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N-(3-Acyloxy-2-benzylpropyl)-*N*′-(4-hydroxy-3-methoxybenzyl) thiourea Derivatives as Potent Vanilloid Receptor Agonists and Analgesics

Jeewoo Lee,[a,*] Jiyoun Lee,[a] Jiyoung Kim,[a] Soo Yeon Kim,[a] Moon Woo Chun,[a] Hawon Cho,[b] Sun Wook Hwang,[b] Uhtaek Oh,[b] Young Ho Park,[c] Victor E. Marquez,[d] Maryam Beheshti,[e] Tamas Szabo[e] and Peter M. Blumberg[e]

[a]*Laboratory of Medicinal Chemistry, College of Pharmacy, Seoul National University, Shinlim-Dong, Kwanak-Ku, Seoul 151-742, South Korea*

[b]*Sensory Research Group, CRI, College of Pharmacy, Seoul National University, Shinlim-Dong, Kwanak-Ku, Seoul 151-742, South Korea*

[c]*Pacific R&D Center, 314-1, Bora-Ri, Kiheung-Eup, Yougin-Si, Kyounggi-Do 449-900, South Korea*

[d]*Laboratory of Medicinal Chemistry, Division of Basic Sciences, National Cancer Institute, National Institute of Health, Bethesda, MA 20892, USA*

[e]*Laboratory of Cellular Carcinogenesis and Tumor Promotion, Division of Basic Sciences, National Cancer Institute, National Institute of Health, Bethesda, MA 20892, USA*

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Abstract—A series of *N*-(3-acyloxy-2-benzylpropyl)-*N*′-(4-hydroxy-3-methoxybenzyl)thiourea derivatives were investigated as vanilloid receptor ligands in an effort to discover a novel class of analgesics. The proposed pharmacophore model of resiniferatoxin, which includes the C_{20}-homovanillic moiety, the C_3-carbonyl and the orthoester phenyl ring as key pharmacophoric groups, was utilized as a guide for drug design. The compounds were synthesized after several steps from diethylmalonate and evaluated in vitro in a receptor binding assay and in a capsaicin-activated channel assay. Additional evaluation of analgesic activity, anti-inflammatory activity and pungency was conducted in animal models by the writhing test, the ear edema assay, and the eye-wiping test, respectively. Among the new compounds, **23** and **28** were found to be the most potent receptor agonists of the series with K_i values of 19 nM and 11 nM, respectively. Their strong in vitro potencies were also reflected by an excellent analgesic profile in animal tests with ED_{50} values of 0.5 μg/kg for **23** and 1.0 μg/kg for **28**. Relative to capsaicin these compounds appear to be ca. 600 and 300 times more potent. Both **23** and **28** were found to be less pungent than capsaicin based on the eye-wiping test. However, the compounds did not show significant anti-inflammatory activity. A molecular modeling study comparing the energy-minimized structures of resiniferatoxin and **35** demonstrated a good correlation in the spatial disposition of the corresponding key pharmacophores. The thioureas described in this investigation, which were designed as simplified resiniferatoxin surrogates, represent a novel class of potent vanilloid receptor agonists endowed with potent analgesic activity and reduced pungency.

Introduction

The vanilloid receptor (VR) (or capsaicin receptor) is a specific neuronal membrane recognition site for capsaicin (CAP) and related irritant compounds.[1] It is expressed almost exclusively by primary sensory neurons involved in nociception and neurogenic inflammation.[2] The receptor functions as a cation-selective ion channel with a preference for calcium, and its functional subtype VR1, activated by both CAP and noxious heat, has recently been cloned.[3] Its desensitization caused by specific ligands has been recognized as a promising therapeutic approach to mitigate neuropathic pain and other pathological conditions in which neuropeptides released from primary sensory neurons play a crucial role.[3–6]

Most exogenous VR agonists which are being developed or used as analgesics are structurally related to CAP

*Corresponding author. Tel.: +82-2-880-7846; fax: +82-2-888-06469; e-mail: jeewoo@snu.ac.kr

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(i.e., Zostrix, Olvanil, SDZ-249482, and DA-5018) and resiniferatoxin.[7] Their structures include a common vanilloid ring which appears to be important for agonist activity. However, in a recent report, it was demonstrated that compounds lacking the vanilloid moiety, such as sesquiterpenoid unsaturated dialdehydes or triprenyl phenol, may also activate the receptor.[8] Although receptor antagonists are fewer, several compounds such as capsazepine, which acts competitively at the CAP binding site,[9] the channel blocker ruthenium red[10] and capsazocaine[11] have been reported.

Resiniferatoxin (RTX), a tricyclic diterpene isolated from *Euphorbia resinifera*, has been regarded as an ultrapotent CAP analogue (Chart 1). Indeed, the specific binding of RTX to the CAP binding site in dorsal root ganglia has been demonstrated with labelled [^{3}H]RTX.[12] RTX is being developed as an ultrapotent sensory neuron desensitising agent for the treatment of urinary urge incontinence and the pain associated with diabetic neuropathy.[13] Recently, a totally enantiocontrolled synthesis[14] and a conformational analysis of RTX[15] have been reported. However, the pharmacophoric groups of RTX have not yet been clearly defined, although structure–activity studies suggest that the C_{20}-homovanillic moiety, the C_3-keto group, and the orthoester phenyl group on ring C are crucial structural elements responsible for the extremely high potency of RTX.[16–24] The lower potency of CAP relative to RTX may be rationalized by the lack of some of these critical pharmacophoric groups, especially the C_3-keto group. Although a number of vanilloid agonist based on the structures of CAP and RTX have been reported as potential analgesics,[7] CAP-like analogues are limited by their intrinsic lower potency and narrow therapeutic index. RTX, on the other hand, is of limited availability from natural sources and is difficult to obtain synthetically due to its structural complexity (Fig. 1).

As part of an ongoing program in our laboratory aimed at the discovery of novel analgesic agents targeted to the vanilloid receptor, we have directed our synthetic efforts toward the preparation of simplified analogues of RTX according to our proposed RTX-pharmacophore model depicted in Chart 2. This pharmacophore model was derived from previous structure–activity relationship studies of RTX that included the C_{20}-homovanillic moiety (encompassing the carbonyl of the C_{20}-ester and

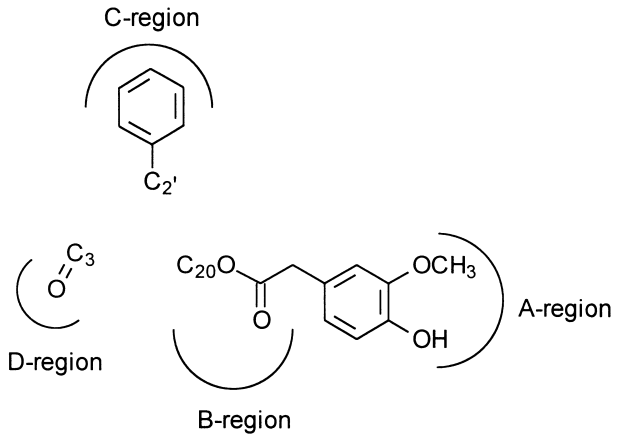
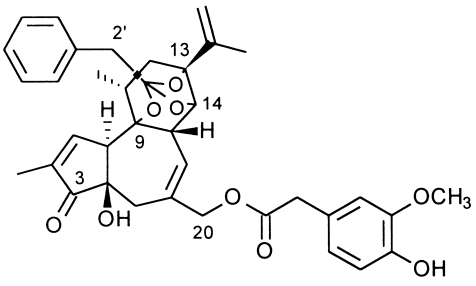


Chart 2.

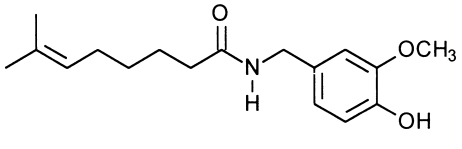
the hydroxyl group of the vanilloid ring), the C_3-carbonyl, and the orthoester phenyl moiety as essential groups for recognition and binding.[16]

We have recently reported a series of 3-acyloxy-2-benzylpropyl homovanillate analogues (Chart 3, templates I and II) designed as simplified vanilloid receptor ligands of RTX that were based on our pharmacophore model.[25] The amide analogues proved to be quite potent vanilloid agonists in terms of the receptor binding assay and the CAP-activated single channels assay, and thus represent good leads for the development of a novel class of analgesics that function as VR agonists. Previous structure–activity relationship studies on the amide region of CAP, the so-called B-region, have shown that the thiourea moiety confers high potency to most CAP analogues in terms of CAP-like activities.[26] This high potency could be explained by the capacity of the thiourea moiety to engage in multiple hydrogen bonding interactions with the receptor and its increased conformational rigidity. However, unlike CAP analogues, thiourea surrogates of RTX were reported to exhibit much diminished potency in receptor binding affinity and other biological responses.[19]

In this paper, we wish to describe the results of a structure activity relationship study of *N*-(3-acyloxy-2-benzylpropyl)-N′-(4-hydroxy-3-methoxybenzyl)thioureas (Chart 3, template III) as vanilloid receptor agonists and potential analgesics which can be considered as thiourea surrogates of template I.



Resiniferatoxin (RTX)



Capsaicin

Chart 1.

Chemistry

The syntheses of 3-acyloxy-2-benzylpropyl thiourea derivatives (**18**–**29**) are outlined in Schemes 1 and 2. Monoalkylation of diethyl malonate with various benzyl halides, followed by $LiAlH_4$-reduction, produced the corresponding diols (**2a**–**d**). Monoacetylation of **2**,[27] conversion of the remaining alcohol function to an azide, and deprotection of the acetyl group afforded the intermediate 3-azido-2-benzyl-1-propanols (**5a**–**d**). Acylation of **5a**–**d** with various acyl halides produced the corresponding esters **6**–**17**, which following the reduction of the azide group were condensed with 4-methoxymethyloxy-3-methoxybenzylisothiocyanate and hydrolyzed to the final targets **18**–**29**, respectively. The alternative ether analogues, represented by the 3-benzyloxy-2-benzylpropyl thiourea derivatives, **32** and **33**, were synthesized from **5a**,**c** as shown in Scheme 2. Two chiral analogues of **18** (**35** and **36**) were synthesized from (*R*)-3-acetoxy-2-benzyl-1-propanol ((***R***)-**3a**)-obtained from the enantioselective enzymatic acetylation of racemic 2-benzyl-1,3-propanediol[28] following the same procedure for the synthesis of **18** (Scheme 3). For the synthesis of the conformationally rigid analogues of **18** and **22** (**38** and **39**) bis-1,2-chloromethylbenzene was employed to alkylate diethylmalonate under similar conditions to those described above for Scheme 1 (Scheme 4).

Results and Discussion

Receptor binding affinity and CAP-activated channel assay

The CAP-like activity of the target compounds was measured by an in vitro receptor binding assay[20] and a CAP-activated single channel activation assay.[29] In the receptor binding assay, the compounds were evaluated for their ability to compete with [^{3}H]RTX for binding to the receptor. The results are expressed in terms of K_i values (mean±SEM, three experiments) which represent the concentration of the non-radioactive ligand that would give half-maximal receptor occupancy. In the CAP-activated single channel activation assay, the increase of inward current resulting from the non-selective cation influx was measured following the extracellular application of the compounds to cultured neonatal rat dorsal root ganglion (DRG) neurons. The activities of the compounds are expressed in terms of the relative difference in ion conductance compared to CAP

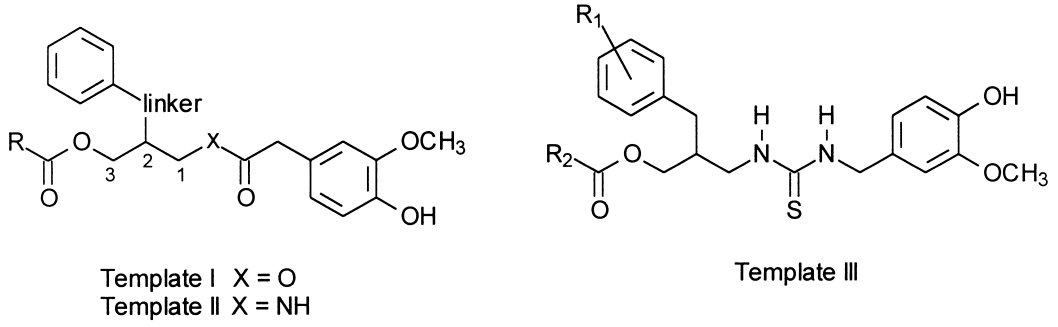
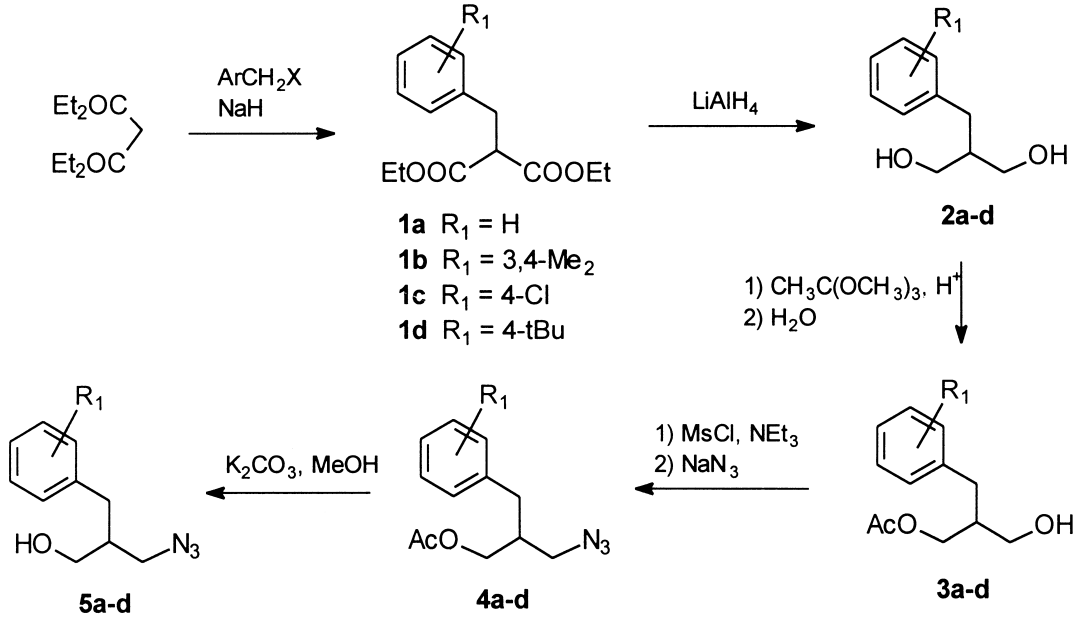


Chart 3.

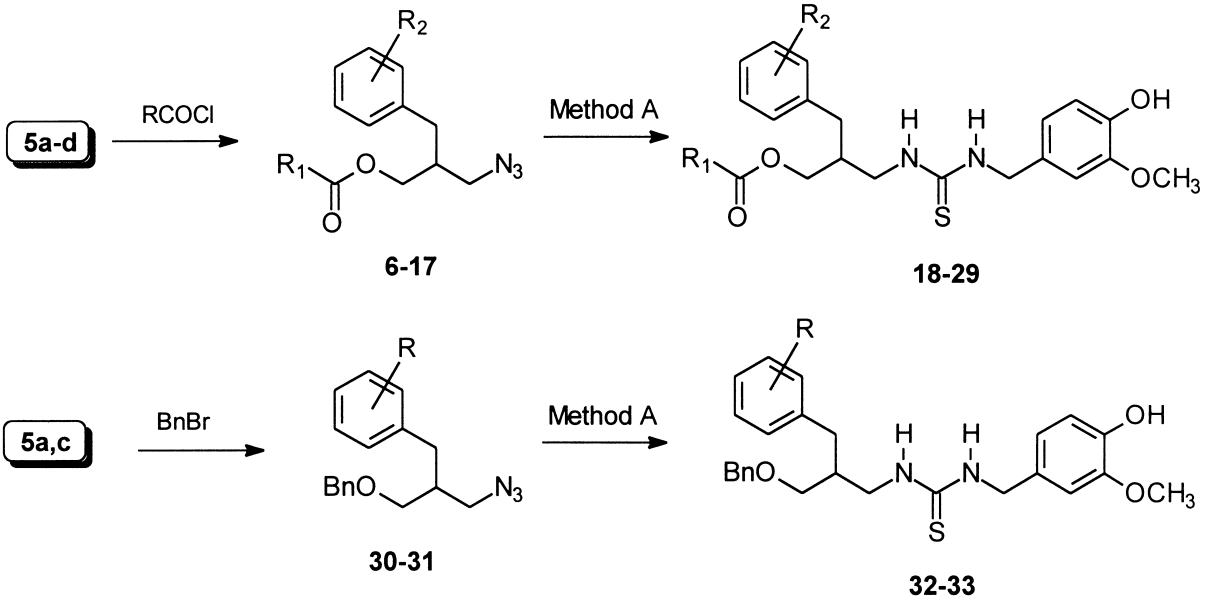


Scheme 1.

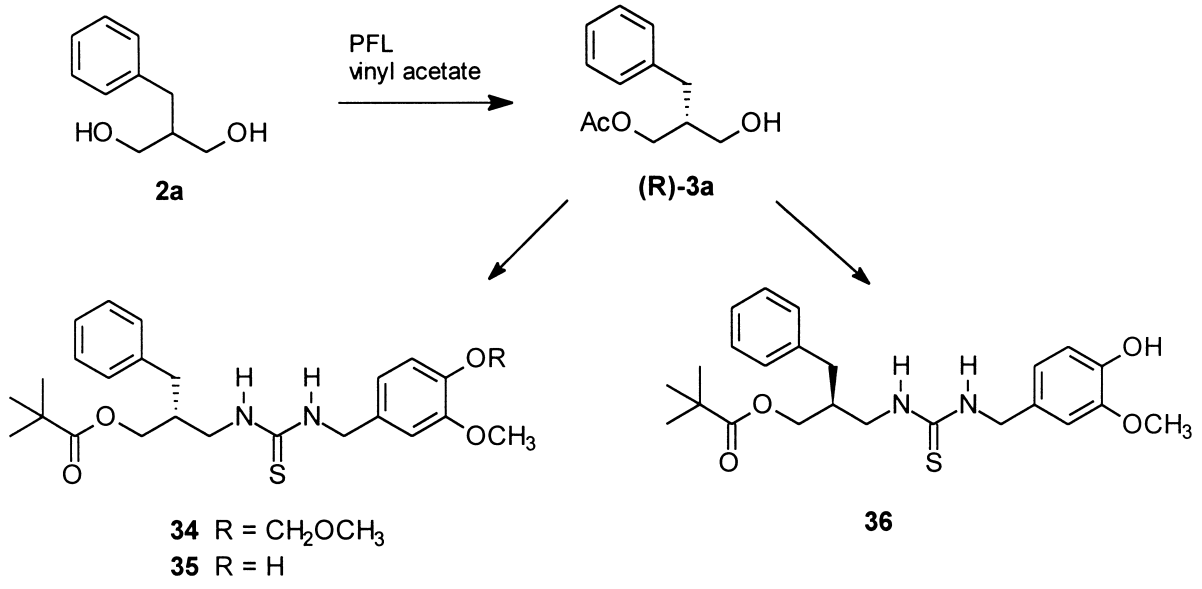
as a control. The results are presented in Table 1 and can be summarized as follows: (1) all the compounds tested showed stronger agonist activity and receptor binding affinity than CAP; (2) the thiourea analogue **18** ($K_i = 96$ nM) exhibited more potent agonist activity than the CAP-related amide analogue ($K_i = 404$ nM) reported in our earlier study;[25] (3) the ester group in R_1, which was designed to mimic the C_3-carbonyl of RTX, appears to be important for potent agonist activity relative to the benzyl ether analogues in R_1 (compare **22** ($K_i = 31.2$ nM) with **32** ($K_i = 148$ nM) and **27** ($K_i = 149$ nM) with **33** ($K_i = 660$ nM)) suggesting an important role for the carbonyl moiety in hydrogen-bonding to the receptor; (4) the pivaloyl group at R_1 and the 4-*t*-butylbenzyl group at R_2 proved to be effective hydrophobic groups resulting in optimal agonist activity for compounds **23** and **28** ($K_i = 19$ and 10.8 nM, respectively) which are ca. 280 and 490 times more potent than CAP; (5) binding of thiourea to the receptor appears to be quite stereospecific as the two optically active enantiomers of **18** showed substantial differences in receptor binding affinity relative to the racemate (compare **18** ($K_i = 96$ nM) with **35** ($K_i = 18.4$ nM) and **36** ($K_i = 74$ nM)); and (6) a conformationally restricted benzyl moiety in R_2 diminishes agonistic potency (compare **18** ($K_i = 96$ nM)) with **38** ($K_i = 480$ nM) and **22** ($K_i = 31.2$ nM) with **39** ($K_i = 1820$ nM)).

Analgesic and anti–inflammatory activity

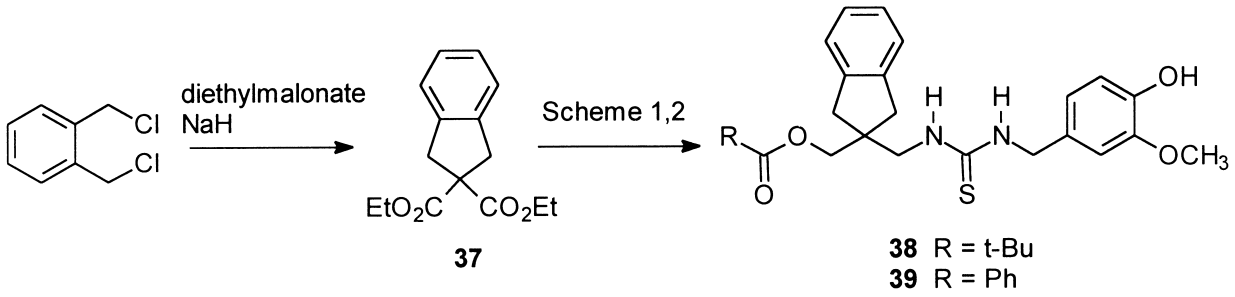
The analgesic activities of some of the most potent CAP agonists, selected from the receptor binding and single channel assays, were evaluated in the PBQ-induced writhing assay and the results are shown in Table 2. As expected, two of the most potent compounds based on



Scheme 2. Method A: (1) H_2, Lindler's cat; (2) $SCNCH_2PL(3\text{-}OMOM, 4\text{-}OCH_3)$; (3) SF_3COOH.

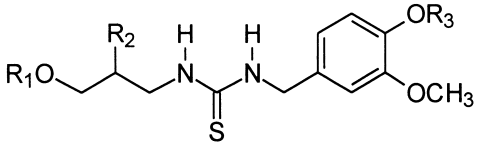


Scheme 3.



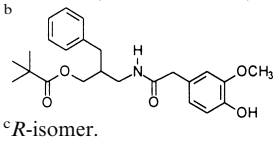
Scheme 4.

Table 1. Affinities and channel activations



Compound	R_1	R_2	R_3	Affinity, K_i (nM)	Activations[a]
CAP				5310 (±370)	
RTX				0.023	
Amide[b]				404 (±37)	+ +
18	$(CH_3)_3CCO$	CH_2Ph	H	96 (±31)	+ + +
19	$(CH_3)_2CHCO$	CH_2Ph	H	133 (±75)	+ + +
20	$CH_3(CH_2)_4CO$	CH_2Ph	H	632 (±48)	+ +
21	$CH_3(CH_2)_{16}CO$	CH_2Ph	H	1870 (±350)	+
22	PhCO	CH_2Ph	H	31.2 (±9.0)	+ +
23	$(CH_3)_3CCO$	$CH_2Ph(3,4\text{-}Me_2)$	H	19 (±4.3)	+ +
24	PhCO	$CH_2Ph(3,4\text{-}Me_2)$	H	410 (±180)	+ +
25	$(3,4\text{-}Me_2)PhCO$	$CH_2Ph(3,4\text{-}Me_2)$	H	184 (±69)	+ + +
26	$(CH_3)_3CCO$	$CH_2Ph(4\text{-}Cl)$	H	54 (±16)	+ +
27	PhCO	$CH_2Ph(4\text{-}Cl)$	H	149 (±11)	+ +
28	$(CH_3)_3CCO$	$CH_2Ph(4\text{-}t\text{-}Bu)$	H	10.8 (±4.0)	+ +
29	PhCO	$CH_2Ph(4\text{-}t\text{-}Bu)$	H	60 (±10)	+ +
32	$PhCH_2$	CH_2Ph	H	148 (±11)	+ +
33	$PhCH_2$	$CH_2Ph(4\text{-}Cl)$	H	660 (±290)	+ +
34	$(CH_3)_3CCO$	CH_2Ph	CH_2OCH_3	146 (±48)	+
35[c]	$(CH_3)_3CCO$	CH_2Ph	H	18.4 (±5.1)	+ + +
36[d]	$(CH_3)_3CCO$	CH_2Ph	H	74 (±16)	+ +
38	$(CH_3)_3CCO$	gem-$(CH_2)_2Ph$	H	480 (±170)	+ +
39	PhCO	gem-$(CH_2)_2Ph$	H	1820 (±790)	+ +

[a] + = CAP, + + = 10 CAP, + + + = 100 CAP.

[b] 

[c] *R*-isomer.

[d] *S*-isomer.

the in vitro assay, **23** and **28**, exhibited excellent analgesic activities with ED_{50} values of 0.5 and 1 μg/kg, respectively. Such values make these compounds 300 to 600 times more potent than CAP. Both compounds were likewise more potent than olvanil or DA-5018, which are either currently available in the market or undergoing clinical trial as topical analgesics. However, in the TPA-induced ear edema assay,[31] even the most potent agonists of this series showed weak antiinflammatory activity compared to the strong anti-edema activity of CAP (Table 2). We had originally speculated that the thiourea analogues, with their higher intrinsic potency, might dramatically reduce the TPA-induced ear edema. In this respect, their activity resembles that of olvanil or DA-5018, both of which display weak topical activity in the TPA ear edema assay. Poor bioavailability through transcutaneous penetration may explain in part the weak topical antiinflammatory properties observed with these compounds.

Analgesic and anti-inflammatory activity

The analgesic activities of some of the most potent CAP agonists, selected from the receptor binding and single channel assays, were evaluated in the PBQ-induced writhing assay and the results are shown in Table 2. Though

they displayed distinctive potencies, tested compounds could attain the same maximal antinociceptive activity (100% inhibition) as did capsaicin. As expected, two of the most potent compounds based on the in vitro assay, **23** and **28**, exhibited excellent analgesic activities with ED_{50} values of 0.5 and 1 μg/kg, respectively. Such values make these compounds 300 to 600 times more potent than CAP. It should be noted that the biological data in Table 2 are not adjusted for their respective molecular weights of tested compounds. Both compounds were likewise more potent than olvanil or DA-5018, which is a orally active capsaicin analogue with less pungency or undergoing clinical trial as topical analgesics. However, in the TPA-induced ear edema assay,[31] even the most potent agonists of this series showed weak antiinflammatory activity compared to the strong anti-edema activity of CAP (Table 2). We had originally speculated that the thiourea analogues, with their higher intrinsic potency, might dramatically reduce the TPA-induced ear edema. In this respect, their activity resembles that of olvanil or DA-5018, both of which display weak topical activity in the TPA ear edema assay. Poor bioavailability through transcutaneous penetration may explain in part the weak topical anti-inflammatory properties observed with these compounds.

Pungency and tachyphylaxis

The focus of medicinal chemistry in developing vanilloid-derived therapeutics has been the improvement of the bioavailability and the reduction of the excitatory properties. In line with these objectives, the rat eye-wiping test was employed as an in vivo pungency test to assess the pain-producing effects of the compounds.[32] As shown in Table 3, pungency profiles of selected potent agonists, **22**, **23** and **28** did not follow the trend of the intrinsic agonistic activity measured by the two in vitro assays. In this assay, RTX, an ultrapotent capsaicin analogue, was only 3-fold more potent than CAP and the selected compounds from our series were much less potent in evoking acute pain. A possible explanation for the reduced excitatory properties of these synthetic compounds (**23** and **28**) could lie in their rate of excitation of the sensory neuron.[33] Separation of intrinsic agonist activity from acute irritancy should confer an important therapeutic advantage.

To check for the development of tachyphylaxis or cross-tachyphylaxis, a group of rats treated with the selected synthetic compounds were challenged 6 h postreatment with 10 μg/mL CAP.[8] CAP, RTX and the synthetic analogues displayed an attenuated eye-wiping response in the test challenge of 10 μg/mL CAP, a result that is indicative of cross-tachyphylaxis between these classes of compounds. However, it should be noted that for CAP analogues a prior eye-wiping response is a prerequisite for the development of after-desensitization. In other words, non-irritating doses of CAP analogues were without effect to protect the eye-wiping movement by test treatment with 0.001% CAP.

Molecular modelling

A search for a common pharmacophore pattern between the thiourea analogues and RTX was performed by molecular modelling using the program Sybyl 6.4 from Tripos on a Silicon Graphics R4600 INDY workstation. Energy-minimized structures of RTX and **35** were obtained from molecular dynamics simulation. Four pharmacophoric groups in **35**, namely the carbonyl of the ester, the sulfur of the thiourea, the centre of vanilloid ring and the centre of phenyl ring were matched with the C_3-keto carbonyl, the C_{20}-ester carbonyl, the centre of vanilloid ring and the centre of orthoester phenyl in RTX, respectively. The fitting demonstrated a good correlation in the spatial disposition of the corresponding key pharmacophores with 1.494 Å rms values. The result indicated that target thiourea compounds serve as good surrogates of RTX by containing their crucial pharmacophores.

Table 2. Writhing test and ear edema assay

Compound	Writhing test ED_{50} (μg/kg)	Relative potency	Ear edema assay ID_{50} (μg/ear)	Relative potency
CAP	300	1	3	1
RTX	0.01	30,000	0.002	1500
Olvanil	30	10	30	0.1
DA-5018	3	100	200	0.015
Indomethacin	400	0.75		
Aspirin	3500	0.086		
Morphine	1000	0.3		
18	2	150	25	0.12
19	15	20		
20	5	60		
21	20	15		
22	20	15	20	0.15
23	0.5	600	18	0.17
24	7	43	50	0.06
25	2	150	50	0.06
26	5	60	22	0.14
27	12	25	17	0.18
28	1	300	5	0.6
29	1.5	200	30	0.1
35	8	38		
36	9	33		

Table 3. Eye-Wiping Test[a–d]

Compound	MPP (μg/mL)	RPP	Tachyphylaxis
CAP	3	100	55 (1000)
			35 (100)
RTX	1	300	85 (100)
			20 (10)
Olvanil	WP	—	0 (1000)
DA-5018	30	10	90 (1000)
			35 (100)
22	WP	—	0 (1000)
23	20	15	90 (1000)
			30 (100)
			5 (10)
28	30	10	NT[e]

[a]MPP, moderate pain-producing potency.
[b]RPP, relative pain-producing potency.
[c]WP, weakly pungent (less pungent than 3 μg/mL capsaicin at 100 μg/mL).
[d]Tachyphylaxis percent reduction of eye wiping number after test challenge of 10 μg/mL capsaicin. The number in parentheses indicates concd (μg/mL) of desensitising challenge.
[e]NT, not tested.

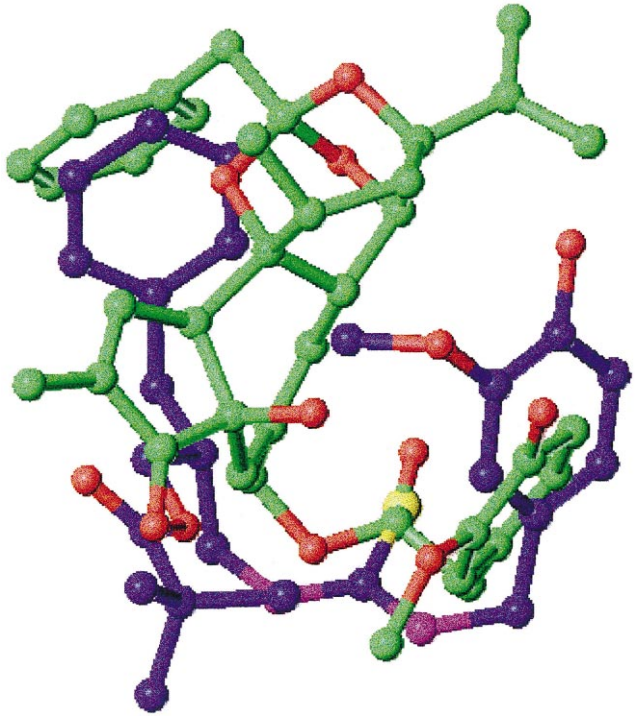


Figure 1. Superposition of **35** on resiniferatoxin.

Conclusion

A series of *N*-(3-acyloxy-2-benzylpropyl)-N′-(4-hydroxy-3-methoxybenzyl)thiourea analogues were constructed as novel agonists of the vanilloid receptor with the intent of mimicking the spatial disposition of the C_{20}-homovanillate, the C_3-carbonyl, and the phenyl ortho-ester side chain of RTX. Two compounds, **23** and **28**, showed very potent CAP-like activities in terms of the binding assay and CAP-activated single channel assay. Both compounds also showed excellent analgesic activity as shown by the PBQ-induced writhing test and were less pungent than CAP. However, all the thioureas had weak antiinflammatory activities. These compounds, however, represent excellent leads for the development of novel VR ligands as analgesics.

Experimental

General

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 and Carbon NMR spectra were recorded on a JEOL JNM-LA 300 at 300 MHz and JEOL JNM-GCX 400 at 100 MHz, respectively. Chemical shifts are reported in ppm units with Me_4Si as a reference standard. Infrared spectra were recorded on a Perkin-Elmer 1710 Series FTIR. Mass spectra were recorded on a VG Trio-2 GC-MS. Elemental analyses were performed with an EA 1110 Automatic Elemental Analyzer, CE Instruments.

General procedure for the synthesis of 1a–d. 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 the corresponding substituted benzyl chlorides (48 mmol) and stirred overnight at room temperature. The mixture was diluted with H_2O and extracted with EtOAc several times. The combined organic layer was 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:hexane (1:10) as eluant to give **1**.

Diethyl 3,4-dimethylbenzylmalonate (1b). 70% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.9–7.05 (m, 3H), 4.14 (m, 4H, 2×CO_2CH_2), 3.61 (t, 1H, CH), 3.25 (d, 1H, CH_2Ar), 3.14 (d, 1H, CH_2Ar), 2.2–2.25 (m, 6H, 2×CH_3), 1.21 (m, 6H, 2×$CO_2CH_2C\underline{H}_3$).

Diethyl 4-chlorobenzylmalonate (1c). 74% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.25 (d, 2H), 7.16 (d, 2H), 4.14 (m, 4H, 2×CO_2CH_2), 3.60 (t, 1H, CH), 3.18 (d, 2H, CH_2Ar), 1.21 (t, 6H, 2×$CO_2CH_2C\underline{H}_3$).

Diethyl 4-*t*-butylbenzylmalonate (1d). 74% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.29 (d, 2H), 7.13 (d, 2H), 4.16 (q, 4H, 2×CO_2CH_2), 3.63 (t, 1H, CH), 3.18 (d, 2H, CH_2Ar), 1.30 (s, 9H, $C(CH_3)_3$), 1.20 (t, 6H, 2×$CO_2CH_2C\underline{H}_3$).

General procedure for the synthesis of 2a–d. 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 **1** (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 3.5 mL of H_2O, 7 mL of 15% NaOH solution, and 10.5 mL of H_2O. The mixture was filtered by washing with EtOAc and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography over silica gel with EtOAc:hexane (3:1) as eluant to give **2**.

2-Benzyl-1,3-propanediol (2a). 98% yield, white solid, mp = 67 °C; ^{1}H NMR ($CDCl_3$) δ 7.15–7.30 (m, 5H, phenyl), 3.74 (dd, 2H, CH_2OH), 3.62 (dd, 2H, CH_2OH), 2.9–3.0 (bs, 2H, OH), 2.58 (d, 2H, CH_2Ph), 2.03 (m, 1H, CH).

2-(3,4-Dimethylbenzyl)-1,3-propandiol (2b). 92% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.88–7.06 (m, 3H, phenyl), 3.6–3.8 (m, 4H, 2×CH_2OH), 2.5–2.7 (bs, 2H, OH), 2.60 (d, 1H, CH_2Ph), 2.52 (d, 1H, CH_2Ph), 2.2–2.28 (m, 6H, 2×CH_3), 2.02 (m, 1H, CH).

2-(4-Chlorobenzyl)-1,3-propandiol (2c). 75% yield, white solid, mp = 64 °C; ^{1}H NMR ($CDCl_3$) δ 7.24 (d, 2H), 7.10 (d, 2H), 3.75 (dd, 2H, CH_2OH), 3.61 (dd, 2H,

CH_2OH), 2.85 (bs, 2H, OH), 2.58 (d, 2H, CH_2Ph), 1.97 (m, 1H, CH).

2-(4-*t*-Butylbenzyl)-1,3-propandiol (2d). 80% yield, white solid, mp = 67 °C; ^1H NMR ($CDCl_3$) δ 7.31 (d, 2H), 7.11 (d, 2H), 3.81 (dd, 2H, CH_2OH), 3.68 (dd, 2H, CH_2OH), 2.58 (d, 2H, CH_2Ph), 2.23 (bs, 2H, OH), 2.05 (m, 1H, CH), 1.30 (s, 9H, $C(CH_3)_3$).

General procedure of the synthesis of 3a–d. A mixture of **2** (20 mmol), trimethylorthoacetate (3.6 g, 30 mmol) and a catalytic amount of *p*-toluenesulfonic acid in CH_2Cl_2 (40 mL) was stirred for 2 h at room temperature and then treated with H_2O (0.54 g, 30 mmol). After stirring overnight at room temperature, the reaction mixture was concentrated in vacuo. The residue was purified by flash column chromatography over silica gel with EtOAc: hexane (1:2) as eluant to give **3**.

2-Benzyl-3-hydroxypropyl acetate (3a). 97% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 7.1–7.25 (m, 5H, phenyl), 4.06 (ddd of AB, 2H, CH_2OAc), 3.48 (m, 2H, CH_2OH), 2.58 (ddd of AB, 2H, CH_2Ph), 2.10 (m, 1H, CH), 2.01 (s, 3H, $COCH_3$).

(*R*)-2-Benzyl-1,3-propanediol ((R)-3a). This compound was obtained from **2a** by a published literature procedure.[28]

2-(3,4-Dimethylbenzyl)-3-hydroxypropyl acetate (3b). 90% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 6.88–7.06 (m, 5H, phenyl), 4.13 (m, 2H, CH_2OAc), 3.54 (m, 2H, CH_2OH), 2.62 (m, 2H, CH_2Ph), 2.2–2.3 (m, 6H, 2× CH_3), 2.10 (m, 1H, CH), 2.07 (s, 3H, $COCH_3$)

2-(4-Chlorobenzyl)-3-hydroxypropyl acetate (3c). 86% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 7.25 (d, 2H), 7.12 (d, 2H), 4.12 (ddd of AB, 2H, CH_2OAc), 3.53 (ddd of AB, 2H, CH_2OH), 2.63 (ddd of AB, 2H, CH_2Ph), 2.10 (m, 1H, CH), 2.06 (s, 3H, $COCH_3$).

2-(4-*t*-Butylbenzyl)-3-hydroxypropyl acetate (3d). 84% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 7.30 (d, 2H), 7.11 (d, 2H), 4.13 (ddd of AB, 2H, CH_2OAc), 3.56 (ddd of AB, 2H, CH_2OH), 2.62 (ddd of AB, 2H, CH_2Ph), 2.28 (s, 1H, OH), 2.10 (m, 1H, CH), 2.01 (s, 3H, $COCH_3$), 1.30 (s, 9H, $C(CH_3)_3$).

General procedure of the synthesis of 4a–d. A cooled solution of **3** (12 mmol) and triethylamine (3.64 g, 36 mmol) in CH_2Cl_2 (20 mL) at 0 °C was treated dropwise with methanesulfonylchloride (2.06 g, 18 mmol). After stirring for 6 h at room temperature, the mixture was diluted with CH_2Cl_2. The organic layer was washed with 1N HCl, 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:hexane (1:3) as eluant to give the corresponding mesylate as an oil. The mesylate was dissolved in DMF (10 mL) and treated with sodium azide (2.2 g, 34 mmol). After 8 h of stirring at 80 °C, the reaction mixture was diluted with H_2O and extracted with EtOAc several times. The combined organic layer was 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:hexane (1:15) as eluant to give **4**.

3-Azido-2-benzylpropyl acetate (4a). 92% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 7.15–7.35 (m, 5H, phenyl), 4.05 (ddd of AB, 2H, CH_2OAc), 3.34 (m, 2H, CH_2N_3), 2.68 (d, 2H, CH_2Ph), 2.22 (m, 1H, CH), 2.08 (s, 3H, $COCH_3$)

3-Azido-2-(3,4-dimethylbenzyl)propyl acetate (4b). 85% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 6.87–7.07 (m, 3H), 4.04 (m, 2H, CH_2OAc), 3.33 (m, 2H, CH_2N_3), 2.69 (d, 1H, CH_2Ph), 2.60 (d, 1H, CH_2Ph), 2.1–2.3 (m, 7H, 2×CH_3 and CH), 2.07 (s, 3H, $COCH_3$).

3-Azido-2-(4-chlorobenzyl)propyl acetate (4c). 88% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 7.26 (d, 2H), 7.12 (d, 2H), 4.03 (ddd of AB, 2H, CH_2OAc), 3.34 (m, 2H, CH_2N_3), 2.65 (d, 2H, CH_2Ph), 2.17 (m, 1H, CH), 2.07 (s, 3H, $COCH_3$).

3-Azido-2-(4-*t*-butylbenzyl)propyl acetate (4d). 92% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 7.32 (d, 2H), 7.08 (d, 2H), 4.05 (ddd of AB, 2H, CH_2OAc), 3.35 (ddd of AB, 2H, CH_2N_3), 2.64 (d, 2H, CH_2Ph), 2.20 (m, 1H, CH), 2.07 (s, 3H, $COCH_3$), 1.30 (s, 9H, $C(CH_3)_3$).

General procedure for the synthesis of 5a–d. A solution of **4** (8 mmol) and a catalytic amount of K_2CO_3 in MeOH (10 mL) was treated with a couple of drops of H_2O. After stirring for 2 h at room temperature, the reaction mixture was quenched with several drops of acetic acid and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc:hexane (1:3) as eluant to give **5**.

3-Azido-2-benzyl-1-propanol (5a). 98% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 7.15–7.35 (m, 5H, phenyl), 3.65 (m, 2H, CH_2OH), 3.40 (ddd of AB, 2H, CH_2N_3), 2.67 (ddd of AB, 2H, CH_2Ph), 2.04 (m, 1H, CH).

3-Azido-2-(3,4-dimethylbenzyl)-1-propanol (5b). 92% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 6.88–7.08 (m, 5H, phenyl), 3.65 (m, 2H, CH_2OH), 3.42 (m, 2H, CH_2N_3), 2.68 (dd, 1H, CH_2Ph), 2.60 (dd, 1H, CH_2Ph), 2.2–2.3 (m, 6H, 2×CH_3), 2.03 (m, 1H, CH).

3-Azido-2-(4-chlorobenzyl)-1-propanol (5c). 88% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 7.25 (d, 2H), 7.10 (d, 2H), 3.58 (m, 2H, CH_2OH), 3.36 (m, 2H, CH_2N_3), 2.63 (dd, 2H, CH_2Ph), 2.1 (bs, 1H, OH), 1.98 (m, 1H, CH).

3-Azido-2-(4-*t*-butylbenzyl)-1-propanol (5d). 98% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 7.32 (d, 2H), 7.10 (d, 2H), 3.63 (ddd of AB, 2H, CH_2OH), 3.39 (ddd of AB, 2H, CH_2N_3), 2.62 (m, 2H, CH_2Ph), 2.02 (m, 1H, CH), 1.78 (bs, 1H, OH), 1.30 (s, 9H, $C(CH_3)_3$).

General procedure for the synthesis of 6–17. A cooled solution of **5** (1 mmol), triethylamine (4 mmol) and a catalytic amount of 4-dimethylaminopyridine in CH_2Cl_2 (5 mL) at 0 °C was treated with the corresponding acyl

chloride (2 mmol). After 2–12 h of stirring at room temperature, the mixture was diluted with CH_2Cl_2. The organic layer was washed with 1N HCl, H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by flash column chromatography over silica gel with EtOAc:hexane (1:10–20) as eluant to give **6**–**17**, respectively.

3-Azido-2-benzylpropyl pivalate (6). 92% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.15–7.35 (m, 5H, phenyl), 4.04 (ddd of AB, 2H, CH_2OCO), 3.34 (ddd of AB, 2H, CH_2N_3), 2.68 (d, 2H, CH_2Ph), 2.21 (m, 1H, CH), 1.23 (s, 9H, $C(CH_3)_3$)

3-Azido-2-benzylpropyl 2-methylpropanoate (7). 93% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.15–7.35 (m, 5H, phenyl), 4.05 (ddd of AB, 2H, CH_2OCO), 3.34 (m, 2H, CH_2N_3), 2.68 (d, 2H, CH_2Ph), 2.58 (m, 1H, $CHMe_2$), 2.21 (m, 1H, CH), 1.18 (dd, 6H, 2×CH_3).

3-Azido-2-benzylpropyl hexanoate (8). 90% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.14–7.35 (m, 5H, phenyl), 4.05 (ddd of AB, 2H, CH_2OCO), 3.34 (m, 2H, CH_2N_3), 2.68 (d, 2H, CH_2Ph), 2.32 (t, 2H, $OOCCH_2$), 2.20 (m, 1H, CH), 1.64 (m, 2H), 1.2–1.4 (m, 4H), 0.88 (distorted t, 3H).

3-Azido-2-benzylpropyl stearate (9). 78% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.14–7.35 (m, 5H, phenyl), 4.05 (ddd of AB, 2H, CH_2OCO), 3.33 (m, 2H, CH_2N_3), 2.68 (d, 2H, CH_2Ph), 2.32 (t, 2H, $OOCCH_2$), 2.20 (m, 1H, CH), 1.2–1.7 (m, 30H), 0.88 (distorted t, 3H).

3-Azido-2-benzylpropyl benzoate (10). 90% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 8.04 (m, 2H), 7.15–7.60 (m, 8H), 4.35 (dd, 1H, J = 5.1 and 11.2 Hz, $BzOCH_2$), 4.26 (dd, 1H, $BzOCH_2$), 3.43 (ddd of AB, 2H, J = 5.61, 5.85 and 12.42 Hz, CH_2N_3), 2.78 (m, 2H, CH_2Ph), 2.35 (m, 1H, CH).

3-Azido-2-(3,4-dimethylbenzyl)propyl pivalate (11). 94% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 6.86–7.07 (m, 3H), 4.04 (m, 2H, CH_2OCO), 3.36 (m, 2H, CH_2N_3), 2.70 (d, 1H, CH_2Ph), 2.61 (d, 1H, CH_2Ph), 2.1–2.3 (m, 7H, 2×CH_3 and CH), 1.23 (s, 9H, $C(CH_3)_3$).

3-Azido-2-(3,4-dimethylbenzyl)propyl benzoate (12). 95% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 8.02 (m, 2H), 7.57 (m, 1H), 7.46 (m, 2H), 6.8–7.07 (m, 3H), 4.32 (m, 2H, CH_2OCO), 3.45 (m, 2H, CH_2N_3), 2.80 (d, 1H, CH_2Ph), 2.71 (d, 1H, CH_2Ph), 2.2–2.4 (m, 7H, 2×CH_3 and CH).

3-Azido-2-(3,4-dimethylbenzyl)propyl 3,4-dimethylbenzoate (13). 84% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.77 (m, 2H), 7.21 (m, 1H), 6.9–7.07 (m, 3H), 4.30 (m, 2H, CH_2OCO), 3.44 (m, 2H, CH_2N_3), 2.79 (d, 1H, CH_2Ph), 2.70 (d, 1H, CH_2Ph), 2.2–2.4 (m, 13H, 4×CH_3 and CH).

3-Azido-2-(4-chlorobenzyl)propyl pivalate (14). 86% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.27 (d, 2H), 7.09 (d, 2H), 4.02 (ddd of AB, 2H, CH_2OCO), 3.33 (m, 2H, CH_2N_3), 2.66 (d, 2H, CH_2Ph), 2.18 (m, 1H, CH), 1.23 (s, 9H, $C(CH_3)_3$).

3-Azido-2-(4-chlorobenzyl)propyl benzoate (15). 92% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 8.01 (m, 2H), 7.58 (m, 1H), 7.46 (m, 2H), 7.28 (d, 2H), 7.13 (d, 2H), 4.29 (ddd of AB, 2H, CH_2OCO), 3.43 (m, 2H, CH_2N_3), 2.75 (d, 2H, CH_2Ph), 2.33 (m, 1H, CH).

3-Azido-2-(4-*t*-butylbenzyl)propyl pivalate (16). 99% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.31 (d, 2H), 7.08 (d, 2H), 4.04 (ddd of AB, 2H, CH_2OCO), 3.35 (m, 2H, CH_2N_3), 2.65 (d, 2H, CH_2Ph), 2.20 (m, 1H, CH), 1.30 (s, 9H, $C(CH_3)_3$), 1.23 (s, 9H, $C(CH_3)_3$).

3-Azido-2-(4-*t*-butylbenzyl)propyl benzoate (17). 88% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 8.04 (m, 2H), 7.57 (m, 1H), 7.45 (m, 2H), 7.32 (d, 2H), 7.12 (d, 2H), 4.31 (ddd of AB, 2H, CH_2OCO), 3.44 (m, 2H, CH_2N_3), 2.75 (d, 2H, CH_2Ph), 2.35 (m, 1H, CH), 1.30 (s, 9H, $C(CH_3)_3$).

General procedure for the synthesis of 18–29. A solution of **6**–**17** (0.5 mmol) and Lindler's catalyst (50 mg) in EtOH (5 mL) was hydrogenated under a hydrogen balloon for 2 h. The reaction mixture was filtered and the filtrate was concentrated in vacuo. The residue was dissolved in CH_2Cl_2 (5 mL) and treated with 4-[(methoxylmethyl)oxy)]-3-methoxybenzyl isothiocyanate (0.5 mmol). After stirring overnight at room temperature, the reaction mixture was concentrated and the residue was purified by flash column chromatography on silica gel with EtOAc:hexane (1:1) as eluant to give the corresponding thiourea. The thiourea was dissolved in CH_2Cl_2 (2 mL) and treated with trifluoroacetic acid (1 mL). After 1 h of stirring at room temperature, the mixture was quenched with solid $NaHCO_3$, filtered, and the filtrate was concentrated. 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:hexane (1:1) as eluant to give **18**–**29**.

2-Benzyl-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl pivalate (18). 40% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.1–7.32 (m, 5H, phenyl), 6.75–6.9 (m, 3H, Ar), 6.27 (bt, 1H, NH), 6.05 (bs, 1H, NH), 5.63 (s, 1H, OH), 4.40 (bd, 2H, J = 4.38 Hz, $NHCH_2Ar$), 4.17 (dd, 1H, J = 3.9 and 11.46 Hz, CH_2OCO), 3.87 (s, 3H, OCH_3), 3.7–3.85 (m, 2H, 1H of CH_2OCO and $CHCH_2NHC{=}S$), 3.24 (ddd, 1H, J = 5.37, 8.07 and 13.89 Hz, $CHCH_2NHC{=}S$), 2.61 (ddd of AB, 2H, CH_2Ph), 2.33 (m, 1H, CH), 1.23 (s, 9H, $C(CH_3)_3$); IR (neat): 3362, 1715, 1278, 1157; MS m/e 445 ($M^+ + 1$). Anal. calcd for $C_{24}H_{32}N_2O_4S$: C, 64.84; H, 7.25; N, 6.30; S, 7.21. Found: C, 65.12; H, 7.28; N, 6.32; S, 7.19.

2-Benzyl-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl 2-methylpropanoate (19). 35% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.13–7.30 (m, 5H, phenyl), 6.75–6.87 (m, 3H, Ar), 6.34 (bt, 1H, NH), 6.30 (bs, 1H, NH), 5.75 (s, 1H, OH), 4.40 (bs, 2H, $NHCH_2Ar$), 4.17 (dd, 1H, J = 3.9 and 10.95 Hz, CH_2OCO), 3.84 (s, 3H,

OCH_3), 3.65–3.85 (m, 2H, CH_2OCO and $CHCH_2NHC=S$), 3.28 (m, 1H, $CHCH_2NHC=S$), 2.60 (m, 3H, CH_2Ph and $CHMe_2$), 2.30 (m, 1H, $CHCH_2Ph$), 1.18 (dd, 6H, $2\times CH_3$); IR (neat): 3361, 1715, 1273, 1157; MS *m/e* 430 (M^+). Anal. calcd for $C_{23}H_{30}N_2O_4S$: C, 64.16; H, 7.02; N, 6.51; S, 7.45. Found: C, 64.42; H, 7.05; N, 6.49; S, 7.42.

2-Benzyl-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl hexanoate (20). 34% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 7.1–7.3 (m, 5H, phenyl), 6.75–6.9 (m, 3H, Ar), 6.23 (bt, 1H, NH), 6.11 (bs, 1H, NH), 5.66 (s, 1H, OH), 4.40 (bs, 2H, $NHCH_2Ar$), 4.15 (dd, 1H, $J=3.9$ and 11.43 Hz, CH_2OCO), 3.86 (s, 3H, OCH_3), 3.65–3.85 (m, 2H, CH_2OCO and $CHCH_2NHC=S$), 3.29 (m, 1H, $CHCH_2NHC=S$), 2.61 (m, 2H, CH_2Ph), 2.30 (m, 3H, CH_2COO and CH), 1.65 (m, 2H), 1.2–1.4 (m, 4H), 0.88 (distorted t, 3H); IR (neat): 3360, 1715, 1274, 1122; MS *m/e* 458 (M^+). Anal. calcd for $C_{25}H_{34}N_2O_4S$: C, 65.47; H, 7.47; N, 6.11; S, 6.99. Found: C, 65.72; H, 7.50; N, 6.09; S, 6.96.

2-Benzyl-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl stearate (21). 30% yield, colorless oil; ^1H NMR ($CDCl_3$) δ 7.1–7.3 (m, 5H, phenyl), 6.75–6.9 (m, 3H, Ar), 6.28 (bt, 1H, NH), 6.17 (bs, 1H, NH), 5.69 (s, 1H, OH), 4.39 (bs, 2H, $NHCH_2Ar$), 4.14 (dd, 1H, $J=3.9$ and 11.67 Hz, CH_2OCO), 3.87 (s, 3H, OCH_3), 3.65–3.85 (m, 2H, CH_2OCO and $CHCH_2NHC=S$), 3.30 (m, 1H, $CHCH_2NHC=S$), 2.60 (m, 2H, CH_2Ph), 2.30 (m, 3H, CH_2COO and CH), 1.2–1.7 (m, 30H), 0.88 (distorted t, 3H); IR (neat): 3363, 1731, 1274, 1031; MS *m/e* 626 (M^+). Anal. calcd for $C_{37}H_{58}N_2O_4S$: C, 70.89; H, 9.32; N, 4.47; S, 5.11. Found: C, 71.15; H, 9.36; N, 4.45; S, 5.10.

2-Benzyl-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl benzoate (22). 50% yield, white solid, mp = 90 °C; ^1H NMR ($CDCl_3$) δ 7.98–8.02 (m, 2H), 7.58 (m, 1H), 7.4–7.5 (m, 2H), 7.1–7.32 (m, 5H, phenyl), 6.72–6.85 (m, 3H, Ar), 6.42 (bt, 1H, NH), 6.31 (bs, 1H, NH), 5.73 (s, 1H, OH), 4.34–4.42 (m, 3H, $NHCH_2Ar$ and CH_2OCO), 4.05 (dd, 1H, CH_2OCO), 3.7–3.85 (m, 4H, OCH_3 and $CHCH_2NHC=S$), 3.38 (m, 1H, CH $CH_2NHC=S$), 2.6–2.78 (m, 2H, CH_2Ph), 2.46 (m, 1H, CH); IR (neat): 3360, 1714, 1274, 1121; MS *m/e* 464 (M^+). Anal. calcd for $C_{26}H_{28}N_2O_4S$: C, 67.22; H, 6.07; N, 6.03; S, 6.90. Found: C, 67.50; H, 6.10; N, 6.01; S, 6.88.

2-(3,4-dimethylbenzyl)-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl pivalate (23). 40% yield, white solid, mp = 47 °C; ^1H NMR ($CDCl_3$) δ 6.93–7.05 (m, 3H), 6.77–6.9 (m, 3H, Ar), 6.22 (m, 1H, NH), 5.98 (bs, 1H, NH), 5.61 (s, 1H, OH), 4.37 (bs, 2H, $NHCH_2Ar$), 4.17 (ddd of AB, 1H, CH_2OCO), 3.87 (s, 3H, OCH_3), 3.7–3.85 (m, 2H, CH_2OCO and $CHCH_2NHC=S$), 3.27 (m, 1H, $CHCH_2NHC=S$), 2.60 (m, 2H, CH_2Ph), 2.2–2.32 (m, 7H, $2\times CH_3$ and CH), 1.22 (s, 9H, $C(CH_3)_3$); IR (neat): 3360, 1714, 1279, 1159; MS *m/e* 474 ($M^+ + 2$). 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, 7.71; N, 5.91; S, 6.76.

2-(3,4-Dimethylbenzyl)-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl benzoate (24). 45% yield, white solid, mp = 44 °C; ^1H NMR ($CDCl_3$) δ 8.02 (m, 2H), 7.59 (m, 1H), 7.46 (m, 2H), 6.9–7.06 (m, 3H), 6.72–6.85 (m, 3H, Ar), 6.33 (m, 1H, NH), 6.13 (bs, 1H, NH), 5.65 (s, 1H, OH), 4.35–4.44 (m, 3H, $NHCH_2Ar$ and CH_2OCO), 4.05 (m, 1H, CH_2OCO), 3.7–3.85 (m, 4H, OCH_3 and $CHCH_2NHC=S$), 3.40 (m, 1H, $CHCH_2NHC=S$), 2.6–2.78 (m, 2H, CH_2Ph), 2.44 (m, 1H, CH), 2.2-2.3 (m, 6H, $2\times CH_3$); IR (neat): 3360, 1713, 1274, 1121; MS *m/e* 492 (M^+). Anal. calcd for $C_{28}H_{32}N_2O_4S$: C, 68.27; H, 6.55; N, 5.69; S, 6.51. Found: C, 68.53; H, 6.58; N, 5.67; S, 6.49.

2-(3,4-Dimethylbenzyl)-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl 3,4-dimethylbenzoate (25). 55% yield, white solid, mp = 58 °C; ^1H NMR ($CDCl_3$) δ 7.75 (m, 2H), 7.20 (m, 1H), 6.9–7.06 (m, 3H), 6.74–6.85 (m, 3H, Ar), 6.39 (m, 1H, NH), 6.07 (bs, 1H, NH), 5.63 (s, 1H, OH), 4.35–4.43 (m, 3H, $NHCH_2Ar$ and CH_2OCO), 4.05 (m, 1H, CH_2OCO), 3.7–3.85 (m, 4H, OCH_3 and $CHCH_2NHC=S$), 3.37 (m, 1H, $CHCH_2$ $NHC=S$), 2.6–2.74 (m, 2H, CH_2Ph), 2.41 (m, 1H, CH), 2.2–2.35 (m, 12H, $4\times CH_3$); IR (neat): 3373, 1711, 1266, 1124; MS *m/e* 520 (M^+). Anal. calcd for $C_{30}H_{36}N_2O_4S$: C, 69.20; H, 6.97; N, 5.38; S, 6.16. Found: C, 69.47; H, 7.00; N, 5.36; S, 6.14.

2-(4-Chlorobenzyl)-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl pivalate (26). 40% yield, white solid, mp = 62 °C; ^1H NMR ($CDCl_3$) δ 7.25 (d, 2H), 7.10 (d, 2H), 6.77–6.90 (m, 3H, Ar), 6.38 (t, 1H, NH), 6.25 (bs, 1H, NH), 5.72 (s, 1H, OH), 4.42 (bs, 2H, NH CH_2Ar), 4.16 (dd of AB, 1H, CH_2OCO), 3.86 (s, 3H, OCH_3), 3.7–3.85 (m, 2H, CH_2OCO and $CHCH_2$ $NHC=S$), 3.19 (m, 1H, $CHCH_2NHC=S$), 2.59 (ddd of AB, 2H, CH_2Ph), 2.32 (m, 1H, CH), 1.22 (s, 9H, $C(CH_3)_3$); IR (neat): 3360, 1714, 1279, 1159; MS *m/e* 479 (M^+). Anal. calcd for $C_{24}H_{31}ClN_2O_4S$: C, 60.17; H, 6.52; N, 5.85; S, 6.69. Found: C, 60.40; H, 6.55; N, 5.83; S, 6.66.

2-(4-Chlorobenzyl)-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl benzoate (27). 55% yield, white solid, mp = 57 °C; ^1H NMR ($CDCl_3$) δ 8.00 (m, 2H), 7.60 (m, 1H), 7.47 (m, 2H), 7.27 (d, 2H), 7.15 (d, 2H), 6.76–6.90 (m, 3H, Ar), 6.36 (t, 1H, NH), 6.15 (bs, 1H, NH), 5.61 (s, 1H, OH), 4.40 (m, 3H, $NHCH_2Ar$ and CH_2OCO), 4.00 (dd of AB, 1H, CH_2OCO), 3.86 (s, 3H, OCH_3), 3.85 (m, 1H, $CHCH_2NHC=S$), 3.31 (m, 1H, $CHCH_2NHC=S$), 2.67 (ddd of AB, 2H, CH_2Ph), 2.46 (m, 1H, CH); IR (neat): 3373, 1711, 1266, 1124; MS *m/e* 499 (M^+). Anal. calcd for $C_{26}H_{27}ClN_2O_4S$: C, 62.58; H, 5.45; N, 5.61; S, 6.43. Found: C, 62.83; H, 5.47; N, 5.59; S, 6.40.

2-(4-*t*-Butylbenzyl)-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl pivalate (28). 40% yield, yellow solid, mp = 52 °C; ^1H NMR ($CDCl_3$) δ 7.30 (d, 2H), 7.09 (d, 2H), 6.75–6.90 (m, 3H, Ar), 6.27 (t, 1H, NH), 6.10 (bs, 1H, NH), 5.66 (s, 1H, OH), 4.40 (bs, 2H, $NHCH_2Ar$), 4.15 (dd of AB, 1H, CH_2OCO), 3.86 (s, 3H, OCH_3), 3.7–3.85 (m, 2H, CH_2OCO and $CHCH_2$ $NHC=S$), 3.27 (m, 1H, $CHCH_2NHC=S$), 2.58 (ddd of AB, 2H, CH_2Ph), 2.30 (m, 1H, CH), 1.29 (s, 9H,

$C(CH_3)_3$), 1.22 (s, 9H, $C(CH_3)_3$); IR (neat): 3360, 1714, 1277, 1158; MS *m/e* 500 (M^+). Anal. calcd for $C_{28}H_{40}N_2O_4S$: C, 67.17; H, 8.05; N, 5.59; S, 6.40. Found: C, 67.42; H, 8.08; N, 5.57; S, 6.38.

2-(4-*t*-Butylbenzyl)-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl benzoate (29). 50% yield, white solid, mp = 54 °C; ^{1}H NMR ($CDCl_3$) δ 8.02 (m, 2H), 7.60 (m, 1H), 7.46 (m, 2H), 7.32 (d, 2H), 7.14 (d, 2H), 6.75–6.90 (m, 3H, Ar), 6.28 (t, 1H, NH), 6.06 (bs, 1H, NH), 5.61 (s, 1H, OH), 4.40 (m, 3H, NHCH2Ar and CH_2 OCO), 4.08 (dd of AB, 1H, CH_2OCO), 3.86 (s, 3H, OCH_3), 3.80 (m, 1H, $CHCH_2NHC=S$), 3.40 (m, 1H, $CHCH_2NHC=S$), 2.68 (m, 2H, CH_2Ph), 2.46 (m, 1H, CH), 1.29 (s, 9H, $C(CH_3)_3$); IR (neat): 3360, 1714, 1272, 1124; MS *m/e* 520 (M^+). Anal. calcd for $C_{30}H_{36}N_2O_4S$: C, 69.20; H, 6.97; N, 5.38; S, 6.16. Found: C, 69.48; H, 6.99; N, 5.36; S, 6.14.

General procedure for the synthesis of 30–31. A solution of **5a** (or **5c**) (0.1 g, 0.52 mmol) in THF (10 mL) was treated with sodium hydride (60% dispersion, 0.032 g, 0.78 mmol) followed by benzyl bromide (0.92 mL, 0.78 mmol) and tetrabutylammonium iodide (0.096, 0.26 mmol), and stirred for 3 h at room temperature. The mixture was diluted with ether, filtered through a short pad of silica gel and the filtrate was concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with EtOAc:hexane (1:20) as eluant to give **30** (or **31**).

3-Azido-2-benzylpropyl benzyl ether (30). 82% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.1–7.4 (m, 10H, phenyl), 4.49 (dd of AB, 2H, $PhCH_2O$), 3.33–3.50 (m, 4H, BnO CH_2 and CH_2N_3), 2.68 (m, 2H, CH_2Ph), 2.14 (m, 1H, CH).

3-Azido-2-(4-chlorobenzyl)propyl benzyl ether (31). 84% yield, colorless oil; ^{1}H NMR ($CDCl_3$) δ 7.23–7.4 (m, 5H, phenyl), 7.21 (d, 2H), 7.05 (d, 2H), 4.47 (dd of AB, 2H, $PhCH_2O$), 3.30–3.45 (m, 4H, $BnOCH_2$ and CH_2 N_3), 2.64 (m, 2H, CH_2Ph), 2.08 (dd, 1H, CH).

***N*-[2-Benzyl-3-(benzyloxy)propyl]-*N'*-(4-hydroxy-3-methoxybenzyl)thiourea (32).** This compound was prepared from **30** by following the general procedure for the synthesis of **18**–**29** in 42% yield as a colorless oil. ^{1}H NMR ($CDCl_3$) δ 7.10–7.30 (m, 10H, 2×Ph), 6.81 (d, 1H, Ar), 6.60 (bs, 1H, Ar), 6.56 (d, 1H, Ar), 6.54 (bs, 1H, NH), 5.64 (s, 1H, OH), 4.35 (m, 4H, $NHCH_2Ar$ and $PhCH_2O$), 3.82 (s, 3H, OCH_3), 3.50 (m, 2H, $BnOCH_2$), 3.37 (bt, 2H, $CHCH_2NHC=S$), 2.64 (m, 2H, CH_2Ph), 2.20 (m, 1H, CH); IR (neat): 3288, 1274, 1123; MS *m/e* 450 (M^+). Anal. calcd for $C_{26}H_{30}N_2O_3S$: C, 69.30; H, 6.71; N, 6.22; S, 7.12. Found: C, 69.59; H, 6.74; N, 6.20; S, 7.10.

***N*-[3-Benzyloxy-2-(4-chlorobenzyl)propyl]-*N'*-(4-hydroxy-3-methoxybenzyl)thiourea (33).** This compound was prepared from **30** by following the general procedure for the synthesis of **18**–**29** in 46% yield as a white solid. mp = 37.5 °C; ^{1}H NMR ($CDCl_3$) δ 7.0–7.3 (m, 9H), 6.5–6.84 (m, 3H, Ar), 6.54 (bs, 1H, NH), 6.30 (bs, 1H, NH), 5.68 (s, 1H, OH), 4.32 (m, 4H, $NHCH_2Ar$ and $PhCH_2O$), 3.82 (s, 3H, OCH_3), 3.48 (m, 2H, $BnOCH_2$), 3.34 (m, 2H, $CHCH_2NHC=S$), 2.58 (m, 2H, CH_2Ph), 2.16 (m, 1H, CH); IR (neat): 3340, 1273, 1153; MS *m/e* 484 (M^+). Anal. calcd for $C_{26}H_{29}ClN_2O_3S$: C, 64.38; H, 6.03; N, 5.78; S, 6.61. Found: C, 64.62; H, 6.02; N, 5.75; S, 6.58.

(*R* or *S*)-2-Benzyl-3-{[(4-hydroxy-3-methoxybenzyl)amino]carbothioyl}propyl pivalate (35, 36). 35, (*R*)-enantiomer of **18** and **36**, (*S*)-enantiomer of **18** were obtained from (***R***)-**3a**[28] following the same general procedure for **18**; **35**: $[\alpha]_D = -714$ (c, 0.22, $CHCl_3$), **36**: $[\alpha]_D = +695$ (c, 0.08, $CHCl_3$).

Diethyl 1,3-dihydro-2H-indene-2,2-dicarboxylate (37). This compound was prepared from bis-1,2-chloromethylbenzene by following general procedures for the synthesis of **1** in 58% yield as a white solid. mp = 68 °C; ^{1}H NMR ($CDCl_3$) δ 7.12–7.26 (m, 4H, phenyl), 4.20 (q, 4H), 3.56 (s, 4H), 1.26 (t, 6H).

{2-[({[(4-Hydroxy-3-methoxybenzyl)amino]carbothioyl}amino)methyl]-2,3-dihydro-1H-inden-2-yl}methyl pivalate (38). This compound was prepared from **37** by following general procedures of **18** in 28% yield as a white solid. mp = 60.5 °C; ^{1}H NMR ($CDCl_3$) δ 7.14 (s, 4H, aromatic), 6.79–6.89 (m, 3H, Ar), 6.45 (bt, 1H, NH), 6.12 (bs, 1H, NH), 5.62 (s, 1H, OH), 4.38 (bs, 2H, $NHCH_2Ar$), 3.97 (s, 2H, $O=COCH_2$), 3.88 (s, 3H, OCH_3), 3.69 (bs, 2H, $CCH_2NHC=S$), 2.92 (d, 2H, *J* = 12 Hz, CH_2C), 2.74 (d, 2H, *J* = 12 Hz, CH_2C), 1.21 (s, 9H, $C(CH_3)_3$); IR (neat): 3356, 1715, 1553, 1514, 1276, 1155; MS *m/e* 456 (M^+). Anal. calcd for $C_{25}H_{32}N_2O_4S$: C, 65.76 ; H, 7.06; N, 6.14; S, 7.02. Found: C, 66.00; H, 7.09; N, 6.11; S, 7.00.

{2-[({[(4-Hydroxy-3-methoxybenzyl)amino]carbothioyl}amino)methyl]-2,3-dihydro-1H-inden-2-yl}methyl benzenecarboxylate (39). This compound was prepared from **37** by following general procedures of **22** in 30% yield as a white solid. mp = 62.6 °C; ^{1}H NMR ($CDCl_3$) δ 8.01 (d, 2H, *J* = 5.5 Hz, Bz), 7.60 (t, 1H, *J* = 5.6 Hz, Bz), 7.46 (t, 2H, *J* = 5.8 Hz, Bz), 7.16 (s, 4H, aromatic), 6.78–6.87 (m, 3H, Ar), 6.52 (bt, 1H, NH), 6.15 (bs, 1H, NH), 5.61 (s, 1H, OH), 4.40 (bs, 2H, $NHCH_2Ar$), 4.22 (s, 2H, $O=COCH_2$), 3.86 (s, 3H, OCH_3), 3.79 (bs, 2H, $CCH_2NHC=S$), 3.00 (d, 2H, *J* = 12 Hz, CH_2C), 2.85 (d, 2H, *J* = 12 Hz, CH_2C); IR (neat): 3354, 1715, 1553, 1514, 1273, 1113; MS *m/e* 476 (M^+). Anal. calcd for $C_{27}H_{28}N_2O_4S$: C, 68.04; H, 5.92; N, 5.88; S, 6.73. Found: C, 68.30; H, 5.94; N, 5.86; S, 6.71.

Molecular modelling

Modelling studies on RTX and **35** were performed on a Silicon Graphics R4600 INDY workstation. The molecules were constructed using SYBYL 6.4, TRIPOS. The conformational space available was explored using molecular dynamic (MD) simulations. The MD simulations were accomplished using the SYBYL Dynamic program. Thus, each compound was submitted to the following protocol: A starting structure was energy-

minimized using the Tripos force field and the conjugate gradient method until the rms derivative of the energy was below 0.005 kcal/mol/Å. The system was then 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. For each compound, the 501 resulting minimized structures were analyzed by superposition of the four pharmacophore points (RTX: centre of the vanilloid ring, centre of the orthoester phenyl, carbonyl oxygen of the C_{20}-ester, and carbonyl oxygen of the C_3-carbonyl; **35**: centre of the vanilloid ring, centre of the phenyl ring, sulfur of the thiourea, and carbonyl oxygen of ester).

[$_3$H]Resiniferatoxin binding assay

[^{3}H]RTX (37 Ci/mmol) was synthesized by the Chemical Synthesis and Analysis Laboratory, NCI-FCRDC. Nonradioactive RTX and capsazepine were purchased from LC Laboratories (Woburn, MA).

Inhibition of [^{3}H]RTX binding in the presence of competing ligands was determined on membrane preparations from rat spinal cord. Membrane preparations were prepared as described.[34] Briefly, animals were euthanized under general anesthesia and the entire spinal cord was removed aseptically. Samples were disrupted with the aid of an Omni 2000 tissue homogenizer in ice-cold 10 mM HEPES, pH 7.4, containing 5 mM KCl, 5.8 mM NaCl, 2 mM $MgCl_2$, 0.75 mM $CaCl_2$, 12 mM D-glucose, and 137 mM sucrose (buffer A). Homogenates were centrifuged at $1000\times g$ for 10 min at 4 °C; pellets were resuspended in Buffer A and recentrifuged at $35{,}000\times g$ for 40 min at 4 °C. The pellets from the second centrifugation were resuspended in the same buffer at an approximate protein concentration of 2 mg/mL, quick frozen on dry ice as small aliquots, and stored at −70 °C until assayed.

Experiments were designed to assess inhibition of specific [^{3}H]RTX binding to membranes by non-radioactive compounds. [^{3}H]RTX (100 pM) was incubated in the presence of competing ligand in a total volume of 300 μL with 100 μg membrane protein for 60 min at 37 °C in Buffer A supplemented with 0.25 mg/mL bovine serum albumin (type V, Sigma) . The bovine serum albumin was included to reduce nonspecific adsorption of RTX to surfaces. At the end of the incubation, tubes were chilled on ice and 100 μg of 'α_1-acid glycoprotein' (AGP, Sigma) in a 50 μL volume was added to each tube to reduced nonspecific binding. Bound and free [^{3}H]RTX were then separated by pelleting the membranes by centrifugation at $10{,}000\times g$ for 15 min at 4 °C. The tips of the tubes containing the pellets were cut off, and the bound radioactivity was determined by scintillation counting. Nonspecific binding was determined in the presence of 1 mM non-radioactive RTX. Measurements of binding were determined in triplicate in each experiment, and each experiment was performed at least three times. In each experiment, competition curves were determined typically using 5–6 concentrations of competing ligand.

Binding was expressed as fmol/mg protein. Protein concentration was measured using the Bio-Rad protein assay according to the manufacturer's protocol (Bio-Rad Laboratories, CA). Samples were equilibrated in scintillation fluid for 10 h before scintillation counting began and each sample was counted for 5 min. Binding data were analyzed by fitting to the following equation:

$$B = ((L_{\mathrm{H}} + L_{\mathrm{C}} * K_{\mathrm{d}}K_{\mathrm{I}})^{(n-1)} (K_{\mathrm{d}}^{n} + (L_H + L_{\mathrm{C}} * K_{\mathrm{d}}K_{\mathrm{I}})^{n}))(L_H^{n-1}(K_{\mathrm{d}}^{n} + L_H^{n})) \quad (1)$$

where L_{C} is the concentration of the non-radioactive ligand and K_{I} is the concentration of the free non-radioactive ligand at which it occupies half of the binding sites. B represents specifically bound [^{3}H]RTX, $B_{\max}$ is the receptor density, L_{H} is the concentration of free [^{3}H]RTX, K_{d} is the concentration of [^{3}H]RTX at which half of the receptors are occupied and n is the cooperativity index referred to as the Hill coefficient.

CAP-activated single channel assay

Cell preparation. Cultured DRG neurons were prepared as described previously.[29] Briefly, DRGs were dissected from all levels of lower cervical, thoracic and lumbar spinal cord of 1- or 2-day-old neonatal rats. DRGs were collected in cold culture medium (4 °C) containing DMEM/F-12 mixture (Gibco, Grand Island, NY), fetal bovine serum (10%, Gibco), 1 mM sodium pyruvate, 25 ng/mL nerve growth factor (Sigma, St. Louis, MO), and 100 units/mL of penicillin/streptomycin (Sigma). Ganglia were washed three times with DMEM/F-12 medium and incubated for 30 min in the DMEM/F-12 medium containing 1 mg/mL collagenase (Type II, Worthington Biomedical, Freehold, NJ). The ganglia were then washed three times with Mg^{2+}- and Ca^{2+}-free Hank's solution and incubated with gentle shaking in the warm (37 °C) Hank's solution containing 2.5 mg/mL trypsin (Gibco). The solution was centrifuged at 1000 rpm for 10 min, and the pellet was washed two or three times with the culture medium to inhibit the enzyme. The pellet was suspended in the culture medium and gently triturated with a Pasteur pipette. The suspension was plated on round glass coverslips (Fisher, Pittsburgh, PA) placed in small Petri dishes. The glass coverslips were treated overnight with poly-L-lysine (Sigma) and dried before use. Cells were incubated at 37 °C in 95% air/5% CO_2 gas mixture. Cells were used 2–4 days after plating.

Current recording. Borosilicate glass pipettes (Narishige Scientific Instrument Lab., Tokyo) were pulled and coated with Sylgard (Dow Corning Co., Midland, MI). Tip resistances were about 2 and 5 Mohms for whole-cell and single-channel current recordings, respectively. After gigaseals were formed with the glass pipettes, cell-attached and inside-out patch configurations were used to study single-channel currents as described by Hamill et al.[30] A salt bridge (1% agar in 300 mM KCl) immersed in the bath and an Ag/AgCl reference electrode in the

pipette solution was used to minimize changes in junctional potentials. Junctional potentials were cancelled before gigaseals were formed. For whole-cell recording, the cell membrane under a glass pipette was ruptured by a gentle suction. After forming a whole-cell patch, the capacitative transient was cancelled. Single-channel currents were recorded using a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA) and filtered at 5 KHz with an 8-pole, low-pass Bessel filter. Data were digitized at 37 KHz with a digital data recorder (VR-10B, Instrutech, Great Neck, NY) and stored on videotapes for later analysis. For chart recording, output of the amplifier was filtered at 500 Hz (Frequency Device, Havenhill, MA) and fed into a thermal array chart recorder (TA-240, Gould Instrument System, Valley View, OH). The digitized data stored on videotapes were imported to a personal computer (IBM Pentium-compatible) for computer analysis of single-channel currents.

Channel open probability (P_o), amplitude and mean open time of single-channel currents were obtained using the pCLAMP software (Version 6.02, Axon Instruments). P_o of single channels was obtained from the ratio of the areas under the curves representing open events divided by the sum of the areas under the curves representing open and closed events. The half-amplitude algorithm in FETCHAN (Axon Instruments) was used for the threshold amplitude for detecting open events. Channel activity (NP_o) was calculated as a product of the number of channel (N) in the patch and P_o. NP_o or P_o was collected only from patches that contained power than five functional CAP-activated channels.

Solutions. Solutions in bath and pipette for single-channel recordings contained (in mM) 140 Na^+, 2 Mg^{2+}, 144 Cl^-, 5 EGTA, and 10 HEPES at pH 7.2. For whole cell recording, pipette solution contained (in mM) 140 K^+, 2 Mg^{2+}, 144 Cl^-, 5 EGTA, 10 HEPES, and 4 ATP at pH 7.2. The control perfusion solution for whole-cell recording contained (in mM) 140 Na^+, 5 K^+, 2 Mg^{2+}, 1 Ca^{2+}, 151 Cl^-, and 10 HEPES. The synthesized compounds were dissolved and stored in 100% ethanol to make 10 mM stock solutions. All other reagents used in cell-culture or in electrophysiological experiments were purchased from Sigma.

Mouse writhing antinociceptive assay

Male ICR mice (weight 25 g) were maintained in a controlled lighting environment (12 h on/12 h off). Groups of 10 animals were used for each dose and for each treatment. Animals received an intraperitoneal injection of 0.3 mL of the chemical irritant phenyl-*p*-quinone (4.5 mg/kg dissolved in saline containing 5% ethanol), and 6 min later the number of abdominal constrictions was counted in the subsequent period of 6 min. Animals received the synthesized compounds in 0.2 mL vehicle of ethanol:Tween-80:saline (10:10:80) intraperitoneally 30 min before the injection of phenyl-*p*-quinone. A reduction in the number of writhes responding in the phenyl-*p*-quinone treated group relative to the number responding in the saline control group was considered to be indicative of an antinociceptive effect. The data are expressed as an ED_{50} value representing a 50% reduction in the number of writhes. ED_{50} values were determined by linear regression analysis with at least three data points in the log dose–response curve.

TPA-induced mouse ear edema assay.[31] Male ICR mice (25–30 g) were treated topically on the right ear with 25 μL acetone or synthesized compound in acetone. Groups of 10 animals were used for each dose and for each treatment. Initial treatment of vanilloid agonist caused erythema in the treated mouse ear possibly through release of neuropeptides. Approximately 17 h later, an identical treatment was applied to release residual neuropeptides and desensitize nociceptive sensory neurons which are known to play an important role in neurogenic inflammation. One hour later, 25 μL of 0.5 nmol TPA in acetone was applied to the same ear. Five hours following the application of TPA, the animals were sacrificed and ear punches (6 mm diameter) were weighed to the nearest 0.1 mg on a electrobalance. The increased weight of the ear punch is a measure of inflammation (ear edema). Anti-inflammatory effects were calculated as percent inhibition of swelling in the compound-treated versus the control group. The percent inhibition is defined by the following equation.% inhibition $=(C-T)/C\times100$, where C and T refer to increases in ear weight in TPA-treated and TPA + drug-treated groups, respectively. From this data the IC_{50} value was determined as the dose required to produce a 50% reduction in the TPA-induced inflammation.

Pungency and tachyphylaxis in the rat eye-wiping test

The pain-inducing potency of the compounds was determined in the eye-wiping assay as previously described,[32] and expressed quantitatively as follows. Solutions (20 μL/eye) in 10-fold increasing concentrations in physiological saline, containing at most 5% ethanol, were dropped into the eye of rats weighing 150–180 g, and the number of protective movements (eye-wiping with the foreleg) was counted in the period of 5 min. Each concentration was applied to six rats and the dose–response curves were obtained from the mean values. From the dose–response curves the concentrations having a moderate pain-producing potency (MPP), i.e., inducing an equal response of 10 wipings, were calculated for each compound. On the basis of these concentrations the relative pain-producing potency (RPP) was determined as compared to that of capsaicin, which was taken as 100.

To check for the development of tachyphylaxis or cross-tachyphylaxis, 6 h following the application of the test compound (20 μL/eye), test challenge of 10 μg/mL (20 μL/eye) capsaicin was applied to the same eye. The percent reduction of response to 10 μg/mL CAP in rats treated with the test compounds, compared to the vehicle-treated rats, was estimated to be the index of desensitization.

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