), 53.2 (C3), 51.8 ($OCH_3$), 37.7 (C2), 36.4 (C5), 28.0 ($CH_3$ tBu), 26.3 (C6), 20.0 (C2'), 15.1 (5-$CH_3$), 11.0 (C7). MS: 451.1 [M+1 Anal. Calc. for $C_{24}H_{38}N_2O_6$: C, 63.98; H, 8.50; N, 6.22. Found: C, 63.71; H, 8.20; N, 5.95.

**Methyl (4*R*)-benzyloxycarbonylamino-(3*R*)-[(1'*S*-*tert*-butoxy-carbonyl)eth-1'-yl]amino-(5*S*)-methylheptanoate (16c)**

Data extracted from a diasteroisomeric mixture of **16b,c** 1:2. (EtOAc:hexane, 1:7). Yield: 16 % (syrup), mixture of D2 and D3 in 1:2 ratio. $t_R$ = 3.82 min (15 to 95% A in 5 min). $^1$H NMR ($CDCl_3$) δ D3: 7.34 (m, 5H, Ar), 5.09, 5.05 (d, 1H, *J* = 12.3 Hz, $OCH_2$), 4.67 (d, 1H, *J* = 10.5, 4-NH), 3.64 (m, 1H, H-4), 3.58 (s, 3H, $OCH_3$), 3.20 (m, 1H, H-1'), 3.05 (m, 2H, H-3), 2.41 (m, 2H, H-2), 1.82 (m, 1H, H-5), 1.54 (m, 1H, H-6), 1.44 (s, 9H, $CH_3$ $^t$Bu), 1.37 (m, 1H, H-6), 1.18 (d, 3H, J = 6.9, H-2'), 0.90 (t, 3H, *J* = 7.2, H-7), 0.84 (d, 3H, *J* = 6.8, 5-$CH_3$). $^{13}$C NMR ($CDCl_3$) δ: 175.1, 173.7 (COO), 157.0 (NCO), 136.9 (C Ar), 128.6, 128.3, 128.1 (CH Ar), 82.5 (C $^t$Bu), 66.8 ($OCH_2$), 57.3 (C4), 55.2 (C-1'), 53.9 (C3), 51.7 ($OCH_3$), 37.1 (C2), 34.9 (C5), 28.0 ($CH_3$ $^t$Bu), 27.1 (C6), 19.7 (C2'), 15.7 (5-$CH_3$), 11.5 (C7). MS: 451.5 $[M+1]^+$.

**Methyl (4*R*)-benzyloxycarbonylamino-(3*S*)-[(1'*S*-*tert*-butoxy-carbonyl)eth-1'-yl]amino-(5*S*)-methylheptanoate (16d)**

(EtOAc:hexane, 1:7). Yield: 6 % (syrup). $t_R$ = 3.64 min (15 to 95% A in 5 min). $^1$H NMR ($CDCl_3$) δ: 7.35 (m, 5H, Ar), 5.08 (s, 2H, $OCH_2$), 5.20 (d, 1H, *J* = 10.3 Hz, 4-NH), 3.61 (m, 4H, H-4, $OCH_3$), 3.30 (m, 1H, H-1'), 3.19 (m, 1H, H-3), 2.42 (dd, 1H, *J* = 15.3, 5.5, H-2), ), 2.32 (dd, 1H, *J* = 15.3, 6.3, H-2), 1.56 (m, 2H, H-5, H-6), 1.45 (s, 9H, $CH_3$ $^t$Bu), 1.21 (d, 3H, *J* = 6.9, H-2'), 1.00 (m, 1H, H-6), 0.93 (d, 3H, *J* = 6.5, 5-$CH_3$), 0.90 (t, 3H, *J* = 7.2, H-7). $^{13}$C NMR ($CDCl_3$) δ: 174.9, 173.3 (COO), 157.0 (NCO), 136.8 (C Ar), 128.6, 128.2, 128.1 (CH Ar), 80.9 (C $^t$Bu), 67.0 ($OCH_2$), 59.1(C4), 54.8 (C-1'), 53.3 (C3), 51.7 ($OCH_3$),

36.4 (C2), 35.9 (C5), 28.1 ($CH_3$ $^t$Bu), 24.4 (C6), 19.7 (C2'), 16.5 (5-$CH_3$), 11.5 (C7). MS: 451.1 $[M+1]^+$. Anal. Calc. for $C_{24}H_{38}N_2O_6$: C, 63.98; H, 8.50; N, 6.22. Found: C, 63.86; H, 8.09; N, 6.05.

**Methyl (4*S*)-benzyloxycarbonylamino-(3*S*)-[[N-(1'*S*-*tert*-butoxy-carbonyl)eth-1'-yl]-N-acetil]amino-5-phenylpentanoate (17a)**

A solution of compound **4a** (52 mg, 0.107 mmol) in THF (2 mL) was cooled to 0ºC and treated with propylene oxide (113 µL, 1.61 mmol) and acetyl chloride (29 µL, 0.322 mmol). Stirring at room temperature was continued for three days. After evaporation of the solvent, the resulting residue was purified on a column using EtOAc-hexane (3:1) as eluent. The title compound, 48 mg (86 %) was obtained as a syrup. $t_R$ = 8.52 min (15 to 95% A in 10 min). $^1$H NMR (300 MHz, $CDCl_3$) δ: 7.30-7.00 (m, 10H, Ar), 5.81 (d, 1H, J = 8.5, 4-NH), 4.90 (m, 2H, *OCH*$_2$), 4.59 (m, 1H, H-4), 4.00 (m, 1H, H-2'), 3.65 (m, 1H, H-3), 3.59 (s, 3H, $OCH_3$), 3.12 (m, 1H, H-5), 2.91 (m, 1H, H-5), 2.68 (m, 2H, H-2), 2.15 (s, 3H, $CH_3$ Ac), 1.45 (s, 9H, $CH_3$ $^t$Bu), 1.26 (d, 3H, J = 7.1, H-3'). MS: 527.6 $[M+1]^+$. Anal. Calc. for $C_{29}H_{38}N_2O_7$: C, 66.14; H, 7.27; N, 5.32. Found: C, 65.87; H, 6.95; N, 5.01.

**Calcium microfluorography**

For fluorescence assays, cells expressing TRP channels (TRPV1-SH-SY5Y, TRPM8-HEK and TRPA1-IMR90) were seeded in 96-well plates (Corning Incorporated, Corning, NY) at a cell density of 40,000 cells 2 days before treatment. The day of treatment the medium was replaced with 100 µL of the dye loading solution Fluo-4 NW supplemented with probenecid 2.5 mM. Then the compounds dissolved in DMSO were added at the desired concentrations and the plate(s) were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ for 60 minutes.

The fluorescence was measured using instrument settings appropriate for excitation at 485 nm and emission at 535 nm. (POLARstar Omega BMG LAB tech). A baseline recording of 7 cycles was recorded prior to stimulation with the agonist (10 µM capsaicin for TRPV1, 100 µM menthol for TRPM8, and 100 µM AITC for TRPA1). The corresponding antagonist (10 µM Ruthenium Red for TRPV1 and TRPA1, 100 µM AMTB for TRPM8) was added for the blockade. The changes in fluorescence intensity were recorded during 15 cycles more. DMSO, at the higher concentration used in the experiment, was added to the control wells.

$$\%Blockage = \frac{(F_0 - F_I)}{(Fc_0 - Fc_I)}$$

The degree of blockage (%) of TRP channel activity was calculated by:

Where F0 is the fluorescence after the addition of agonist in the presence of the compound, FI is the fluorescence before the addition of agonist in the presence of the compound, Fc0 is the fluorescence after the addition of agonist in the absence of the compound, FcI is the fluorescence before the addition of agonist in the absence of the compound.

$$Z = 1 - \frac{3 * (SD_{max} + SD_{min})}{Mean_{max} - Mean_{min}}$$

The Z factor was calculated using the following equation:

Where $Mean_{max}$ is the mean of the maximum fluorescence in the presence of agonist, $Mean_{min}$ is the mean of the maximum fluorescence in the presence of agonist and antagonist.

## Acknowledgements

This work was supported by the Spanish MINECO: CSD2008-00005, The Spanish Ion Channel Initiative-CONSOLIDER INGENIO 2010, and BFU2012-39092-C02.

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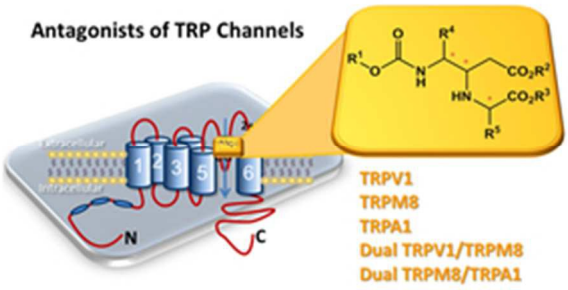


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