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Structure–activity relationships of simplified resiniferatoxin analogues with potent VR1 agonism elucidates an active conformation of RTX for VR1 binding

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Abstract—We previously described a series of *N*-(3-acyloxy-2-benzylpropyl) homovanillate and *N*′-(4-hydroxy-3-methoxybenzyl) thiourea derivatives that were potent VR1 agonists with high-affinities and excellent analgesic profiles. The design of these simplified RTX analogues was based on our RTX-derived pharmacophore model which incorporates the 4-hydroxy-3-methoxyphenyl (A-region), C_{20}-ester (B-region), orthophenyl (C1-region) and C_3-keto (C2-region) groups of RTX. For the purpose of optimizing the spatial arrangement of the four principal pharmacophores on the lead agonists (**1**–**4**), we have modified the distances in the parent C-region, 3-acyloxy-2-benzylpropyl groups, by lengthening or shortening one carbon to vary the distances between the pharmacophores. We find that two of the amides, **4** and **19**, possess EC_{50} values <1 nM for induction of calcium influx in the VR1-CHO cells. As observed previously, the structure–activity relations for inhibition of RTX binding to VR1 and for induction of calcium uptake were distinct, presumably reflecting both intrinsic and methodological factors. In order to find the active conformation of VR1 ligands, the energy-minimized conformations of seven selected agonists were determined and the positions of their four pharmacophores were matched with those of five low energy RTX conformations. The rms values for the overlaps in the pharmacophores were calculated and correlated with the measured binding affinities (K_i) and calcium influx (EC_{50}) values. The binding affinities of the agonists correlated best with the RMS values derived from RTX conformation E ($r^2 = 0.92$), predicting a model of the active conformation of RTX and related vanilloids for binding to VR1. Poorer correlation was obtained between any of the conformations and the EC_{50} values for calcium influx.

1. Introduction

The vanilloid receptor 1 (VR1)[1] is a polymodal nociceptor activated by protons,[2] heat,[3] vanilloids such as capsaicin (CAP)[4] and resiniferatoxin (RTX),[5] and lipid mediators such as anandamide[6] and the lipid metabolic products of lipoxygenases.[7] VR1 has been cloned from rat,[3] human,[8] chicken[9] and guinea pig,[10] and has been shown to be a member of the vanilloid receptor (TRPV) subfamily of transient receptor potential (TRP) ion channels.[11] It is a cation channel with modest selectivity for calcium. Consistent with its complicated regulation, multiple sites of phosphorylation have been identified[12,13] as well as multiple sites mediating acid responsiveness,[14,15] and a site involved in inhibition by phosphoinositides.[16] Since VR1 is present predominantly in nociceptors on primary sensory neuron, it has been an attractive therapeutic target for the treatment of pain and for other indications in which C-fiber sensory neurons are involved. Its validation as a target for analgesia has been confirmed recently by the observation that VR1 knockout mice exhibit deficits in the thermal hyperalgesia that accompanies tissue injury

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and inflammation[17,18] as well as by emerging studies characterizing VR1 antagonists.[19]

VR1 agonists initially activate the receptor to trigger cation influx resulting in excitation of primary sensory neurons. Subsequent desensitization leads to the block of pain perception by the central nervous system. This desensitization forms the basis for the primary therapeutic use of VR1 agonists as potent analgesics.[1,20] Reflecting the complicated pharmacology of VR1 and the complicated nature of the clinical endpoint of pain, agonists such as resiniferatoxin and olvanil have shown that it is possible at least partially to separate the induction of acute pain resulting from the initial channel activation and the subsequent desensitization.[21] Potential therapeutic applications of VR1 agonists include not only chronic pain associated with diabetic neuropathy, post-herpetic neuralgia, arthritis and cluster headache, but also urologic problems, pruritus and bowel dysfunction.[22]

Several types of VR1 agonists have been reported and are being developed: capsaicinoids such as capsaicin (currently marketed) or DA-5018[23] and SDZ-249665[24] (undergoing clinical trial); resiniferatoxin;[25] and natural dialdehydes.[26] Resiniferatoxin (RTX), a natural diterpene isolated from the cactus-like succulent *Euphorbia resinifera*, has been of particular interest both as a therapeutic candidate per se and as a lead compound for designing novel VR1 ligands. Not only does RTX display extraordinarily high potency compared to capsaicin (its binding potency was $K_i = 0.13$ nM in CHO/VR1 versus $K_i = 1{,}700$ nM for capsaicin),[27] but it markedly dissociates desensitization, for which it is ultrapotent, from pungency, for which it is only slightly more potent than capsaicin. From the perspective of medicinal chemistry, the identification of RTX as an ultrapotent vanilloid strongly argued that the vanilloid pharmacophore possessed novel elements not previously appreciated from the studies with capsaicin and its derivatives.

Over the past few years, we have demonstrated that *N*-(3-acyloxy-2-benzylpropyl)-*N*′-(4-hydroxy-3-methoxybenzyl)thiourea (**1**, **2**)[28] and *N*-(3-acyloxy-2-benzylpropyl) homovanillic amide (**3**, **4**)[29,30] possess potent VR1 agonism with high affinity in rat DRG and excellent analgesic activity. These agonists were designed based on an RTX-derived pharmacophore model which incorporates the 4-hydroxy-3-methoxyphenyl (A-region), C_{20}-ester (B-region), orthophenyl (C1-region) and C_3-keto (C2-region) groups of RTX, which are suggested from previously published SAR studies on RTX[31] to be the principal pharmacophores for interaction with the capsaicin binding site of VR1 (Fig. 1) (the structural regions of the vanilloids are denoted based on the terminology of Novartis[32]). The particular structural feature of these compounds is the replacement of the diterpene moiety of resiniferatoxin with a 3-acyloxy-2-benzylpropyl moiety,[28,29] which represents a motif conferring significantly enhanced potency for VR1 agonists compared to capsaicin. Recently, combination of this template together with an antagonistic A-region led to a series of *N*-(3-acyloxyl-2-benzylpropyl)-*N*′-[(4-methylsulfonylamino)benzyl]thiourea analogues, which have shown high affinity antagonism/partial antagonism and agonism on VR1.[33–35]

As part of our ongoing program to clarify the structural interactions of VR1 ligands with the capsaicin binding site of VR1 and eventually to generate simplified, potent RTX surrogates as clinical drug candidates for analgesia, we have modified the 3-acyloxy-2-benzylpropyl group in the C-region of our lead structures. The strategy was that we varied the spatial arrangement of the four pharmacophores in the model by lengthening or shortening one carbon between the C-region pharmacophores, thereby producing seven different sets of distances between the four pharmacophores (Fig. 2). We probed their activities on VR1 both for inhibition of ligand binding (measured by competition of [^{3}H]RTX

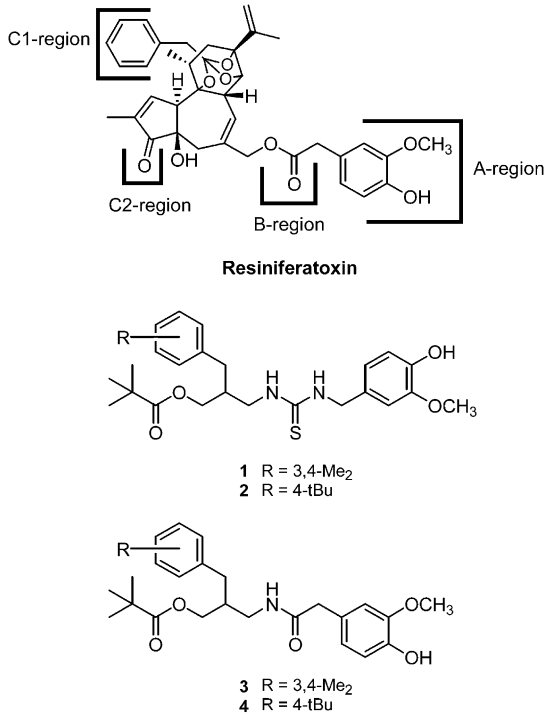


Figure 1. Resiniferatoxin and its simplified analogues.

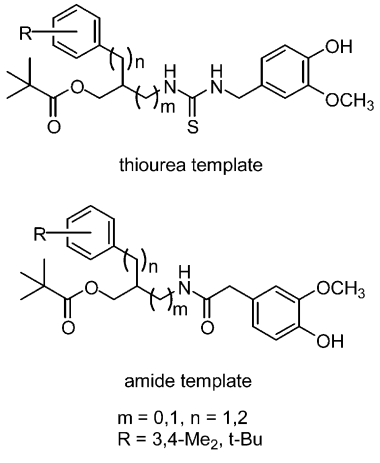
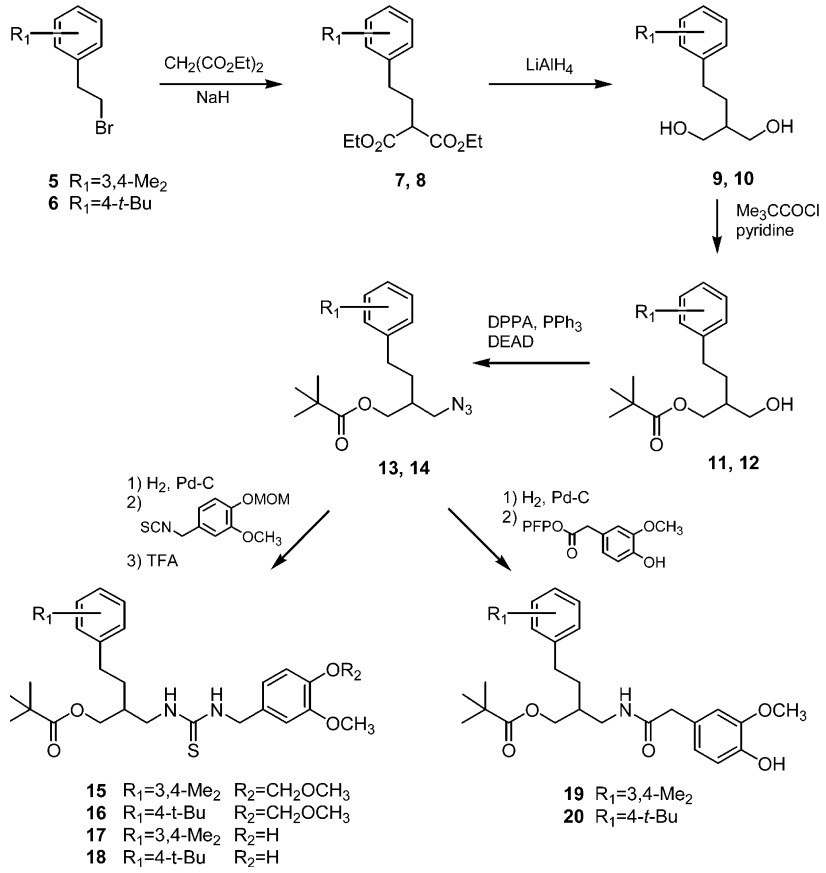


Figure 2. SAR of C region in simplified RTX analogues.



Scheme 1. Synthesis of **17**–**20**.

binding) and for stimulation of calcium uptake. We further determined their energy minimized conformations and correlated the goodness of fit of these conformations relative to the preferred conformations of RTX with the biological potency data. Our studies advance our understanding of vanilloid structure activity relationships and identify a preferred conformation of the pharmacophoric elements in the binding to VR1.

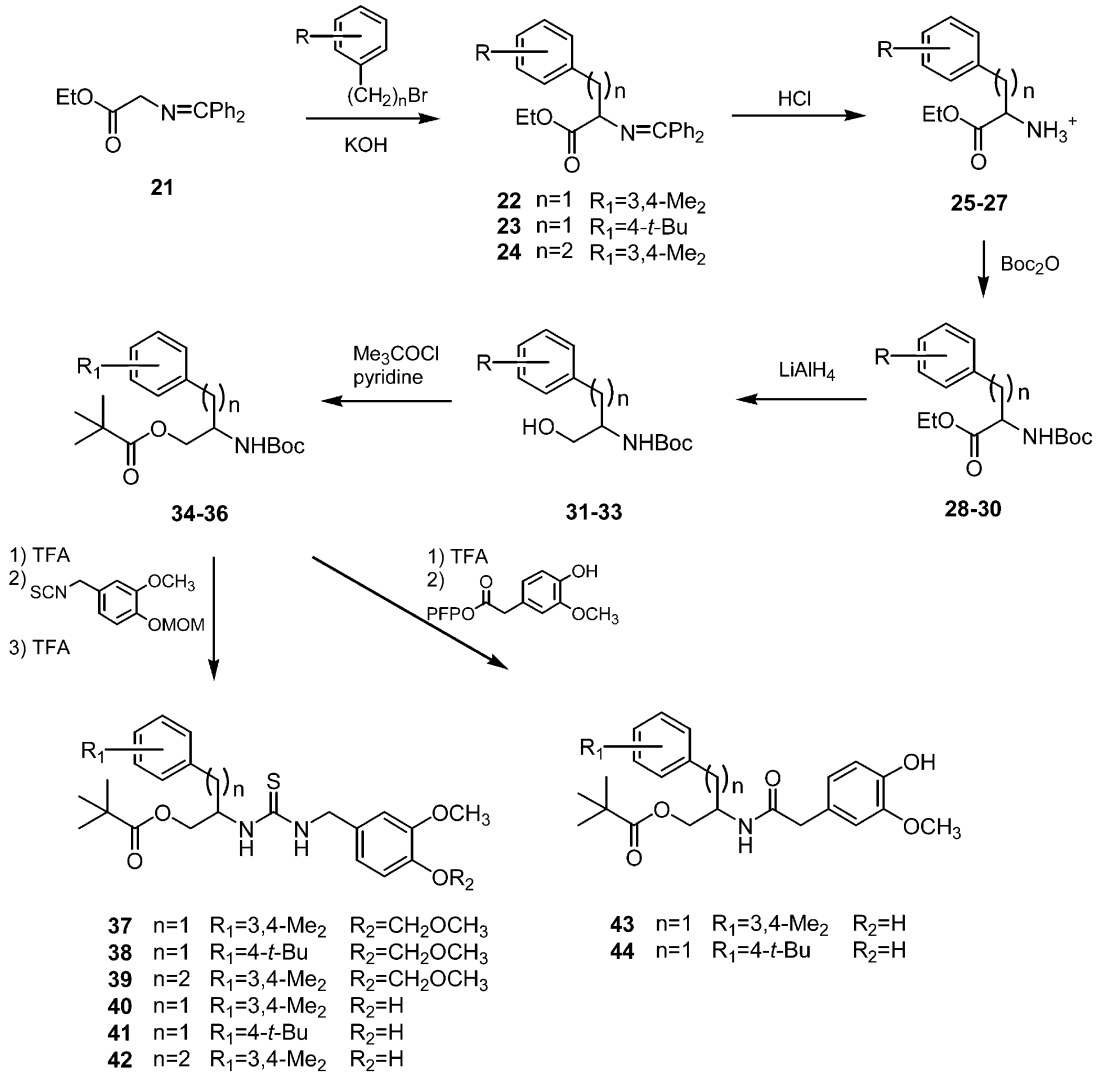
2. Chemistry

The syntheses of *N*-[3-pivaloyloxy-2-(2-phenylethyl)-propyl] thiourea and amide analogues (**17**–**20**) are outlined in Scheme 1. Alkylation of diethyl malonate with 2-(3,4-dimethylphenyl)ethyl bromide (**5**) or 2-(4-*t*-butylphenyl)ethyl bromide (**6**), prepared from 3,4-dimethylbenzyl chloride and 4-*tert*-butylstyrene by the conventional route, produced mono-alkylated malonate (**7**, **8**). $LiAlH_4$-reduction followed by selective mono-pivaloylation afforded monoesters (**11, 12**) and, subsequently, intact free hydroxyl was converted into the corresponding azides (**13, 14**) using PPh_3, DEAD, and diphenylphosphoryl azide.[36] Azides of **13, 14** were reduced to the amines, which, without further purification, were directly condensed with 4-methoxymethoxy-3-methoxybenzyl isothiocyanate[29] or pentafluorophenyl homovanillate[30] to produce thiourea (**15**, **16**) and final amide (**19**, **20**) analogues. The ensuing MOM deprotection of **15**, **16** afforded the final thioureas (**17, 18**). The syntheses of *N*-[2-acyloxy-1-(benzyl or phenylethyl)-ethyl] thiourea and amide analogues (**40**–**44**) are shown in Scheme 2. Alkylation of *N*-(diphenylmethylene)glycine ethyl ester with the corresponding halides under potassium hydroxide in DMSO provided **22**–**24**. The diphenylmethylene group of **22**–**24** was hydrolyzed under acidic condition and then reprotected with *t*-Boc group to afford **28**–**30**. $LiAlH_4$-reduction followed by pivaloylation produced **34**–**36**. The ensuing deprotection of *t*-Boc furnished the corresponding amines, which were utilized using the condensation method described in Scheme 1 to provide thioureas (**40**–**42**) and amides (**43**–**44**).

3. Results and discussion

3.1. Biological results

The agonistic activities of the synthesized VR1 agonists were assessed in vitro by a $^{45}Ca^{2+}$ influx assay, which was carried out using VR1 heterologously expressed in Chinese hamster ovary (CHO) cells as previously described.[27,33] The potencies of the compounds were expressed as their EC_{50} values. Their receptor binding affinities were determined by competition of binding of [^{3}H]RTX to VR1 in CHO cells and were expressed as their K_i values. All values represent the mean of at least three experiments (Table 1). Since only the agonistic activities of the parent agonists (**1**–**4**) in rat DRG were reported previously,[28,30] their binding affinities and potencies for induction of calcium uptake were reevaluated in our CHO/VR1 system for direct comparison.



Scheme 2. Synthesis of **40**–**44**.

In the CHO/VR1 system, the parent thioureas, **1** and **2**, showed high binding affinities with K_i values of 17.4 and 6.35 nM, respectively, and potent agonism for calcium uptake with EC_{50} values of 1.97 and 2.83 nM, respectively, as expected. Their potencies thus represented, respectively, 100- and 300-fold enhancements in binding affinity and 20- and 15-fold enhancements in agonism compared to capsaicin. The parent amides, **3** and **4**, also showed high binding affinities, $K_i = 157$ and 15 nM, and potent agonism, $EC_{50} = 12.7$ and 0.29 nM. These values represented 10- and 150-fold increases in binding affinity and 3.5- and 150-fold increases in agonism compared to capsaicin. In particular, the high agonism of **4** ($EC_{50} = 0.29$ nM) in the CHO/VR1 cells approaches that of RTX ($EC_{50} = 0.27$ nM in this system).

One-carbon lengthening of the thiourea lead compounds, **1** and **2**, providing 3-pivaloyloxy-2-phenethylpropyl analogues, **17** and **18** ($m = 1$, $n = 2$ in thiourea), led to modestly reduced potencies both in binding affinities with values of $K_i = 25.0$ and 18.3 nM (1.5-fold in **17**, 3-fold in **18**) and in agonism with values of $EC_{50} = 6.3$ and 18.7 nM (3-fold in **17**, 6.5-fold in **18**) as compared to the parent thioureas, **1** and **2**, respectively. One-carbon shortening ($m = 0$, $n = 1$ in thiourea) produced 2-pivaloyloxy-1-benzylethyl analogues, **40** and **41**, in which their binding affinities and potencies as agonists were also reduced to a similar extent as that of lengthening: $K_i = 45$ and 34.6 nM (2.5-fold in **40**, 5.5-fold in **41**), $EC_{50} = 19.6$ and 5.7 nM (10-fold in **40**, 2-fold in **41**). Interestingly, **42** ($m = 0$ and $n = 2$ in thiourea), modified both in m and n, showed a complicated result. Whereas its binding affinity was reduced by 53-fold compared to the parent **1**, its potency as an agonist was unaltered. This finding is yet another example that receptor binding affinity is not fully correlated with agonistic activity. Such divergence between binding affinity and agonism has been described by us previously.[27,37] Since we have shown that RTX binding, like calcium uptake, is mediated through VR1, we assume that the divergence reflects both the influence of factors differentially mediating the intrinsic SAR for the two assays, as well as some contribution from methodological differences in the two assays.[17,27]

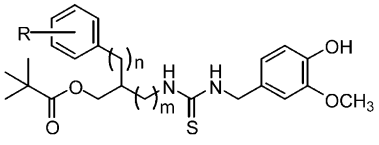
In the similar manner, one-carbon elongation of parent amides **3** and **4** produced compounds **19** and **20** ($m=1$, $n=2$ in amide). As found with **42**, compound **19** displayed high agonistic potency for calcium uptake with a value of $EC_{50}=0.78$ nM, which is 16- and 60-fold more potent, respectively, than parent compound **3** and capsaicin; its binding affinity, with a value of $K_i=153$ nM, was virtually the same as parent compound **3** ($K_i=157$ nM). Once again, this result highlights the distinct structure activity relations of VR1 ligands for binding to VR1 and for stimulation of calcium uptake. Compound **20** displayed a 2-fold reduction in binding affinity with a value of $K_i=36$ nM and a 30-fold reduction in potency as an agonist with a value of $EC_{50}=8.4$ nM as compared to the parent **4**. Analogues shortened by one-carbon, **43** and **44** ($m=0$, $n=1$ in amide), showed significantly reduced binding affinities (45-fold in **43**, 60-fold in **44**) and potencies as agonists (40-fold in **43**, 550-fold in **44**) compared to parent compounds. We conclude that the in vitro receptor activities of amide analogues were more sensitive than those of thiourea analogues to the C1-region modifications. We further note that appropriately substituted amides were similar to or more potent than thiourea analogues, in contrast to the conclusion for some other series of derivatives with different C regions.[38]

3.2. Conformational analysis

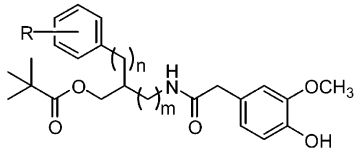
The search for the principal pharmacophores of VR1 ligands and their active conformation is of particular importance for the discovery of potent and selective clinical candidates because of the difficulty in obtaining X-ray structures of membrane proteins such as the vanilloid receptor. To date, several structural and conformational analyses of VR1 ligands have been reported. A conformational rationale for agonism or antagonism of capsaicin analogues was proposed by Walpole et al.[39] based on NMR, X-ray and molecular modeling studies. Quantitative structure activity relationships of capsaicin analogues were studied using MULTICASE methology by Klopman et al.[40] 3-D-QSAR analysis of simplified RTX analogues was investigated using the CoMFA and the COMSIA methods by Kim.[41] Nevertheless, analysis of the active conformation of RTX on binding to the receptor has not been investigated despite the very potent binding affinity of RTX itself.[27] Through the conformational analysis of RTX using NMR and computational methods,[42] Vander Velde et al. proposed four energetically stable conformations of RTX in solution; unfortunately, this approach cannot address the nature of the actual conformation(s) of RTX when bound to VR1.

One strategy to identify an active conformation of VR1 agonists is through correlation between the rms values calculated from respective pharmacophoric matchings of selected agonists on each of multiple stable RTX conformations and their in vitro activities. Seven agonists (**1**, **17**, **40**, **42** in thiourea, **3**, **19**, **43** in amide) were selected by reason of containing in common a 3,4-dimethylphenyl group as a C1-region. Their energy-minimized structures were obtained through conformational analysis by random search using the program Sybyl 6.5 (Tripos). Five stable conformations of RTX (**RTX-A**$\sim$**E**) were utilized for the comparision. The conformations of RTX-A,B,C,D obtained by NMR and molecular modeling studies were imported from the study of Vander Velde and co-workers.[42] The fifth conformation (RTX-E) was derived by us through molecular dynamics simulation using the program Sybyl 6.5 (Tripos) and is illustrated in Figure 6.

Table 1. Potencies of vanilloid agonists for binding to rat VR1 and for inducing calcium influx in CHO/VR1 cells



THIOUREA



AMIDE

	m	n	R	RTX binding (K_i = nM)	Ca^{2+} Influx (EC_{50} = nM)
Capsaicin				1810 (±270)	44.8(±3.8)
Thiourea					
1	1	1	3,4-Me_2	17.4 (±4.1)	1.97 (±0.56)
2	1	1	4-*t*-Bu	6.35 (±0.48)	2.83 (±0.55)
17	1	2	3,4-Me_2	25.0 (±4.4)	6.3 (±2.1)
18	1	2	4-*t*-Bu	18.3(±5.6)	18.7 (±4.2)
40	0	1	3,4-Me_2	45 (±18)	19.6 (±4.9)
41	0	1	4-*t*-Bu	34.6 (±9.6)	5.7 (±2.1)
42	0	2	3,4-Me_2	917 (±430)	2.36 (±0.39)
Amide					
3	1	1	3,4-Me_2	157 (±56)	12.7 (±3.5)
4	1	1	4-*t*-Bu	15.0 (±3.8)	0.29 (±0.1)
19	1	2	3,4-Me_2	153 (±7)	0.78 (±0.25)
20	1	2	4-*t*-Bu	36 (±12)	8.4 (±2.9)
43	0	1	3,4-Me_2	7000 (±4000)	482 (±74)
44	0	1	4-*t*-Bu	910 (±210)	161 (±26)

The center points of the four pharmacophoric groups in the lowest energy conformations of the seven VR1 agonists, including the center of the vanilloid ring (A-region), the sulfur of the thiourea or the oxygen of the amide (B-region), the center of the phenyl ring (C1-region) and the carbonyl of the ester (C2-region), were matched with the center of the vanilloid ring, the C_{20}-ester carbonyl, the center of the orthoester phenyl, and the C_3-keto carbonyl, respectively, in the five conformations of RTX, as indicated in Figure 3. The respective rms values after fitting were calculated for the five conformations (Table 2). For initial comparision, the ranking of the ligands based on quality of fit to each RTX conformation was determined (see numbers in parentheses, Table 2, lowest number indicates the best fit). The ranking of the ligands in terms of their in vitro activities was also indicated. The rank order of binding affinities of the agonists correlated very well with RTX-E; for agonistic potencies, no clear correlation with one of the five RTX conformations was found.

Detailed analysis was performed by calculating the correlation between the rms values in each row of RTX conformations and in vitro activities, binding affinity or agonistic potency, using linear regression. The best-fit lines and regression coefficients for binding affinity and for potency for inducing calcium influx were shown in Figures 4 and 5, respectively. The analyses indicated that the rms values of the agonists generally correlated better with the corresponding binding affinities (K_i values) than with the values for calcium influx (EC_{50} values). This is hardly surprising, since the RTX binding affinity is a more robust measure of receptor interaction than are the calcium uptake values (but see further discussion below).

Among the RTX conformations, the rms values of RTX-E correlated best with the binding affinities of the agonists, yielding a regression coefficient of $r^2 = 0.92$ (Fig. 4e). Consequently, the RTX-E conformation would appear from this analysis to be the most probable

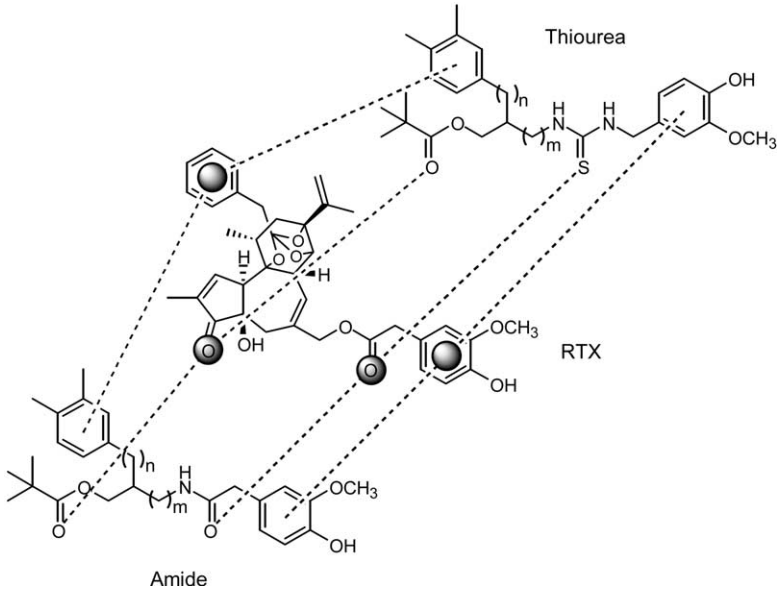


Figure 3. Pharmacophoric matching between RTX and simplified RTX analogues.

Table 2. RMS values of VR agonists after overlay on five conformers of RTX

	RTX–E	1	17	40	42	3	19	43
RTX–A	3.000	3.222 (7)[a]	2.244 (3)	2.768 (6)	2.246 (4)	2.318 (5)	1.564 (1)	2.03 (2)
RTX–B	2.993	3.554 (6)	1.445 (1)	3.958 (7)	2.981 (4)	3.121 (5)	2.173 (2)	2.382 (3)
RTX–C	2.222	2.838 (4)	1.419 (1)	3.787 (7)	3.176 (6)	3.081 (5)	2.625 (2)	2.683 (3)
RTX–D	2.368	3.03 (7)	2.129 (3)	2.49 (6)	2.211 (5)	2.004 (2)	1.942 (1)	2.137 (4)
RTX–E	0.000	1.722 (1)	1.913 (2)	2.429 (3)	3.394 (7)	2.532 (4)	3.091 (5)	3.334 (6)
K_i	0.13[c]	17.4 (1)	25 (2)	45 (3)	917 (6)	157 (5)	153 (4)	7,000 (7)
Ca^{2+}	0.27	1.97 (2)	6.3 (4)	19.6 (6)	2.36 (3)	12.7 (5)	0.78 (1)	482 (7)
Log P[b]	6.86	5.78	6.27	5.29	5.78	5.46	5.95	4.97

[a] The number in the parenthesis indicates the rank of the activity in the corresponding row.
[b] KOWWIN Program (http://esc.syrres.com/).
[c] Ref 27.

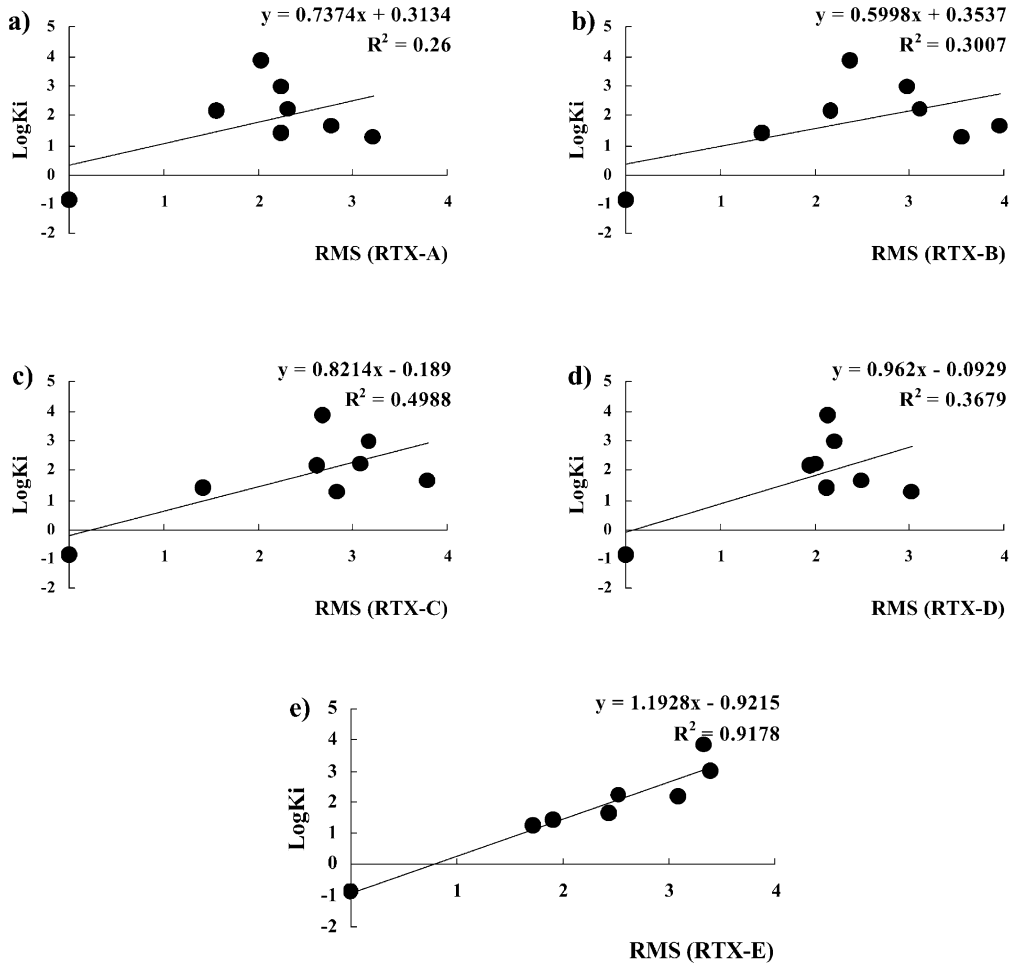


Figure 4. Correlation between rms and binding affinity.

active conformation of RTX on binding to the capsaicin binding site of VR1. We therefore propose the conformation of RTX-E as a plausible model of the active conformation of VR1 ligands based on the four pharmacophore model as shown in Figure 6. In this model, the pharmacophoric distances between the four pharmacophores, the center of the vanilloid ring (A-region), the C_{20}-ester carbonyl (B-region), the center of the orthoester phenyl (C1-region), and the C_3-keto carbonyl (C2-region) are: d(A-B) = 3.9 Å, d(B-C1) = 6.8 Å, d(C1–C2) = 8.2 Å, d(C2-A) = 5.9 Å. Future experience will provide the ultimate measure of the utility of this model for the design of novel VR1 ligands by the pharmacophore-based approach.

An on-going complication in the field of vanilloid pharmacology is that the interplay between ligand binding and gating of the VR1 channel is highly complex. We use ligand binding as a relatively robust measure of ligand–receptor interaction. This assay has the advantage of being conducted with long incubation times, facilitating equilibrium conditions, is not distorted by spare receptors, and is relatively insensitive to perturbation by co-regulators. The calcium influx assay for measuring agonism integrates multiple factors, such as rate of uptake or stability, desensitization, modulation by co-factors, spare receptors, and the pattern of activation of individual cells, all of which are important for this cellular response but which complicate analysis of receptor–ligand interactions. A further complication in vanilloid pharmacology is that the extent of the differences in structure activity relationships for binding and calcium uptake strongly suggests that the two assays detect different pools of VR1. Ultimately, of course, the complete structure activity relations for VR1 as a function of its different co-regulators, its different functional pools, and its different extents of agonism and antagonism will all need to be understood. Clarification of the basis for the structure activity relations as determined for ligand binding to VR1 represents one piece of this challenging and important enterprise.

4. Conclusions

The structure activity relationships of *N*-(3-acyloxy-2-benzylpropyl) homovanillates and *N*′-(4-hydroxy-3-methoxybenzyl) thioureas, high-affinity VR1 agonists, have been investigated through the modification of the C-region and have provided a series of potent agonists with high agonism. The pharmacophoric matching between these agonists and stable RTX conformations suggests a putative conformation of RTX, RTX-E conformation, involved in binding to VR1. Finally, the data

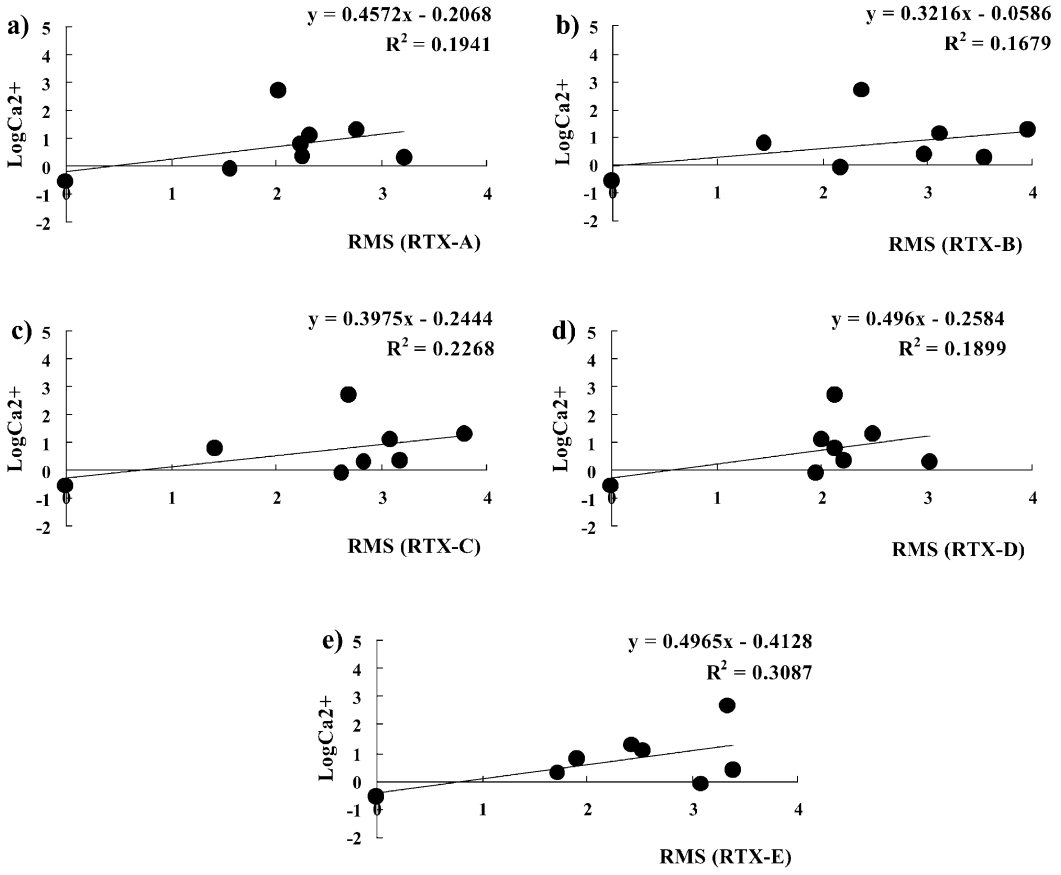


Figure 5. Correlation between rms and calcium influx.

further illustrate the conclusion that the ligand binding and calcium uptake assays for vanilloids reflect somewhat distinct structure activity relations.

5. Experimental

5.1. General method

All chemical reagents were commercially available. Melting points were determined on a Melting Point Büchi B-540 apparatus and are uncorrected. Silica gel column chromatography was performed on silica gel 60, 230–400 mesh, Merck. Proton NMR spectra were recorded on a JEOL JNM-LA 300 at 300 MHz. Chemical shifts are reported in ppm units with Me_4Si as a reference standard. Mass spectra were recorded on a VG Trio-2 GC-MS. Combustion analyses were performed on an EA 1110 Automatic Elemental Analyzer, CE Instruments, and were within 0.4% of the calculated values unless otherwise noted.

5.1.1. 4-(2-Bromoethyl)-1,2-dimethylbenzene (5). A mixture of 3,4-dimethylbenzyl chloride (10 g, 64.7 mmol) and NaCN (15.85 g, 323.3 mmol) in DMF (15 mL) was heated at 100 °C for 16 h. The reaction mixture was cooled, diluted with H_2O and extracted with CH_2Cl_2 several times. The combined organic layers were washed with H_2O, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:10) as eluant to afford 2-(3,4-dimethylphenyl)acetonitrile (9.395 g, 100%) as a colorless oil.; ^1H NMR ($CDCl_3$) δ 6.95–7.25 (m, 5H), 3.60 (s, 2H, CH_2CN), 2.25 (d, 3H, $J = 12.9$ Hz, CH_3), 2.20 (d, 3H, $J = 12.9$ Hz, CH_3).

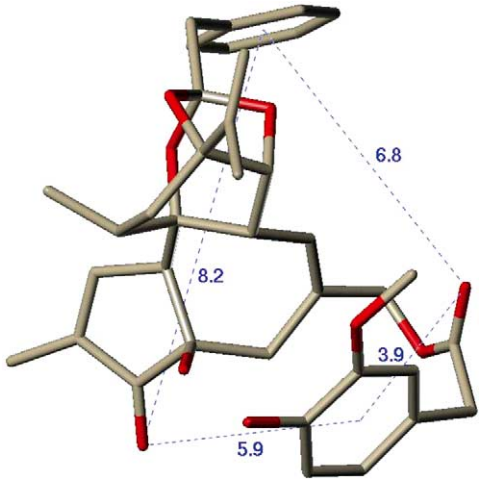


Figure 6. Proposed active conformation of RTX (RTX-E).

A mixture of the above nitrile (9.395 g, 64.7 mmol) and concentrated H_2SO_4 (3.5 mL, 64.7 mmol) in MeOH (25 mL) was refluxed for 3 days. The reaction mixture was cooled, alkalinized with solid $NaHCO_3$ and con-

centrated in vacuo. The residue was partitioned between H_2O and CH_2Cl_2 and the aqueous layer was extracted with CH_2Cl_2 several times. The combined organic layers were washed with H_2O, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:10) as eluant to afford methyl 2-(3,4-dimethylphenyl)acetate (11.285 g, 98%) as a yellow oil; ^{1}H NMR ($CDCl_3$) δ 6.95–7.1 (m, 5H), 3.66 (s, 3H, CO_2CH_3), 3.54 (s, 2H, CH_2CO_2), 2.25 (d, 3H, J = 13.4 Hz, CH_3), 2.20 (d, 3H, J = 9.8 Hz, CH_3).

To a cooled suspension of lithium aluminum hydride (4.8 g, 126.6 mmol) in ether (50 mL) at −10 °C was added dropwise a solution of the above ester (11.285 g, 63.3 mmol) in ether (30 mL) and the reaction mixture was stirred for 30 min at room temperature. The mixture was cooled in an ice-bath, quenched by successive addition of H_2O (5 mL), 15% aqueous NaOH (10 mL), and H_2O (15 mL) and stirred for 1 h at room temperature. The resulting suspension was filtered after addition of EtOAc, and the combined filtrate was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:3) as eluant to afford 2-(3,4-dimethylphenyl)-1-ethanol (5.705 g, 60%) as a colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.95–7.1 (m, 5H), 3.85 (m, 2H, CH_2OH), 2.81 (t, 2H, J = 6.6 Hz, CH_2Ar), 2.25 (m, 6H, 2×CH_3).

A cooled solution of the above alcohol (3.285 g, 21.9 mmol) and triphenylphosphine (6.883 g, 26.2 mmol) in THF (20 mL) at 0 °C was treated with carbon tetrabromide (8.703 g, 26.2 mmol) and stirred for 3 h at room temperature. The reaction mixture was diluted with ether, filtered through a Celite pad and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with hexanes as eluant to afford **5** (4.24 g, 91%) as a colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.95–7.1 (m, 5H), 3.54 (t, 2H, J = 7.3 Hz, CH_2Br), 3.10 (t, 2H, J = 7.8 Hz, CH_2Ar), 2.25 (m, 6H, 2×CH_3).

5.1.2. 4-(2-Bromoethyl)-4-*tert*-butylbenzene (6). A cooled solution of 4-*tert*-butylstyrene (4.81 g, 30 mmol) and sodium borohydride (0.34 g, 9 mmol) in THF (30 mL) was treated dropwise with a solution of boron trifluoride diethyl etherate (1.84 mL, 15 mmol) in THF (10 mL) and stirred for 2 h at room temperature. The reaction mixture was quenched with water (10 mL) carefully and allowed to stand until no further hydrogen was evolved. The solution was made alkaline by the addition of dilute sodium hydroxide solution (15 mL), followed by 30% hydrogen peroxide solution (15 mL) in 2–3 mL portions. The reaction mixture was poured into ice water and extracted with ether several times. The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:5) as eluant to afford 2-(4-*tert*-butylphenyl)-1-ethanol (4.17 g, 78%) as a colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.34 (d, 2H, J = 8.0 Hz), 7.16 (d, 2H, J = 8.0 Hz), 3.86 (dd, 2H, J = 6.4, 12.7 Hz, CH_2OH), 2.85 (t, 2H, J = 6.6 Hz, CH_2Ar), 1.31 (s, 9H, $C(CH_3)_3$).

Compound **6** was prepared from the above alcohol by following the procedure described for the synthesis of **5** as a colorless oil in 90% yield; ^{1}H NMR ($CDCl_3$) δ 7.35 (d, 2H, J = 8.3 Hz), 7.14 (d, 2H, J = 8.3 Hz), 3.55 (t, 2H, J = 7.3 Hz, CH_2Br), 3.10 (t, 2H, J = 7.8 Hz, CH_2Ar), 1.31 (s, 9H, $C(CH_3)_3$).

5.2. General procedure for the synthesis of 7–8

A cooled solution of diethylmalonate (6.4 g, 40 mmol) in DMF (20 mL) at 0 °C was treated with sodium hydride (60%, 1.92 g, 48 mmol) portionwise and stirred for 40 min at room temperature. The reaction mixture was treated with **5–6** (48 mmol) and stirred for 16 h at room temperature. The mixture was diluted with H_2O and extracted with EtOAc several times. The combined organic layers were washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:10) as eluant to afford **7–8**.

5.2.1. Diethyl 2-(3,4-dimethylphenethyl)malonate (7). 76% Yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.9–7.1 (m, 3H), 4.20 (q, 4H, J = 7.1 Hz, 2×$CO_2CH_2CH_3$), 3.34 (t, 1H, J = 7.6 Hz, CH), 2.59 (t, 2H, J = 6.5 Hz, CH_2Ar), 2.1–2.3 (m, 6H, 2×CH_3 and CH_2CH_2Ar), 1.27 (t, 6H, J = 7.1 Hz, 2×$CO_2CH_2CH_3$).

5.2.2. Diethyl-2-(4-*tert*-butylphenethyl)malonate (8). 98% Yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.31 (d, 2H, J = 8.3 Hz), 7.12 (d, 2H, J = 8.3 Hz), 4.19 (q, 4H, J = 7.1 Hz, 2×$CO_2CH_2CH_3$), 3.35 (t, 1H, J = 7.6 Hz, CH), 2.63 (t, 2H, J = 7.3 Hz, CH_2Ar), 2.21 (m, 2H, CH_2CH_2Ar), 1.31 (s, 9H, $C(CH_3)_3$), 1.27 (t, 6H, J = 7.1 Hz, 2×$CO_2CH_2CH_3$).

5.3. General procedure for the synthesis of 9–10

A cooled solution of lithium aluminium hydride (3.64 g, 96 mmol) in diethyl ether (80 mL) at 0 °C was treated dropwise with a solution of **7–8** (24 mmol) in diethyl ether (20 mL). After stirring for 3 h at room temperature, the reaction mixture was cooled over an ice-bath and treated successively by the dropwise addition of H_2O (3.5 mL), 15% NaOH solution (7 mL), and H_2O (10.5 mL). The mixture was filtered by washing with EtOAc and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (3:1) as eluant to afford **9–10**.

5.3.1. 2-(3,4-Dimethylphenethyl)-1,3-propanediol (9). 74% Yield, white solid, mp = 70 °C; ^{1}H NMR ($CDCl_3$) δ 6.9–7.1 (m, 3H), 3.85 (m, 2H, CH_2OH), 3.71 (m, 2H, CH_2OH), 2.60 (t, 2H, J = 7.3 Hz, CH_2Ar), 2.23 (dd, 6H, 2×CH_3), 1.82 (m, 1H, CH), 1.5–1.65 (m, 2H, CH_2CH_2Ar).

5.3.2. 2-(4-*tert*-Butylphenethyl)-1,3-propanediol (10). 86% Yield, white solid, mp = 68 °C; ^{1}H NMR ($CDCl_3$) δ 7.30 (d, 2H, J = 8.3 Hz), 7.12 (d, 2H, J = 8.3 Hz), 3.86 (m, 2H, CH_2OH), 3.72 (m, 2H, CH_2OH), 2.64 (t, 2H, J = 7.3 Hz, CH_2Ar), 1.82 (m, 1H, CH), 1.5–1.65 (m, 2H, CH_2CH_2Ar), 1.31 (s, 9H, $C(CH_3)_3$).

5.3.3. General procedure for the synthesis of 11–12. A cooled solution of 9–10 (15 mmol) and pyridine (16.5 mmol, 1.33 mL) in CH_2Cl_2 (50 mL) was treated with pivaloyl chloride (16.5 mmol, 2.02 mL) at 0 °C. After being stirred for 30 min at 0 °C, the reaction mixture was quenched with ice and extracted with EtOAc several times. The combined organic layers were washed with H_2O and brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by column chromatography on silica gel with EtOAc/hexanes (1:4) as eluant to afford **11–12**.

5.3.4. 4-(3,4-Dimethylphenyl)-2-(hydroxymethyl)butyl pivalate (11). 84% Yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.9–7.1 (m, 3H), 4.26 (dd of AB, 1H, J = 4.4, 11.4 Hz, CH_2OCO), 4.14 (dd of AB, 1H, J = 6.1, 11.4 Hz, CH_2OCO), 3.56 (m, 2H, CH_2OH), 2.62 (t, 2H, J = 7.8 Hz, CH_2Ar), 2.23 (dd, 6H, 2×CH_3), 2.03 (t, 1H, OH), 1.85 (m, 1H, CH), 1.55–1.75 (m, 2H, CH_2CH_2Ar), 1.21 (s, 9H, C(CH_3)$_3$).

5.3.5. 4-(4-*tert*-Butylphenyl)-2-(hydroxymethyl)butyl pivalate (12). 80% Yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.33 (d, 2H, J = 8.2 Hz), 7.14 (d, 2H, J = 8.2 Hz), 4.29 (dd of AB, 1H, J = 4.3, 11.4 Hz, CH_2OCO), 4.17 (dd of AB, 1H, J = 6.0, 11.4 Hz, CH_2OCO), 3.63 (m, 1H, CH_2OH), 3.55 (m, 1H, CH_2OH), 2.68 (t, 2H, J = 7.8 Hz, CH_2Ar), 2.08 (bs, 1H, OH), 1.90 (m, 1H, CH), 1.6–1.75 (m, 2H, CH_2CH_2Ar), 1.33 (s, 9H, C(CH_3)$_3$), 1.24 (s, 9H, COC(CH_3)$_3$).

5.4. General procedure for the synthesis of 13–14

A mixture of **11–12** (10 mmol), triphenylphosphine (20 mmol, 5.25 g), diethyl azodicarboxylate (20 mmol, 3.15 mL) in THF (70 mL) was treated with diphenylphosphorylazide (20 mmol, 4.32 mL) and stirred at room temperature for 18 h. The solvent was removed in vacuo and the residue was purified by column chromatography on silica gel with EtOAc/hexanes (1:10) as eluant to afford **13–14**.

5.4.1. 2-(Azidomethyl)-4-(3,4-dimethylphenyl)butyl pivalate (13). 82% Yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.9–7.1 (m, 3H), 4.10 (m, 2H, CH_2OCO), 3.38 (d, 2H, J = 5.8 Hz, CH_2N_3), 2.60 (t, 2H, J = 7.4 Hz, CH_2Ar), 2.23 (dd, 6H, 2×CH_3), 1.95 (m, 1H, CH), 1.6–1.7 (m, 2H, CH_2CH_2Ar), 1.21 (s, 9H, C(CH_3)$_3$).

5.4.2. 2-(Azidomethyl)-4-(4-*tert*-butylphenyl)butyl pivalate (14). 84% Yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.32 (d, 2H, J = 8.5 Hz), 7.11 (d, 2H, J = 8.5 Hz), 4.12 (dd of AB, 1H, J = 4.9, 11.2 Hz, CH_2OCO), 4.08 (dd of AB, 1H, J = 4.4, 11.4 Hz, CH_2OCO), 3.38 (d, 2H, J = 5.8 Hz, CH_2N_3), 2.64 (t, 2H, J = 7.3 Hz, CH_2Ar), 1.95 (m, 1H, CH), 1.65–1.75 (m, 2H, CH_2CH_2Ar), 1.31 (s, 9H, C(CH_3)$_3$), 1.21 (s, 9H, COC(CH_3)$_3$).

5.5. General procedure for the synthesis of 15–18

A suspension of **13–14** (1 mmol) and Lindler's catalyst (100 mg) in EtOH (10 mL) was hydrogenated under a hydrogen balloon for 2 h. The reaction mixture was filtered and the filtrate was concentrated in vacuo to afford the corresponding amine in a quantitative yield, which was used for the next step without further purification. A solution of amine (1 mmol) in CH_2Cl_2 (10 mL) was treated with isothiocyanate (1 mmol) and stirred overnight at room temperature. After the mixture was concentrated in vacuo, the residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:1) as eluant to afford **15–16**. A cooled solution of **15–16** (1 mmol) in CH_2Cl_2 (4 mL) at 0 °C was treated with trifluoroacetic acid (2 mL) and stirred for 1 h at room temperature. The mixture was quenched with solid $NaHCO_3$, filtered, and the filtrate was concentrated in vacuo. The residue was diluted with EtOAc, washed with $NaHCO_3$, H_2O and brine, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:1) as eluant to afford **17–18**.

5.5.1. *N*-[4-(3,4-Dimethylphenyl)-2-(pivaloyloxymethyl)-butyl] - *N*′ - [3 - methoxy - 4 - (methoxymethoxy)benzyl]-thiourea (15). 85% yield, white solid, mp = 42 °C; ^{1}H NMR ($CDCl_3$) δ 6.8–7.15 (m, 6H), 6.32 (t, 1H, NH), 6.01 (bs, 1H, NH), 5.21 (s, 2H, OCH_2O), 4.50 (d, 2H, J = 5.2 Hz, CSNHCH_2Ar), 4.18 (dd, 1H, J = 3.7, 11.5 Hz, CH_2OCO), 3.95 (dd, 1H, J = 5.1, 11.5 Hz, CH_2OCO), 3.86 (s, 3H, OCH_3), 3.76 (m, 1H, CHCH_2NHCS), 3.50 (s, 3H, OCH_3), 3.23 (m, 1H, CHCH_2NHCS), 2.65 (t, 2H, J = 8.3 Hz, CH_2CH_2Ar), 2.15–2.3 (m, 7H, 2×CH_3), 1.94 (m, 1H, CH), 1.60 (m, 2H, CH_2CH_2Ar), 1.20 (s, 9H, COC(CH_3)$_3$).

5.5.2. *N*-[4-*tert*-Butylphenyl-2-(pivaloyloxymethyl)butyl]-*N*′-[3-methoxy - 4 - (methoxymethoxy)benzyl]thiourea (16). 89% Yield, white solid, mp = 45 °C; ^{1}H NMR ($CDCl_3$) δ 7.30 (d, 2H, J = 8.3 Hz), 7.1–7.15 (m, 3H), 6.8–6.9 (m, 2H), 6.41 (bt, 1H, NH), 6.35 (bs, 1H, NH), 6.10 (bs, 1H, NH), 5.21 (s, 2H, OCH_2O), 4.51 (d, 2H, J = 4.1 Hz, CSNHCH_2Ar), 4.18 (dd, 1H, J = 3.7, 11.5 Hz, CH_2OCO), 3.95 (dd, 1H, J = 5.1, 11.5 Hz, CH_2OCO), 3.86 (s, 3H, OCH_3), 3.75 (m, 1H, CHCH_2NHCS), 3.50 (s, 3H, OCH_3), 3.25 (m, 1H, CHCH_2NHCS), 2.69 (t, 2H, J = 7.8 Hz, CH_2CH_2Ar), 2.03 (m, 1H, CH), 1.60 (m, 2H, CH_2CH_2Ar), 1.30 (s, 3H, C(CH_3)$_3$), 1.19 (s, 9H, COC(CH_3)$_3$).

5.5.3. *N*-[4-(3,4-Dimethylphenyl)-2-(pivaloyloxymethyl)-butyl]-*N*′-[4-hydroxy-3-methoxybenzyl]thiourea (17). 73% Yield, yellow oil; ^{1}H NMR ($CDCl_3$) δ 6.8–7.1 (m, 6H), 6.29 (bt, 1H, NH), 6.00 (bs, 1H, NH), 5.60 (s, 1H, OH), 4.48 (bs, 2H, CSNHCH_2Ar), 4.20 (dd, 1H, J = 3.7, 11.5 Hz, CH_2OCO), 3.96 (dd, 1H, J = 5.1, 11.5 Hz, CH_2OCO), 3.87 (s, 3H, OCH_3), 3.78 (bs, 1H, CHCH_2NHCS), 3.25 (m, 1H, CHCH_2NHCS), 2.65 (t, 2H, J = 8.3 Hz, CH_2CH_2Ar), 2.2–2.3 (m, 6H, 2×CH_3), 2.00 (m, 1H, CH), 1.58 (m, 2H, CH_2CH_2Ar), 1.19 (s, 9H, COC(CH_3)$_3$); IR (neat) 3355, 2962, 1715, 1557, 1516, 1274, 1156 cm^{-1}; MS (FAB) m/z 487 (MH^+). Anal. calcd for $C_{27}H_{38}N_2O_4S$: C, 66.63; H, 7.87; N, 5.76; S, 6.59. Found: C, 66.83; H, 7.90; N, 5.74; S, 6.57.

5.5.4. *N*-[4-*tert*-Butylphenyl-2-(pivaloyloxymethyl)butyl]-*N'* - [4 - hydroxy - 3 - methoxybenzyl]thiourea (18). 78% Yield, yellow oil; ^{1}H NMR ($CDCl_3$) δ 7.33 (d, 2H, J = 8.2 Hz), 7.14 (d, 2H, J = 8.2 Hz), 6.8–6.9 (m, 3H), 6.34 (bt, 1H, NH), 6.10 (bs, 1H, NH), 5.63 (s, 1H, OH), 4.50 (bs, 2H, $CSNHCH_2Ar$), 4.20 (dd, 1H, J = 3.7, 11.6 Hz, CH_2OCO), 3.97 (dd, 1H, J = 5.1, 11.6 Hz, CH_2OCO), 3.89 (s, 3H, OCH_3), 3.79 (bs, 1H, $CHCH_2NHCS$), 3.28 (m, 1H, $CHCH_2NHCS$), 2.71 (t, 2H, J = 8.1 Hz, CH_2CH_2Ar), 2.04 (m, 1H, CH), 1.62 (m, 2H, CH_2CH_2Ar), 1.33 (s, 3H, $C(CH_3)_3$), 1.21 (s, 9H, $COC(CH_3)_3$; IR (neat) 3355, 2962, 1715, 1557, 1516, 1274, 1156 cm^{-1}; MS (FAB) m/z 515 (MH^+). Anal. calcd for $C_{29}H_{42}N_2O_4S$: C, 67.67; H, 8.22; N, 5.44; S, 6.23. Found: C, 67.90; H, 8.24; N, 5.43; S, 6.20.

5.6. General procedure for the synthesis of 19–20

A suspension of **13–14** (1 mmol) and Lindler's catalyst (100 mg) in EtOH (10 mL) was hydrogenated under a hydrogen balloon for 2 h. The reaction mixture was filtered and the filtrate was concentrated in vacuo to afford the corresponding amine in a quantitative yield, which was used for the next step without further purification. A solution of amine (1 mmol) was treated with pentafluorophenyl ester (1 mmol) in CH_2Cl_2 (10 mL), stirred overnight at room temperature and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:1) as eluant to afford **19–20**.

5.6.1. *N* - [4 - (3,4 - Dimethylphenyl) - 2 - (pivaloyloxymethyl)butyl]-2-[4-hydroxy-3-methoxyphenyl]acetamide (19). 59% Yield, pink oil; ^{1}H NMR ($CDCl_3$) δ 6.7–7.05 (m, 6H), 5.76 (bt, 1H, NH), 5.63 (s, 1H, OH), 4.02 (dd, 1H, J = 4.4, 11.5 Hz, CH_2OCO), 3.95 (dd, 1H, J = 5.4, 11.5 Hz, CH_2OCO), 3.86 (s, 3H, OCH_3), 3.49 (s, 2H, $OCCH_2Ar$), 3.29 (m, 1H, CH_2NH), 3.13 (m, 1H, CH_2NH), 2.58 (m, 2H, CH_2CH_2Ar), 2.1–2.3 (m, 6H, 2×CH_3), 1.84 (m, 1H, CH), 1.54 (m, 2H, CH_2CH_2Ar), 1.17 (s, 9H, $COC(CH_3)_3$); IR (neat) 3445, 2962, 1725, 1716, 1650, 1556, 1538, 1516 cm^{-1}; MS (FAB) m/z 456 (MH^+). Anal. calcd for $C_{27}H_{37}NO_5$: C, 71.18; H, 8.19; N, 3.07. Found: C, 71.39; H, 8.20; N, 3.05.

5.6.2. *N* - [4 - (4 - *tert* - Butylphenyl) - 2 - (pivaloyloxymethyl)butyl]-2-[4-hydroxy-3-methoxyphenyl]acetamide (20). 55% yield, yellow oil; ^{1}H NMR ($CDCl_3$) δ 7.28 (d, 2H, J = 8.2 Hz), 7.06 (d, 2H, J = 8.3 Hz), 6.7-6.9 (m, 3H), 5.75 (bt, 1H, NH), 5.57 (s, 1H, OH), 4.04 (dd, 1H, J = 4.6, 11.6 Hz, CH_2OCO), 3.94 (dd, 1H, J = 5.1, 11.6 Hz, CH_2OCO), 3.86 (s, 3H, OCH_3), 3.49 (s, 2H, $OCCH_2Ar$), 3.28 (m, 1H, CH_2NH), 3.14 (m, 1H, CH_2NH), 2.60 (dd, 2H, J = 6.3, 9.5 Hz CH_2CH_2Ar), 1.86 (m, 1H, CH), 1.56 (m, 2H, CH_2CH_2Ar), 1.30 (s, 3H, $C(CH_3)_3$), 1.17 (s, 9H, $COC(CH_3)_3$); IR (neat) 3444, 2960, 1726, 1716, 1651, 1557, 1539, 1516 cm^{-1}; MS (FAB) m/z 484 (MH^+). Anal. calcd for $C_{29}H_{41}NO_5$: C, 72.02; H, 8.54; N, 2.90. Found: C, 72.24; H, 8.56; N, 2.88.

5.7. General procedure for the synthesis of 22–24

A mixture of *N*-(diphenylmethylene)glycine ethyl ester (**21**) (3 mmol, 800 mg) and potassium hydroxide (9 mmol, 505 mg) in dimethylsulfoxide (5 mL) was treated with benzyl halide (3 mmol) at 0 °C and stirred for 30 min at room temperature. The reaction mixture was neutralized with 1 N hydrochloric acid and extracted with EtOAc several times. The combined organic layers were washed with water and brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:10) as eluant to afford **22–24**.

5.8. Ethyl 3-(3,4-dimethylphenyl) - 2 - [(diphenylmethylene)amino]propanoate (22)

42% Yield, yellow oil; ^{1}H NMR ($CDCl_3$) δ 7.55 (m, 2H), 7.2–7.45 (m, 6H), 6.6–7.0 (m, 5H), 4.1–4.3 (m, 3H, $NCHCO_2CH_2$), 3.0–3.4 (m, 2H, CH_2Ar), 2.1–2.2 (m, 6H, 2×CH_3),1.26 (m, 3H, $CO_2CH_2CH_3$).

5.8.1. Ethyl 3-(4-*tert*-butylphenyl)-2-[(diphenylmethylene)amino]propanoate (23). 70% yield, white solid, mp = 44 °C; ^{1}H NMR ($CDCl_3$) δ 7.58 (d, 2H, J = 8.3 Hz), 7.15–7.4 (m, 10H), 6.94 (d, 2H, J = 8.0 Hz), 4.1–4.25 (m, 3H, $NCHCO_2CH_2$), 3.24 (dd of AB, 1H, J = 4.1, 13.2 Hz, CH_2Ar), 3.12 (dd of AB, 1H, J = 9.2, 13.2 Hz, CH_2Ar), 1.30 (s, 9H, $C(CH_3)_3$), 1.25 (t, 3H, J = 7.0 Hz, $CO_2CH_2CH_3$).

5.8.2. Ethyl 4-(3,4-dimethylphenyl)-2-[(diphenylmethylene)amino]butanoate (24). 65% Yield, yellow oil; ^{1}H NMR ($CDCl_3$) δ 7.66 (m, 2H), 7.35–7.5 (m, 6H), 7.14 (m, 2H), 6.85–7.0 (m, 3H), 4.0–4.25 (m, 3H, $NCHCO_2CH_2$), 2.4–2.6 (m, 2H, CH_2Ar), 2.15–2.3 (m, 8H, 2×CH_3 and $NCHCH_2$),1.26 (t, 3H, J = 5.0 Hz, $CO_2CH_2CH_3$).

5.9. General procedure for the synthesis of 25–27

A solution of **22–24** (1 mmol) in tetrahydrofuran (10 mL) was adjusted to pH 4 with 1 N hydrochloric acid and stirred for 30 min. The mixture was neutralized with 1 N sodium hydroxide solution and extracted with ethyl acetate several times. The combined organic layers were washed with water and brine, dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:2) as eluant to afford **25–27**.

5.9.1. Ethyl 2-amino-3-(3,4-dimethylphenyl)propanoate (25). 61% yield, yellow oil; ^{1}H NMR ($CDCl_3$) δ 6.9–7.1 (m, 3H), 4.1–4.25 (m, 2H, CO_2CH_2), 3.70 (m, 1H, NH_2CH), 3.0–3.2 (m, 1H, CH_2Ar), 2.80 (m, 1H, CH_2Ar), 2.2–2.3 (m, 6H, 2×CH_3), 1.25 (m, 3H, $CO_2CH_2CH_3$).

5.9.2. Ethyl 2-amino-3-(4-*tert*-butylphenyl)propanoate (26). 81% yield, yellow oil; ^{1}H NMR ($CDCl_3$) δ 7.32 (d, 2H, J = 8.3 Hz), 7.13 (d, 2H, J = 8.3 Hz), 4.17 (q, 2H, J = 7.1 Hz, CO_2CH_2), 3.70 (dd, 1H, J = 5.2, 8.0 Hz, NH_2CH), 3.05 (dd of AB, 1H, J = 5.2, 13.5 Hz, CH_2Ar), 3.82 (dd of AB, 1H, J = 8.0, 13.5 Hz, CH_2Ar), 1.30 (s, 9H, $C(CH_3)_3$), 1.23 (t, 3H, J = 7.1 Hz, $CO_2CH_2CH_3$).

5.9.3. Ethyl 2-amino-4-(3,4-dimethylphenyl)butanoate (27). 82% Yield, yellow oil; ^{1}H NMR ($CDCl_3$) δ 6.9–7.1 (m, 3H), 4.18 (q, 2H, J = 7.1 Hz, CO_2CH_2), 3.45 (dd, 1H,

$J = 5.4$, 7.8 Hz, NH_2CH), 2.6–2.7 (m, 2H, CH_2Ar), 2.3–2.4 (m, 6H, $2\times CH_3$), 2.05 (m, 1H, CH_2CH_2Ar), 1.82 (m, 1H, CH_2CH_2Ar), 1.28 (m, 3H, $J = 7.1$ Hz, $CO_2CH_2CH_3$).

5.10. General procedure for the synthesis of 28–30

A mixture of **25–27** (1 mmol) and di-*tert*-butyl dicarbonate (1.2 mmol) in tetrahydrofuran (5 mL) was stirred for 16 h and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:4) as eluant to afford **28–30**.

5.10.1. Ethyl 2-[(*tert*-butoxycarbonyl)amino]-3-(3,4-dimethylphenyl)propanoate (28). 98% Yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.85–7.1 (m, 3H), 4.96 (m, 1H, NH), 4.50 (m, 1H, NHC*H*), 4.1–4.2 (m, 2H, CO_2CH_2), 3.0–3.2 (m, 2H, CH_2Ar), 2.2–2.35 (m, 6H, $2\times CH_3$), 1.41 (d, 9H, $J = 6.8$ Hz, $CO_2C(CH_3)_3$), 1.2–1.3 (t, 3H, $CO_2CH_2CH_3$).

5.10.2. Ethyl 2-[(*tert*-butoxycarbonyl)amino]-3-(4-*tert*-butylphenyl)propanoate (29). 98% Yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.30 (d, 2H, $J = 8.3$ Hz), 7.06 (d, 2H, $J = 8.3$ Hz), 4.96 (bd, 1H, NH), 4.56 (m, 1H, NHC*H*), 4.16 (q, 2H, $J = 7.1$ Hz, CO_2CH_2), 3.04 (d, 2H, CH_2Ar), 1.42 (s, 9H, $CO_2C(CH_3)_3$), 1.30 (s, 9H, $C(CH_3)_3$), 1.22 (t, 3H, $J = 7.1$ Hz, $CO_2CH_2CH_3$).

5.10.3. Ethyl 2-[(*tert*-butoxycarbonyl)amino]-4-(3,4-dimethylphenyl)butanoate (30). 94% Yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.90–7.1 (m, 3H), 5.08 (d, $J = 7.5$ Hz, 1H, NH), 4.32 (m, 1H, NHC*H*), 4.16 (q, 2H, $J = 7.1$ Hz, CO_2CH_2), 2.5–2.7 (m, 2H, CH_2Ar), 2.2–2.35 (m, 6H, $2\times CH_3$), 2.10 (m, 1H, CH_2CH_2Ar), 1.90 (m, 1H, CH_2CH_2Ar), 1.45 (s, 9H, $CO_2C(CH_3)_3$), 1.23 (t, 3H, $J = 7.1$ Hz, $CO_2CH_2CH_3$).

5.11. General procedure for the synthesis of 31–33

A cooled suspension of lithium aluminium hydride (2 mmol) in diethyl ether (10 mL) at 0 °C was treated dropwise with **28–30** (1 mmol) in diethyl ether (10 mL). After being stirred for 1 h at room temperature, the mixture was cooled and quenched with H_2O (1 mL), 15% NaOH solution (2 mL) and H_2O (3 mL) successively. The suspension was filtered with washing of EtOAc and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (4:1) as eluant to afford **31–33**.

5.11.1. *tert*-Butyl *N*-[3-(3,4-dimethylphenyl)-1- (hydroxymethyl)propyl]carbamate (31). 85% Yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.9–7.1 (m, 3H), 4.70 (bs, 1H, NH), 3.82 (bs, 1H, NHC*H*), 3.5–3.7 (m, 2H, CH_2OH), 2.7–2.9 (m, 2H, CH_2Ar), 2.2–2.3 (m, 6H, $2\times CH_3$), 1.42 (s, 9H, $CO_2C(CH_3)_3$).

5.11.2. *tert*-Butyl *N* - [3 - (4 - *tert* - butylphenyl)-1-(hydroxymethyl)propyl]carbamate (32). 80% Yield, white solid, mp = 48 °C; ^{1}H NMR ($CDCl_3$) δ 7.32 (d, 2H, $J = 8.3$ Hz), 7.13 (d, 2H, $J = 8.3$ Hz), 4.70 (bs, 1H, NH), 3.86 (bs, 1H, NHC*H*), 3.67 (dd, 1H, $J = 3.4$, 11.0 Hz, CH_2OH), 3.55 (dd, 1H, $J = 5.6$, 11.0 Hz, CH_2OH), 2.80 (d, 2H, $J = 7.3$ Hz, CH_2Ar), 1.41 (s, 9H, $CO_2C(CH_3)_3$), 1.30 (s, 9H, $C(CH_3)_3$).

5.11.3. *tert*-Butyl *N*-[2-(3,4-dimethylphenyl)-1- (hydroxymethyl)ethyl]carbamate (33). 96% Yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.9–7.1 (m, 3H), 4.70 (bs, 1H, NH), 3.82 (bs, 1H, NHC*H*), 3.5–3.7 (m, 2H, CH_2OH), 2.7–2.9 (m, 2H, CH_2Ar), 2.2–2.3 (m, 6H, $2\times CH_3$), 2.05 (m, 1H, CH_2CH_2Ar), 1.85 (m, 1H, CH_2CH_2Ar), 1.42 (s, 9H, $CO_2C(CH_3)_3$).

5.12. General procedure for the synthesis of 34–36

A cooled solution of 31-33 (1 mmol), triethylamine (3 mmol) and dimethylaminopyridine (0.1 mmol) at 0 °C in CH_2Cl_2 (10 mL) was treated with pivaloyl chloride (1.5 mmol) and stirred for 1 h at room temperature. The mixture was concentrated in vacuo and the residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:4) as eluant to afford **34–36**.

5.12.1. 2-[(*tert*-Butoxycarbonyl)amino]-3-(3,4-dimethylphenyl)propyl pivalate (34). 99% Yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.9–7.1 (m, 3H), 4.61 (d, 1H, NH), 3.9–4.2 (m, 3H, CH_2OCO and NHC*H*), 2.6–2.8 (m, 2H, CH_2Ar), 2.2–2.3 (m, 6H, $2\times CH_3$), 1.41 (s, 9H, $CO_2C(CH_3)_3$), 1.23 (s, 9H, $COC(CH_3)_3$).

5.12.2. 2-[(*tert*-Butoxycarbonyl)amino]-3-(4-*tert*-butylphenyl)propyl pivalate (35). 99% Yield, white solid, mp = 58 °C; ^{1}H NMR ($CDCl_3$) δ 7.31 (d, 2H, $J = 8.3$ Hz), 7.10 (d, 2H, $J = 8.3$ Hz), 4.57 (bs, 1H, NH), 4.0–4.2 (m, 3H, CH_2OCO and NHC*H*), 2.75–2.9 (m, 2H, CH_2Ar), 1.41 (s, 9H, $CO_2C(CH_3)_3$), 1.30 (s, 9H, $C(CH_3)_3$), 1.23 (s, 9H, $COC(CH_3)_3$).

5.12.3. 2-[(*tert*-Butoxycarbonyl)amino]-4-(3,4-dimethylphenyl)butyl pivalate (36). 87% Yield, white solid, mp = 52 °C; ^{1}H NMR ($CDCl_3$) δ 6.9–7.1 (m, 3H), 4.52 (d, 1H, NH), 3.9–4.2 (m, 3H, CH_2OCO and NHC*H*), 2.6–2.8 (m, 2H, CH_2Ar), 2.2–2.3 (m, 6H, $2\times CH_3$), 1.6–1.8 (m, 2H, CH_2CH_2Ar), 1.45 (s, 9H, $CO_2C(CH_3)_3$), 1.20 (s, 9H, $COC(CH_3)_3$).

5.13. General procedure for the synthesis of 37–42

A cooled solution of **34–36** (1 mmol) in CH_2Cl_2 (8 mL) at 0 °C was treated dropwise with trifluoroacetic acid (2 mL) and stirred for 3 h at room temperature. The mixture was concentrated in vacuo to afford the amine salt which was used for the next step without further purification. A solution of the amine salt (1 mmol) and triethylamine (1.2 mmol) in CH_2Cl_2 (10 mL) was treated with isothiocyanate (1 mmol) and stirred overnight at room temperature. After the mixture was concentrated in vacuo, the residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:1) as eluant to afford **37–39**. A cooled solution of **37–39** (1 mmol) in CH_2Cl_2 (4 mL) at 0 °C was treated with trifluoroacetic acid (2 mL) and stirred for 1 h at room temperature. The mixture was quenched with solid $NaHCO_3$, filtered, and the filtrate was concentrated in

vacuo. The residue was diluted with EtOAc, washed with $NaHCO_3$, H_2O and brine, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:1) as eluant to afford **40–42**.

5.13.1. *N*-[3-(3,4-Dimethylphenyl)-1-pivaloyloxy-2-propyl]-*N*′-[3-methoxy-4-(methoxymethoxy)benzyl]thiourea (37). 97% Yield, yellow oil; ^{1}H NMR ($CDCl_3$) δ 6.7–7.1 (m, 6H), 6.36 (bs, 1H, NH), 6.03 (d, 1H, J = 8.0 Hz, NH), 5.21 (s, 2H, OCH_2O), 4.70 (m, 1H, CH), 4.46 (bs, 2H, $NHCH_2Ar$), 4.15 (dd, 1H, J = 5.1, 11.5 Hz, CH_2OCO), 3.98 (dd, 1H, J = 4.7, 11.5 Hz, CH_2OCO), 3.85 (s, 3H, OCH_3), 3.50 (s, 3H, OCH_3), 2.98 (dd, 1H, J = 5.1, 13.4 Hz, CH_2Ar), 2.68 (dd, 1H, J = 8.3, 13.4 Hz, CH_2Ar), 2.2–2.3 (m, 6H, 2×CH_3), 1.19 (s, 9H, $COC(CH_3)_3$).

5.13.2. *N*-[3-(4-*tert*-Butylphenyl)-1-pivaloyloxy-2-propyl]-*N*′-[3-methoxy-4-(methoxymethoxy)benzyl]thiourea (38). 52% Yield, yellow oil; ^{1}H NMR ($CDCl_3$) δ 7.31 (d, 2H, J = 8.3 Hz), 7.12 (d, 2H, J = 8.3 Hz), 7.10 (d, 1H, J = 8 Hz), 6.88 (d, 1H, J = 1.7 Hz), 6.80 (dd, 1H, J = 1.7, 8 Hz), 6.42 (bs, 1H, NH), 6.04 (d, 1H, J = 7.6 Hz, NH), 5.21 (s, 2H, OCH_2O), 4.70 (bs, 1H, CH), 4.48 (bs, 2H, $NHCH_2Ar$), 4.16 (dd, 1H, J = 6.3, 11.4 Hz, CH_2OCO), 3.98 (dd, 1H, J = 4.6, 11.4 Hz, CH_2OCO), 3.86 (s, 3H, OCH_3), 3.50 (s, 3H, OCH_3), 3.01 (dd, 1H, J = 4.6, 13.4 Hz, CH_2Ar), 2.73 (dd, 1H, J = 8.0, 13.4 Hz, CH_2Ar), 1.30 (s, 9H, $C(CH_3)_3$), 1.18 (s, 9H, $COC(CH_3)_3$).

5.13.3. *N*-[4-(3,4-Dimethylphenyl)-1-pivaloyloxy-2-butyl]-*N*′-[3-methoxy-4-(methoxymethoxy)benzyl]thiourea (39). 64% Yield, white solid, mp = 62 °C; ^{1}H NMR ($CDCl_3$) δ 6.8–7.1 (m, 6H), 6.52 (bs, 1H, NH), 5.94 (bs, 1H, NH), 5.20 (s, 2H, OCH_2O), 4.52 (bs, 2H, $NHCH_2Ar$), 4.26 (dd, 1H, J = 4.4, 11.5 Hz, CH_2OCO), 3.96 (bs, 1H, CH_2OCO), 3.85 (s, 3H, OCH_3), 3.49 (s, 3H, OCH_3), 2.5–2.7 (m, 2H, CH_2Ar), 2.1–2.3 (m, 6H, 2×CH_3), 1.80 (m, 2H, CH_2CH_2Ar), 1.14 (s, 9H, $COC(CH_3)_3$).

5.13.4. *N*-[3-(3,4-Dimethylphenyl)-1-pivaloyloxy-2-propyl]-*N*′-[4-hydroxy-3-methoxybenzyl]thiourea (40). 61% Yield, white solid, mp = 43–48 °C; ^{1}H NMR ($CDCl_3$) δ 6.7–7.05 (m, 6H), 6.35 (bs, 1H, NH), 6.00 (t, 1H, NH), 5.68 (bs, 1H, OH), 4.70 (bs, 1H, CHNH), 4.40 (bs, 2H, $NHCH_2Ar$), 4.13 (m, 1H, CH_2OCO), 4.00 (m, 1H, CH_2OCO), 3.86 (s, 3H, OCH_3), 3.00 (ddd, 1H, CH_2Ar), 2.73 (ddd, 1H, CH_2Ar), 2.2–2.3 (m, 6H, 2×CH_3), 1.19 (s, 9H, $COC(CH_3)_3$); IR (KBr) 3352, 2960, 1728, 1515, 1461, 1364, 1273, 1154, 1033 cm^{-1}; MS (FAB) m/z 459 (MH^+). Anal. calcd for $C_{25}H_{34}N_2O_4S$: C, 65.47; H, 7.47; N, 6.11; S, 6.99. Found: C, 65.66; H, 7.49; N, 6.09; S, 6.96.

5.13.5. *N*-[3-(4-*tert*-Butylphenyl)-1-pivaloyloxy-2-propyl]-*N*′-[4-hydroxy-3-methoxybenzyl]thiourea (41). 62% Yield, white solid, mp = 57–60 °C; ^{1}H NMR ($CDCl_3$) δ 7.30 (d, 2H, J = 8.3 Hz), 7.10 (d, 2H, J = 8.3 Hz), 6.87 (d, 1H, J = 8 Hz, H-5), 6.83 (d, 1H, J = 1.7 Hz, H-2), 6.77 (dd, 1H, J = 1.7, 8 Hz, H-6), 6.36 (bs, 1H, NH), 5.99 (d, 1H, J = 8.0 Hz, NH), 5.65 (bs, 1H, OH), 4.71 (bs, 1H, CH), 4.42 (bs, 2H, $NHCH_2Ar$), 4.16 (dd, 1H, J = 5.1, 11.5 Hz, CH_2OCO), 3.99 (dd, 1H, J = 4.4, 11.4 Hz, CH_2OCO), 3.87 (s, 3H, OCH_3), 3.00 (dd, 1H, J = 4.9, 13.4 Hz, CH_2Ar), 2.72 (dd, 1H, J = 8.0, 13.4 Hz, CH_2Ar), 1.30 (s, 9H, $C(CH_3)_3$), 1.18 (s, 9H, $COC(CH_3)_3$); IR (KBr) 3353, 2960, 1729, 1514, 1462, 1363, 1274, 1154, 1034 cm^{-1}; MS (FAB) m/z 487 (MH^+). Anal. calcd for $C_{27}H_{38}N_2O_4S$: C, 66.63; H, 7.87; N, 5.76; S, 6.59. Found: C, 66.84; H, 7.89; N, 5.72; S, 6.56.

5.13.6. *N*-[4-(3,4-Dimethylphenyl)-1-pivaloyloxy-2-butyl]-*N*′-[4-hydroxy-3-methoxybenzyl]thiourea (42):. 76% yield, white solid, mp = 38-40 °C; ^{1}H NMR ($CDCl_3$) δ 6.7–7.0 (m, 6H), 6.53 (bs, 1H, NH), 5.92 (bs, 1H, NH), 5.69 (s, 1H, OH), 4.46 (bs, 2H, $NHCH_2Ar$), 4.25 (dd, 1H, J = 4.6, 11.5 Hz, CH_2OCO), 3.96 (bs, 1H, CH_2OCO), 3.85 (s, 3H, OCH_3), 2.57 (t, 2H, J = 7.6 Hz, CH_2Ar), 2.1-2.3 (m, 6H, 2×CH_3), 1.80 (m, 2H, CH_2CH_2Ar), 1.14 (s, 9H, $COC(CH_3)_3$); IR (KBr) 3357, 2968, 1727, 1613, 1537, 1514, 1480, 1462, 1432, 1367, 1278, 1237, 1155, 1034 cm^{-1}; MS (FAB) m/z 473 (MH^+). Anal. calcd for $C_{26}H_{36}N_2O_4S$: C, 66.07; H, 7.68; N, 5.93; S, 6.78. Found: C, 66.30; H, 7.70; N, 5.90; S, 6.75.

5.13.7. General procedure for the synthesis of 43–44. A cooled solution of **34–36** (1 mmol) in CH_2Cl_2 (8 mL) at 0 °C was treated dropwise with trifluoroacetic acid (2 mL) and stirred for 3 h at room temperature. The mixture was concentrated in vacuo to afford the amine salt which was used for the next step without further purification. A solution of amine salt (1 mmol) and triethylamine (1.2 mmol)) in CH_2Cl_2 (10 mL) was treated with pentafluorophenyl ester (1 mmol) in CH_2Cl_2 (10 mL) and stirred overnight at room temperature. After the mixture was concentrated in vacuo, the residue was purified by flash column chromatography on silica gel with EtOAc/hexanes (1:1) as eluant to afford **43–44**.

5.13.8. *N*-[3-(3,4-Dimethylphenyl)-1-pivaloyloxy-2-propyl]-2-[4-hydroxy-3-methoxyphenyl]acetamide (43). 78% yield, yellow oil; ^{1}H NMR ($CDCl_3$) δ 6.6-7.05 (m, 6H), 5.58 (d, 1H, J = 8.5 Hz, NH), 4.34 (m, 1H, CH), 3.97 (dd of AB, 2H, CH_2OCO), 3.85 (s, 3H, OCH_3), 3.46 (s, 2H, $OCCH_2Ar$), 2.68 (ddd of AB, 2H, CH_2Ar), 2.2–2.3 (m, 6H, 2×CH_3), 1.14 (s, 9H, $COC(CH_3)_3$); IR (neat) 3295, 2969, 1730, 1650, 1514, 1455, 1366, 1279, 1236, 1155, 1035 cm^{-1}; MS (FAB) m/z 428 (MH^+). Anal. calcd for $C_{25}H_{33}NO_5$: C, 70.23; H, 7.78; N, 3.28. Found: C, 70.47; H, 7.81; N, 3.26.

5.13.9. *N*-[3-(4-*tert*-Butylphenyl)-1-pivaloyloxy-2-propyl]-2-[4-hydroxy-3-methoxyphenyl]acetamide (44). 79% yield, yellow oil; ^{1}H NMR ($CDCl_3$) δ 7.25 (d, 2H, J = 8.3 Hz), 6.94 (d, 2H, J = 8.3 Hz), 6.88 (d, 1H, J = 8 Hz, H-5), 6.69 (d, 1H, J = 2 Hz, H-2), 6.65 (dd, 1H, J = 8, 2 Hz, H-6), 5.57 (d, 1H, J = 8.5 Hz, NH), 5.30 (s, 1H, OH), 4.40 (m, 1H, CH), 3.95 (ddd of AB, 2H, CH_2OCO), 3.86 (s, 3H, OCH_3), 3.47 (s, 2H, $OCCH_2Ar$), 2.72 (ddd of AB, 2H, CH_2Ar), 1.29 (s, 9H, $C(CH_3)_3$), 1.13 (s, 9H, $COC(CH_3)_3$); IR (neat) 3296, 2963, 1729, 1650, 1514, 1462, 1364, 1279, 1236, 1155, 1035 cm^{-1}; MS (FAB) m/z 456 (MH^+). Anal. calcd for $C_{27}H_{37}NO_5$: C, 71.18; H, 8.19; N, 3.07. Found: C, 71.42; H, 8.22; N, 3.05.

5.14. Molecular modeling

The structures of all chemicals, resiniferatoxin (RTX) and thiourea and amide analogues, were built using the Sybyl molecular modeling program (Tripos, Inc.), and then the geometries were fully optimized using the Tripos force field with the following non-default options (method: conjugate gradient, termination: gradient 0.005 kcal/mol·Å, and max iterations: 10,000). The partial atomic charges were calculated by the Gasteiger-Hückel method in the Sybyl program. The available conformational space of RTX was explored using molecular dynamic (MD) simulation. The MD simulation was accomplished using the Sybyl Dynamic program. The active conformation of RTX was generated by the following protocol as previously described:[28] The system was brought to equilibrium, over 1 ps at 300 K, before a molecular simulation study spanning, a further 500 ps (at 300 K) was undertaken. Snap shots taken at 1 ps intervals were minimized using the optimization criteria outlined above. The conformational analyses of thiourea and amide analogues (**1**, **3**, **17**, **19**, **40**, **42**, and **43**) were performed using the Sybyl Random Search method (Maximum Cycles: 2,000 and Chirality: *R* configuration). As a final step, global minima were found for each chemical and used in the further analysis. The pharmacophore model has two hydrophobic ring systems and two hydrogen bond acceptors. The center of vanilloid ring, the center of orthoester phenyl ring, the C_3-keto carbonyl, and the C_{20}-ester carbonyl in RTX were superimposed with the center of vanilloid ring, the center of phenyl ring, the carbonyl of ester, and the sulfur atom of the thiourea or the oxygen atom of the amide in the vanilloid agonist templates. All calculations for thiourea and amide analogues were performed on a Silicon Graphics O_2 R10000 workstation.

5.15. Statistical data analysis

The regression equations of the best-fit line and the values of the correlation coefficient R^2 were measured with Microsoft Excel. Since the data of binding affinity (K_is) and calcium influx (EC_{50}s) have a wide range of values each data point was log-transformed.

5.16. VR1 binding and $^{45}Ca^{2+}$ uptake assay

The methods were described in previous reports.[27,33]

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