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3-Ylidenephthalides as a new class of transient receptor potential channel TRPA1 and TRPM8 modulators

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ABSTRACT

Following the recent identification of the naturally occurring 3-ylidene-4,5-dihydrophthalide ligustilide and its oxidation product dehydroligustilide as novel TRPA1 modulators, a series of seventeen 3-ylidenephthalides was synthesized and tested on TRPA1 and TRPM8 channels. Most of these compounds acted as strong modulators of the two channel types with EC_{50} and/or IC_{50} values distinctly lower than those of the reference compounds.

3-Ylidenephthalides [3-ylidene-1(3*H*)-isobenzofuranones] represent an important group of both naturally occurring and synthetic products endowed with remarkably wide-ranging biological activities (Fig. 1).[1] Most of them exist in the thermodynamically more stable *Z*-configuration. In fact, (*E*)-3-ylidenephthalides have appeared less frequently in the chemical literature, being isolated with difficulty from *E*–*Z* mixtures in which the more stable *Z*-isomers predominated.[2] Typically, the content of (*Z*)-3-butylidene-4,5-dihydrophthalide [(*Z*)-ligustilide] is about ten times higher than that of the *E*-isomer in medicinal plants from the traditional chinese medicine.[3] The biological activity of ligustilide, which is a major component of the aromas of celery (*Apium graveolens*) and lovage (*Levisticum officinale*), has been extensively investigated revealing an impressive pleiotropic pharmacological profile that includes, inter alia, reduction of cerebral infarct volume and improvement of neurobehavioral deficits, attenuation of lipopolysaccaride (LPS)-induced endotoxic shock, inhibition of vascular smooth muscle cell proliferation, antioxidant, antiapoptotic, antithrombotic, antinflammatory, and analgesic effects.[3] The ability of ligustilide to penetrate the brain when administered through the nasal route deserves a special mention, because possibly relevant to its neuroprotective actions.[4]

Recently, ligustilide has been reported to behave as a transient receptor potential ankyrin type-1 (TRPA1) agonist (EC_{50} = 44 μM), with only modest desensitizing properties on mustard oil (MO)-induced activation of this channel.[5] This profile of activity

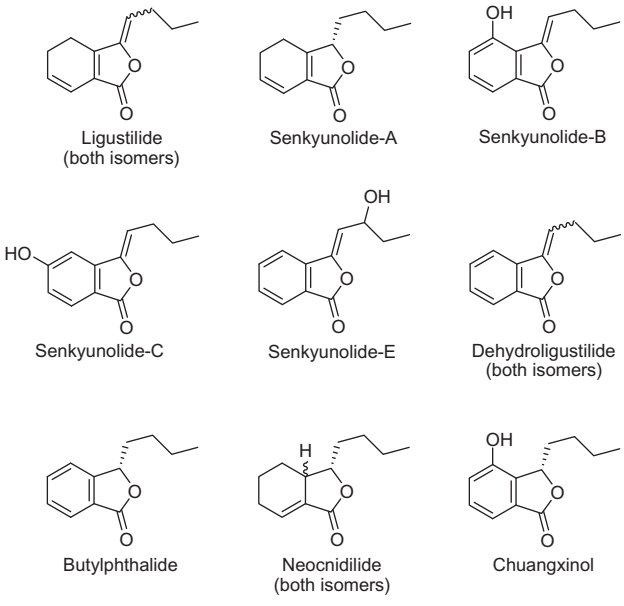

Figure 1. Structures of some representative 3-ylidenephthalides and related compounds.

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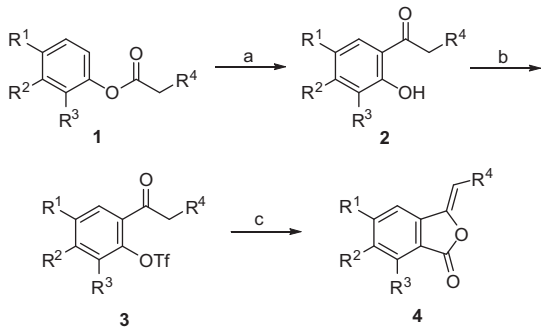


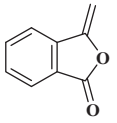
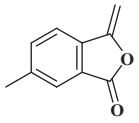
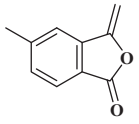
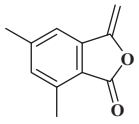
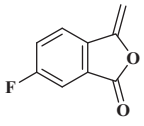
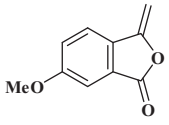
Scheme 1. Synthesis of compounds **4**. Reagents and conditions: (a) $AlCl_3$, 100 °C, 1 h; (b) $(CF_3SO_2)_2O$, 2,6-di-*tert*-butyl-4-methylpyridine, CH_2Cl_2, rt, 16 h; (c) CO, $Pd(OAc)_2$ (dppp), Et_3N (or K_2CO_3), DMF (or toluene), 60 °C (or 100 °C), 1–4 h.

is reversed by aromatization to dehydroligustilide, which acts as a potent blocker of MO-activated currents (IC_{50} = 23 μM). The effects of ligustilide and dehydroligustilide on mouse transient receptor potential vanilloid type-1 (TRPV1) and melastatin type-8 (TRPM8) channels have been tested as well. None of the compounds was able to activate significantly these channels but, when TRPM8 was activated by menthol, a potent inhibition was observed by application of either compound. These observations indicate that 3-ylidenephthalide could be a new and useful template for the identification of TRPA1 and TRPM8 modulators. Therefore, we decided, as a continuation of our ongoing interest in the SAR exploration of natural product ligands of TRP channels,[6] to prepare and test on TRPA1 and TRPM8 channels several representative 3-ylidenephthalides, including dehydroligustilide (compound **4j**, Table 1).

TRPA1 and TRPM8 channels are involved in a wide array of physiological functions and their dysfunction can cause various pathological conditions, including pain, cancer and bladder, respiratory and skeletal disorders.[7] As a consequence, modulation of TRPA1 and TRPM8 channels provides an attractive approach for the treatment of the aforementioned 'channelopathies' and indeed have been among the most actively investigated drug targets within the TRP channel realm over the past few years.

The 3-ylidenephthalides **4** were synthesized by a palladium-catalyzed cyclocarbonylation of 2-triflyloxyacetophenone derivatives **3**, an original approach to 3-ylidenephthalides disclosed by us 20 years ago,[8] which still offers distinct advantages over related

Table 1
Results of TRPA1 and TRPM8 assays of 3-ylidenephthalides **4**[a]

Compound	TRPA1 (efficacy)[b]	TRPA1 (EC_{50}, μM)	TRPA1 (IC_{50}, μM)[c]	TRPM8 (efficacy)[d]	TRPM8 (EC_{50}, μM)	TRPM8 (IC_{50}, μM)[e]	TRPM8 (IC_{50}, μM)[f]
 **4a**	50.5 ± 1.4	24.5 ± 3.2	>100	<10	ND	>200	>200
 **4b**	28.6 ± 1.0	24.2 ± 3.6	>100	<10	ND	>200	>200
 **4c**	11.3 ± 0.4	50.8 ± 1.2	>100	<10	ND	>200	>200
 **4d**	85.1 ± 1.9	17.2 ± 1.3	33.95 ± 1.7	<10	ND	90.1 ± 1.8	128.4 ± 3.7
 **4e**	10.2 ± 0.5	32.2 ± 0.1	>100	<10	ND	>200	>200
 **4f**	10.1 ± 0.2	42.8 ± 0.1	>100	<10	ND	>200	>200

(*continued on next page*)

Table 1 (*continued*)

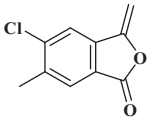
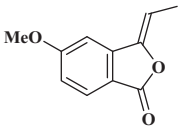
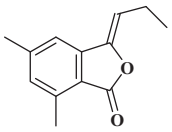
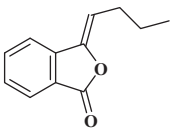
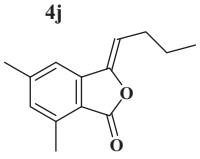
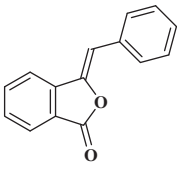
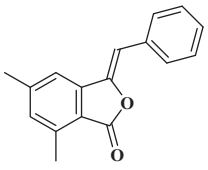
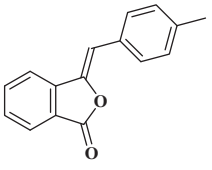
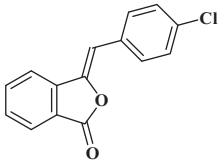
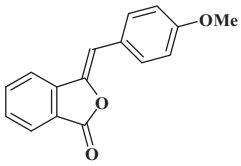
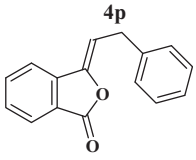
Compound	TRPA1 (efficacy)[b]	TRPA1 (EC_{50}, μM)	TRPA1 (IC_{50}, μM)[c]	TRPM8 (efficacy)[d]	TRPM8 (EC_{50}, μM)	TRPM8 (IC_{50}, μM)[e]	TRPM8 (IC_{50}, μM)[f]
	20.7 ± 0.5	4.7 ± 0.5	46.7 ± 7.2	<10	ND	74.2 ± 1.6	135.5 ± 14.1
4g 	24.6 ± 0.7	21.7 ± 2.9	>100	<10	ND	161.4 ± 3.3	>200
4h 	43.7 ± 1.5	15.8 ± 2.2	36.3 ± 4.3	<10	ND	7.7 ± 0.3	12.1 ± 0.2
4i 	<10	ND	44.5 ± 3.8	<10	ND	140.7 ± 0.3	153.3 ± 5.6
4j 	94.3 ± 1.9	2.8 ± 0.2	3.3 ± 0.3	<10	ND	2.5 ± 0.1	6.8 ± 0.1
4k 	93.6 ± 6.3	3.0 ± 0.9	2.9 ± 0.2	<10	ND	8.3 ± 0.4	10.2 ± 0.1
4l 	117.1 ± 2.9	1.1 ± 0.1	0.80 ± 0.03	<10	ND	1.0 ± 0.1	1.9 ± 0.1
4m 	125.7 ± 2.6	4.3 ± 0.4	2.4 ± 0.1	<10	ND	7.2 ± 0.4	25.1 ± 2.6
4n							

Table 1 (*continued*)

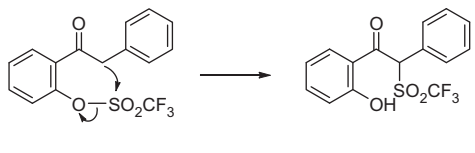
Compound	TRPA1 (efficacy)[b]	TRPA1 (EC_{50}, μM)	TRPA1 (IC_{50}, μM)[c]	TRPM8 (efficacy)[d]	TRPM8 (EC_{50}, μM)	TRPM8 (IC_{50}, μM)[e]	TRPM8 (IC_{50}, μM)[f]
 **4o**	111.6 ± 1.3	2.0 ± 0.1	1.5 ± 0.1	<10	ND	14.9 ± 1.7	48.1 ± 0.3
 **4p**	65.1 ± 0.5	1.3 ± 0.1	2.2 ± 0.2	12.9 ± 2.7	1.8 ± 0.1	3.4 ± 0.5	11.3 ± 0.1
 **4q**	103.0 ± 1.5	3.1 ± 0.2	3.2 ± 0.2	<10	ND	2.4 ± 0.1	4.4 ± 0.5

[a] Data are means ± SEM of $N = 3$ determinations.
[b] As percent of the effect of allyl isothiocyanate (100 μM).
[c] Determined against the effect of allyl isothiocyanate (100 μM).
[d] As percent of the effect of ionomycin (4 μM).
[e] Determined against the effect of icilin (0.25 μM).
[f] Determined against the effect of menthol (50 μM). ND, not determined when efficacy is lower than 10%.

transition metal-catalyzed heteroannulation reactions in terms of starting materials availability (Scheme 1).[9] The cyclocarbonylation of triflates **3a–k,q** was carried out at 60 °C under a CO atmosphere in DMF as solvent, using $Pd(OAc)_2$ as catalyst, 1,3-bis(diphenylphosphino)propane (dppp) as ligand, and Et_3N as base. The reaction of triflates **3l–p** required somewhat different conditions (toluene as solvent, K_2CO_3 as base, 100 °C) because, under standard conditions, **3l** was completely rearranged to the corresponding triflone **5** through a base-catalyzed process (Scheme 2).[8,10] Assignment of (*Z*)-configuration to phthalides **4h–q** was based on ^1H NMR data, in particular the chemical shift of the C-8 olefinic proton.[1h,2b,9,11] In addition, phthalides **4j** and **4l** exhibited physical properties well in agreement with those reported in literature for known *Z*-isomers.[11f,12]

Triflates **3** were in turn obtained by triflation of the corresponding phenols **2**. Non-commercially available 1-(2-hydroxyaryl)alkan-1-ones **2d**, **h–q** were prepared by a $AlCl_3$-promoted Fries rearrangement of aryl esters **1d**, **h–q**.

The 17 phthalides synthesized were tested for their ability to induce Ca^{2+} elevation in HEK293 cells stably transfected with either the rat TRPA1 or the rat TRPM8 cDNAs (Table 1).[13] Control experiments were carried out using non-transfected HEK293 cells. The antagonist or desensitizing activity was evaluated by adding the test compounds 5 min before stimulation of cells with reference agonists.



3l 5

Scheme 2. Base-catalyzed rearrangement of triflate **3l** to triflone **5**.

3-Methylenephthalides **4a–g** produced a moderate activation of TRPA1 with efficiencies between 10.1% and 85.1% and EC_{50} values between 4.7 and 50.8 μM and behaved, with the exception of **4d**, **g**, as weak TRPA1 desensitizers. They were, again with the exception of **4d**, **g**, essentially inactive in TRPM8 activity assays. The best result in terms of activation and subsequent desensitization properties towards TRPA1 was observed with the 5,7-dimethyl-substituted phthalide **4d**, followed by 5-chloro-6-methyl-3-methylenephtahilde **4g**, a result that supports the hypothesis that dehydroligustilide might bind to a hydrophobic pocket via a non-covalent mechanism.[5]

Homologation of the methylene moiety, while maintaining the 5,7-dimethyl substitution (compounds **4i**, **k**), greatly increased the ability to elicit a 'true' antagonistic response on TRPM8 channels (that is, inhibition without agonism per se, and hence not due to desensitization) with IC_{50} values $\leqslant$10 μM. Phthalide **4k** also displayed single-digit micromolar EC_{50} and IC_{50} values (2.8 and 3.3, respectively), on TRPA1 channels.

In comparison with **4k**, and in agreement with literature data,[5] dehydroligustilide (**4j**) which lacks the 5,7-dimethyl substitution, inhibited MO-induced activation of TRPA1 with a IC_{50} value of 44.5 μM, with no appreciable activating capacity towards both TRPA1 and TRPM8 channels. Furthermore, preincubation of TRPM8-HEK293 cells with dehydroligustilide and then continued incubation with either icilin or menthol caused only a rather modest inhibition of the TRPM8 response to these agonists, with IC_{50} values ~150 μM. Compared to literature data,[5] **4j** was thus found to be a weak TRPM8 inhibitor and this discrepancy could be due to the use of TRPM8 from a different species. The large activity gain observed following the introduction of two methyl groups in dehydroligustilide is noteworthy and may be tenta-

tively ascribed to an improvement of the fitting into a lipophilic region of the channel.

A profile similar to that of **4k** was exhibited by the 3-(2-phenylethylidene)phthalide **4q** and by most of the 3-benzylidenephthalides (compounds **4l**–**p**), which acted as robust TRPA1 activators and desensitizers and TRPM8 antagonists. Again, the 5,7-dimethyl substitution proved to be beneficial for both TRPA1 and TRPM8 modulating properties (compare compounds **4l** and **4m**), while *para*-substituents on the benzylidene phenyl ring appeared to exert only little influence on activity, suggesting again a non-covalent binding.

In conclusion, in this Letter we have presented a series of 3-ylidenephtahlides that act as strong modulators of TRPA1 and/or TRPM8 channels with EC_{50} and/or IC_{50} values distinctly lower than those of ligustilide and dehydroligustilide. The 3-ylidenephthalide structure qualifies as a versatile new template for the development of novel TRP channel modulators.

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10. *General procedure for the synthesis of compounds* ***4***. Method A (for triflates **3a**–**k**, **q**): a mixture of triflate **3** (1 mmol), Et_3N (0.28 mL, 2 mmol), $Pd(OAc)_2$ (7 mg, 0.03 mmol), and 1,3-bis(diphenylphosphino)propane (12 mg, 0.03 mmol) in DMF (3 mL) was purged with carbon monoxide for 5 min and then stirred under a CO balloon at 60 °C for 1–4 h. The reaction mixture was then diluted with brine and extracted with AcOEt, washed twice with brine, dried (Na_2SO_4), and evaporated under vacuum. The residue was purified by silica gel column chromatography eluting with light petroleum/AcOEt mixtures. Method B (for triflates **3l**–**p**): a mixture of triflate **3** (1 mmol), K_2CO_3 (138 mg, 1 mmol), $Pd(OAc)_2$ (22 mg, 0.1 mmol), and 1,3-bis(diphenylphosphino)propane (41 mg, 0.1 mmol) in toluene (6 mL) was purged with carbon monoxide for 5 min and then stirred under a CO balloon at 100 °C for 2–4 h. The reaction mixture was then worked-up as in method A. *Data for selected compounds:* compound **4k**: yield 58%; mp 35–37 °C; IR ($CHCl_3$) 1760, 1677, 1457, 1378 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.98 (3H, t, J = 7.5 Hz), 1.53 (2H, sextet, J = 7.5 Hz), 2.41 (2H, q, J = 7.5 Hz), 2.43 (3H, s), 2.63 (3H, s), 5.54 (1H, t, J = 7.5 Hz), 7.05 (1H, s), 7.23 (1H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.80, 17.37, 21.96, 22.61, 27.72, 108.15, 117.24, 119.92, 132.16, 138.86, 140.50, 145.02, 145.60, 167.41. Compound **4l**: yield 82%; mp 92–93 °C; IR ($CHCl_3$) 1769, 1661, 1610, 1474, 1352 cm^{-1}, ^1H NMR (300 MHz, $CDCl_3$) δ 6.43 (1H, s), 7.32–7.95 (9H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 107.07, 119.81, 123.40, 125.57, 128.42, 128.77, 129.77, 130.12, 133.08, 134.48, 140.60, 144.57, 167.06. Compound **4m**: yield 15%; mp 140–142 °C; IR (KBr) 1772, 1654, 1601 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 2.46 (3H, s), 2.65 (3H, s), 6.33 (1H, s), 7.09–7.84 (7H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 17.42, 22.03, 105.83, 117.47, 128.08, 128.71, 129.96, 132.67, 133.42, 139.24, 141.74, 144.62, 145.32, 167.20. Compound **4q**: Yield 34%; mp 88–90 °C; IR (neat) 1768, 1686, 1278, 1045 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 3.81 (2H, d, J = 7.8 Hz), 5.78 (1H, t, J = 7.8 Hz), 7.22–7.90 (9H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 32.06, 107.73, 119.85, 124.57, 125.30, 126.50, 128.58, 128.69, 129.67, 134.34, 140.70, 145.72, 167.01.
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13. *TRPA1 and TRPM8 channel assays.* HEK293 (human embryonic kidney) cells stably over-expressing recombinant rat TRPA1 or rat TRPM8 were grown on 100 mm diameter Petri dishes as mono-layers in minimum essential medium (EMEM) supplemented with non-essential amino acids, 10% foetal bovine serum, and 2 mM glutamine, and maintained at 5% CO_2 at 37 °C. Stable expression of each channel was checked by quantitative PCR (data not shown). The effect of the substances on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined by using Fluo-4, a selective intracellular fluorescent probe for Ca^{2+}. On the day of the experiment, cells were loaded for 1 h at room temperature with the methyl ester Fluo-4-AM (4 μM in dimethyl sulfoxide containing 0.02% Pluronic F-127, Invitrogen) in EMEM without foetal bovine serum, then were washed twice in Tyrode's buffer (145 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-Glucose, and 10 mM HEPES, pH 7.4), resuspended in the same buffer, and transferred (about 100,000 cells) to the quartz cuvette of the spectrofluorimeter (Perkin-Elmer LS50B equipped with PTP-1 Fluorescence Peltier System; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) under continuous stirring. The changes in $[Ca^{2+}]_i$ were determined before and after the addition of various concentrations of test compounds by measuring cell fluorescence (λ_{EX} = 488 nm, λ_{EM} = 516 nm) at 25 °C. Curve fitting (sigmoidal dose–response variable slope) and parameter estimation were performed with GraphPad Prism® (GraphPad Software Inc., San Diego, CA). Potency was expressed as the concentration of test substances exerting a half-maximal agonist effect (i.e., half-maximal increases in $[Ca^{2+}]_i$) (EC_{50}). The effects of TRPA1 agonists are expressed as a percentage of the effect obtained with 100 μM allyl isothiocyanate (AITC). In the case of TRPM8 assays, the efficacy of the agonists was first determined by normalizing their effect to the maximum Ca^{2+} influx effect on $[Ca^{2+}]_i$ observed with application of 4 μM ionomycin (Alexis). When significant, the values of the effect on $[Ca^{2+}]_i$ in wild-type (i.e., not transfected with any construct) HEK293 cells were taken as baseline and subtracted from the values obtained from transfected cells. Antagonist/desensitizing behaviour was evaluated against AITC (100 μM) for TRPA1, icilin (0.25 μM) and menthol (50 μM) for TRPM8, by adding the test compounds in the quartz cuvette 5 min before stimulation of cells with agonists. Data are expressed as the concentration exerting a half-maximal inhibition of agonist-induced $[Ca^{2+}]_i$ elevation (IC_{50}), which was calculated again using GraphPad Prism® software. The effect on $[Ca^{2+}]_i$ exerted by agonist alone was taken as 100%. Dose–response curves were fitted by a sigmoidal regression with variable slope. All determinations were performed at least in triplicate. Statistical analysis of the data was performed by analysis of variance at each point using ANOVA followed by the Bonferroni's test.