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Isovanillin derived *N*-(un)substituted hydroxylamines possessing an *ortho*-allylic group: valuable precursors to bioactive N-heterocycles†

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The intramolecular 1,3-dipolar cycloaddition of isovanillin derived *N*-aryl hydroxylamines possessing *ortho*-allylic dipolarophiles affords novel benzo analogues of tricyclic isoxazolidines that can be readily transformed into functionalized lactams, γ-aminoalcohols and oxazepines. The corresponding *N*-unsubstituted hydroxylamines give rise to tetrahydroisoquinolines. Anxiogenic properties of these compounds are tested in zebra fish.

The development of operationally simple strategies that allow direct and fast access to an array of densely functionalized small molecules of potential pharmacological interest is the central focus of modern organic synthesis. The search for privileged building blocks that allows their broad diversification into pharmacologically relevant scaffolds is therefore of immense importance, and accordingly, we identified isovanillin derived *N*-hydroxylamines as key precursors to hybrid structures combining vanillyl and N-heterocyclic moieties.

Isoxazolidines (**A**, Fig. 1) are important heterocycles[1] commonly found in a diverse array of bioactive natural products (*e.g.* cycloserin, acivicine). They are also valuable intermediates in organic synthesis.[2] Compounds such as capsaicin, the main capsaicinoid in chili peppers containing a vanillyl moiety (**B**, Fig. 1), on the other hand have attracted considerable interest as they target TRPV1 (transient receptor potential vanilloid 1), a molecular integrator for a broad variety of seemingly unrelated noxious stimuli.[3] A few small molecule based TRPV1 antagonists and agonists have been advanced into clinical trials for pain relief.[3] This and our long-standing interest in bioactive fused heterocycles prompted us to design novel molecular scaffolds by combining the isovanillyl moiety and the isoxazolidine ring in a single framework **C** (Fig. 1). We anticipated that isovanilloid based tricyclic drug-like molecules derived from **C** might show pharmacological properties similar to capsaicin. While several methods have been reported for the synthesis of isoxazolidines, the dipolar cycloaddition of nitrile oxides/hydroxylamines to allyl dipolarophiles appears to be attractive.[4,5] The intramolecular version of 1,3-dipolar cycloadditions[6–8] of hydroxylamines to simple allylic or *N*/*O*-allylic dipolarophiles has also been reported.[6] For example, benzo analogues of tricyclic isoxazolidines have been prepared by using this strategy[7] (Scheme 1). While all these methods were not precisely suitable for our purpose these findings however provided a valuable lead to develop a strategically related but unique approach to **C** (Scheme 1). Herein we report the intramolecular 1,3-dipolar cycloaddition of isovanillin derived *N*-substituted hydroxylamines possessing allylic dipolarophiles at the *o*-position. In an extension of this approach the corresponding *N*-unsubstituted hydroxylamines

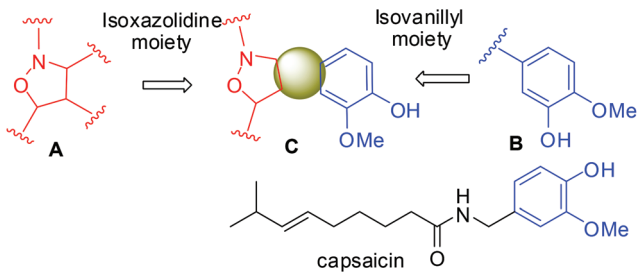

Fig. 1 Design of **C** by combining **A** and **B**.

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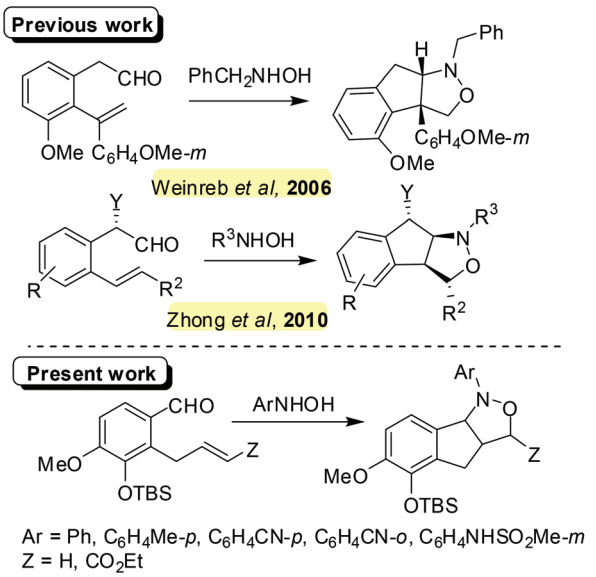
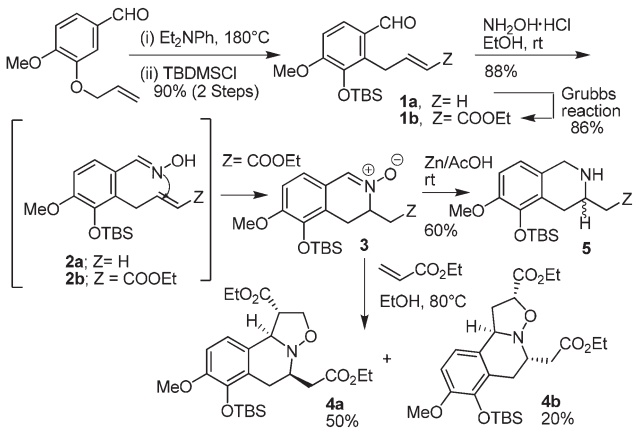
†Electronic supplementary information (ESI) available: Experimental procedures, spectral data for all new compounds, copies of NMR spectra and results of *in vitro* study. CCDC 909838. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c3ob42460j



Scheme 1 Synthesis of benzo analogues of tricyclic isoxazolidine.

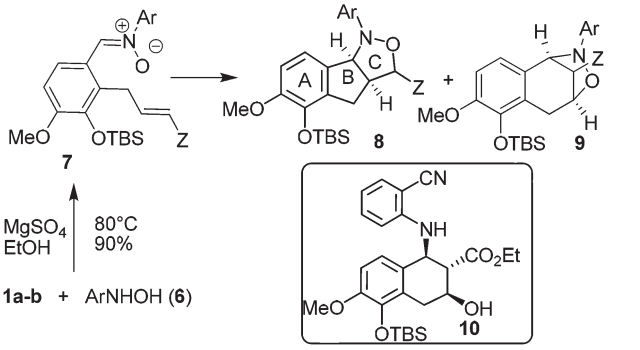


Scheme 2 Preparation of **1a–b** and their reaction with $NH_2OH{\cdot}HCl$. Further chemical transformations of nitrone **3** obtained from **1b**.

give rise to tetrahydroisoquinolines. Further synthetic applications and pharmacological properties of some of these compounds synthesized are presented.

As the starting point, *o*-substituted allyl isovanillins **1a** and **1b** were prepared *via* sequential O-allylation of isovanillin followed by Claisen rearrangement and cross metathesis (Scheme 2).[9] However, neither **1a** nor **1b** afforded the expected isoxazolidines when reacted with $NH_2OH{\cdot}HCl$. While oxime **2a** was obtained from **1a**, the acceptor substituted **1b** reacted further to yield **3** *via* an intramolecular aza-Michael addition of the hydroxylamine nitrogen, being apparently favored over the expected [3 + 2] cycloaddition. Notably, the conversion of **1b** to **3** was not affected significantly by additives like NaOAc or Et_3N or by the reaction temperature. The nitrone **3** can be further transformed, *e.g.* to the regioisomeric tetrahydro-1*H*-isoxazolo[3,2-*a*]isoquinolines **4a** and **4b** when allowed to

Table 1 The reactions of **1a–b** with various *N*-aryl hydroxylamines[a]



Entry	6; Ar=	1; Z=	Products	Yield[b] (%)
1	**6a**; Ph	**1a**; H	**8a : 9a** (1 : 2)[c]	96
2	**6b**; 4-MeC_6H_4	**1a**	**8b : 9b** (1 : 2)[c]	100
3	**6c**; 4-NCC_6H_4	**1a**	**8c : 9c** (1 : 2)[c]	94
4	**6d**; 3-$(MeSO_2NH)C_6H_4$	**1a**	**8d : 9d** (1 : 2)[c]	95
5	**6e**; 2-NCC_6H_4	**1a**	**7e**	88
6	**6a**;	**1b**; CO_2Et	**8f**	78
7	**6e**	**1b**	**10**	58

[a] Reactions were performed using **1a–b** (1 mmol) and **6** (2 mmol) in EtOH (5 mL) at 80 °C for 3–5 h under nitrogen. [b] Isolated overall yield. [c] Ratio was changed to 2 : 1 when the reaction was performed in the absence of any solvent at 130 °C.

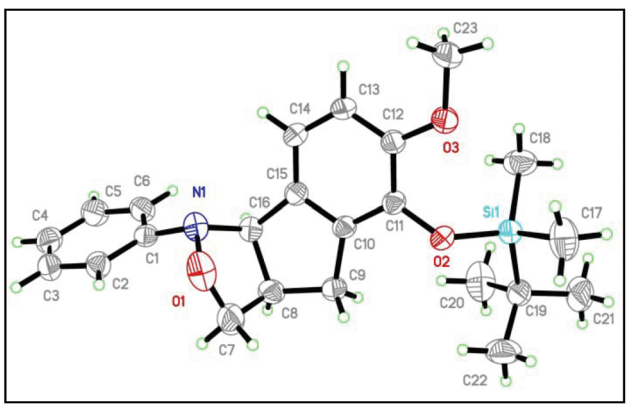


Fig. 2 ORTEP representation of **8a**. Thermal ellipsoids are drawn at the 50% probability level.

undergo intermolecular [3 + 2] cycloaddition with ethyl acrylate or to tetrahydroisoquinoline **5** upon reduction with Zn/AcOH (Scheme 2).

We then focused on the reaction of *N*-aryl hydroxylamines **6** with **1a–b** (Table 1), affording a mixture of two regioisomers **8** and **9** (1 : 2 at 80 °C or 2 : 1 at 130 °C, Table 1) *via* an apparent formation of a *Z*-nitrone[10] **7**. The 2 : 1 mixture of **8** and **9** observed at 130 °C was perhaps due to the reversible formation of **7** from **9** and then to the thermally stable product **8**.[6a] The regioisomers **8** and **9** were separated by column chromatography on silica gel using EtOAc–hexane as an eluant.

The structure of **8a** was unequivocally characterized by single crystal X-ray diffraction studies[11] (Fig. 2) that indicated

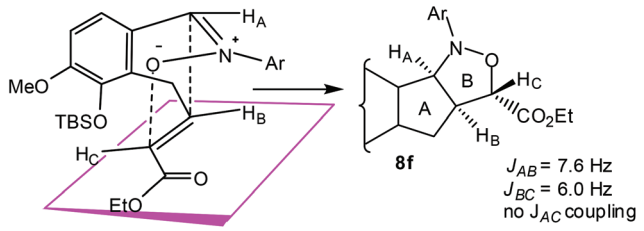
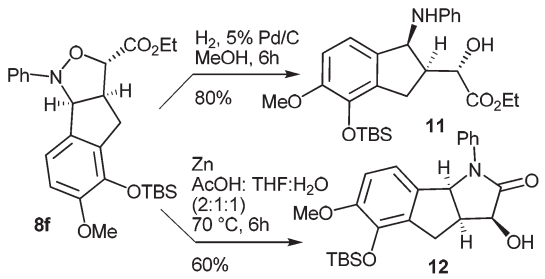


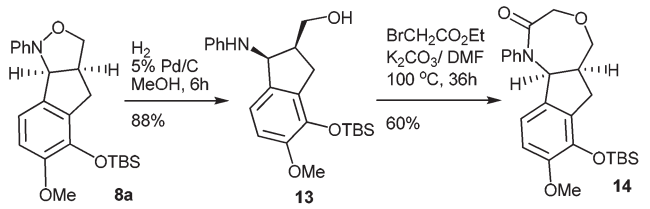
Fig. 3 Endo approach of the C═C moiety to the *Z*-nitrone leading to **8f** with *trans*-relationship of H_C with H_A and H_B (from NOESY spectrum).



Scheme 3 Synthesis of γ-aminoalcohol **11** and lactam **12** from **8f**.

the *cis*-fusion (H_A and H_B) of rings A and B. The reaction of **6e** with **1a** however stopped at the stage of the nitrone **7a** (entry 5, Table 1). While the reason for this observation was not clear, an intramolecular interaction between the $-N^+-O^-$ moiety and –CN group could prevent the reaction from proceeding further. Notably, the aldehyde **1b** afforded **8f** (entry 6, Table 1) as an isolable major product under the same reaction conditions. ^{1}H NMR analysis of **8f** (the NOESY spectrum) revealed the coupling of H_C with the vicinal proton H_B but not with the H_A (long range coupling) indicating the relative *trans*-relationship of H_C with H_A and H_B (Fig. 3). An endo approach of the C═C moiety to the *Z*-nitrone aided by the secondary orbital interactions between the nitrone nitrogen p orbitals and the –C═O group[10] possibly favored the formation of this particular diastereomer (Fig. 3). The reaction of **1b** with **6e** was also successful but afforded a 1,2,3,4-tetrahydronaphthalene **10**[12] possibly *via* the formation of an isomeric bridged cycloadduct (*e.g.* **9**) followed by cleavage of its N–O bond in a single pot. To demonstrate the utility of the present isoxazolidine synthesis, **8f** was converted to a γ-aminoalcohol **11** and densely functionalized lactam **12** separately (Scheme 3). Similarly, **8a** was also converted to another γ-aminoalcohol **13** and then to the oxazepine derivative **14** (Scheme 4).

Based on capsaicin's known anxiogenic activity in various animal models,[13] compounds **15–17** and **19–28** (Fig. 4, see ESI† for compound **18**) obtained after removal of the TBS protecting group (see ESI†) of various previously synthesized compounds *e.g.* **3**, **8**, **9**, **10**, **11**, **12**, **13** and **14** along with a positive control clonidine (a known anxiolytic agent) were tested in the zebrafish model of anxiety[14] (Fig. 5). We used the adult zebrafish model of the light/dark box anxiety test to assess our compounds as this method is simple and does not require the use



Scheme 4 Synthesis of γ-aminoalcohol **13** and oxazepine **14** from **8a**.

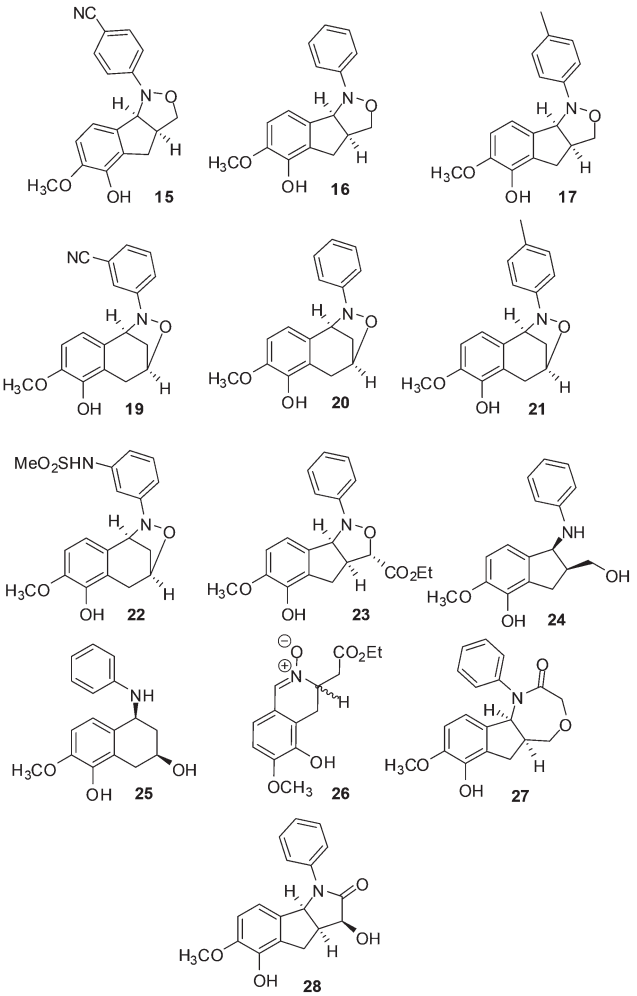


Fig. 4 Structures of compounds **15–17** and **19–28**.

of invasive procedures or euthanasia.[14] We first tested the hypothesis that capsaicin administration should result in anxiogenic behaviour in adult zebrafish. Thereafter, compounds were tested in this model to identify the most potent anxiogenic compounds (Fig. 6). Capsaicin when tested in this model showed clear anxiogenic behaviour, which was evident from the fact that the % time spent in the light box was significantly lower in the capsaicin treated fish as compared to the control at 10 μg kg^{-1} dose and the effect was dose dependent (Fig. 7A). Erratic movements (number and duration) were also observed and they increased in a dose dependent manner in capsaicin treated fish (Fig. 7B & 7C). This suggested that

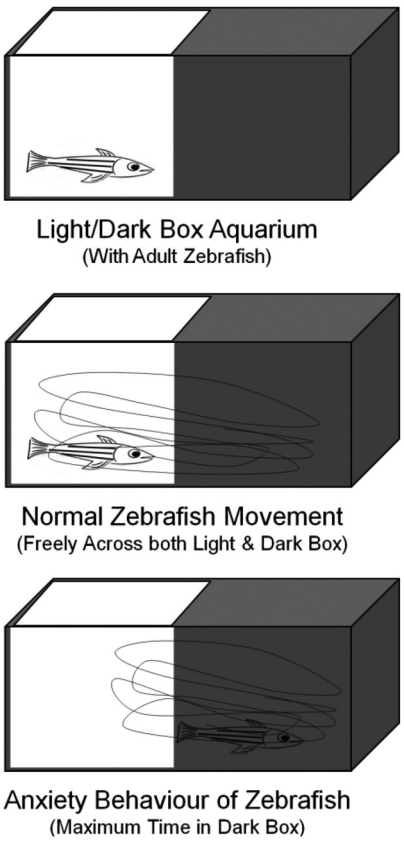


Fig. 5 The figure shows the evaluation of anxiety like behaviour in the adult zebrafish using the light/dark box paradigm. Normal fish move across freely in both light and dark environments; however, when anxious their movements are higher in the dark environment as compared to light.

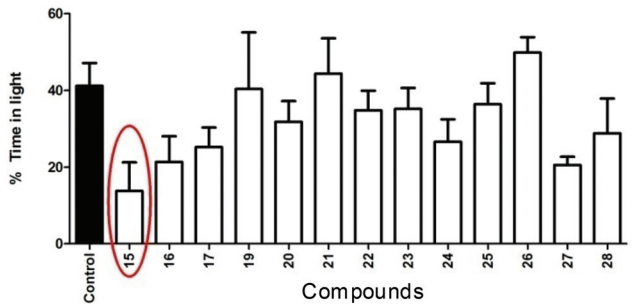


Fig. 6 Screening of compounds for anxiolytic activity. The effect of compounds was assessed at 10 mg kg^{-1} in the light/dark box paradigm to evaluate the parameter of percentage time spent in light. Compound-**15** was found to have the maximum effect in this study.

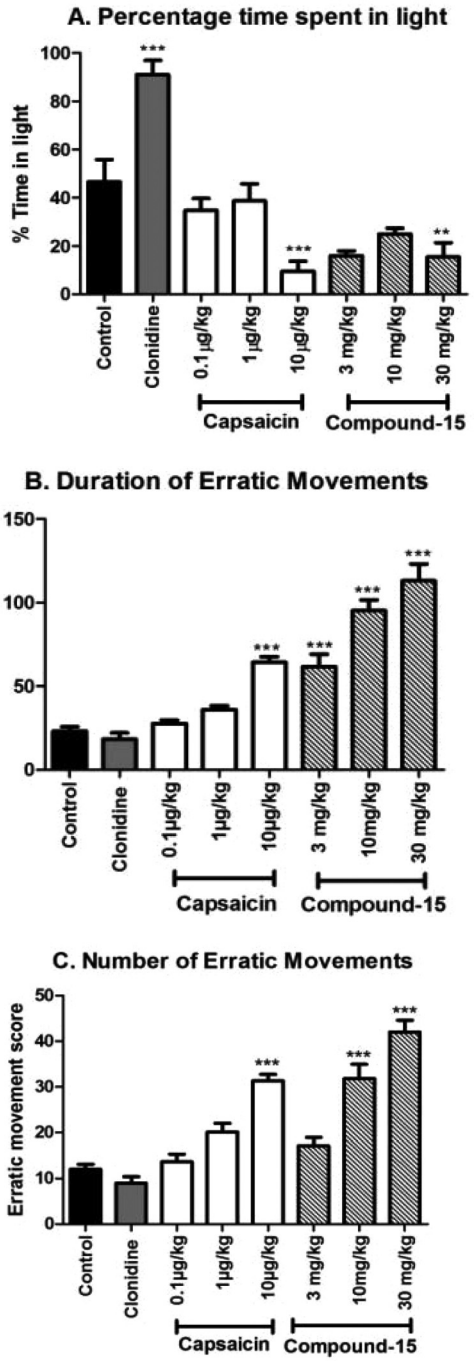


Fig. 7 Multi-dose evaluation of capsaicin and **15** in the adult zebrafish model of anxiety for (A) percentage time spent in light, (B) duration of erratic movements, and (C) the number of erratic movements using the light/dark box paradigm.

capsaicin showed an anxiogenic effect in adult zebrafish that was similar to the effect seen in other mammalian species. Evaluation of the test compound at a single dose was performed and compound **15** obtained from **8c** was identified as most active when tested initially at 10 mg kg^{-1} (Fig. 6). A multi-dose evaluation of **15** confirmed its capsaicin like anxiogenic activity as fish administered **15** showed a significant reduction in the % time spent in the light as compared to control fish (Fig. 7A).[15] Erratic movements (duration and number) were also observed and there was a dose-dependent increase in the compound **15** group (Fig. 7B & 7C).

In conclusion, isovanillin derived *N*-(un)substituted hydroxylamines possessing an *o*-allylic group have been identified as valuable precursors to bioactive N-heterocycles. Thus novel benzo analogues of tricyclic isoxazolidine possessing potential

anxiogenic properties were synthesized for the first time *via* an intramolecular 1,3-dipolar cycloaddition of *N*-aryl hydroxylamines possessing activated/unactivated allylic dipolarophiles at the *o*-position. The corresponding *N*-unsubstituted hydroxylamine afforded tetrahydroisoquinoline. The one-pot methodology presented here is amenable to the synthesis of functionalized lactams, γ-aminoalcohols, oxazepines, *etc.* and may find wide applications in organic synthesis/medicinal chemistry.

Experimental

Chemistry

Representative experimental procedures

*1,3-Dipolar addition of **3** with ethyl acrylate.* To a solution of 5-(*tert*-butyldimethylsilyloxy)-3-(2-ethoxy-2-oxoethyl)-6-methoxy-3,4 dihydroisoquinoline 2-oxide (**3**, 0.393 g, 1 mmol) in ethanol (5 mL) was added ethylacrylate (0.5 g, 5 mmol) and the mixture was refluxed for 3.5 h. After completion of the reaction, the solvent was removed under high vacuum and the residue was purified by column chromatography on silica gel (eluting with 10% EtOAc–hexane) to afford the desired product(s). For the spectral data of the compounds synthesized *e.g.* **4a** and **4b**, see ESI.†

*The reaction of **1a–b** with various* N*-aryl hydroxylamines.* To a solution of aldehyde (**1a–b**, 1.0 mmol) in ethanol (5 mL) was added aryl hydroxyl amine (**6**, 2 mmol) and $MgSO_4$ (5 mmol) under a nitrogen atmosphere. The reaction mixture was refluxed for 3–5 h (for inversion in the yields of regio isomers the reaction mixture was heated at 130 °C for 12 h). After completion of the reaction, ethanol was removed under vacuum and the residue was purified by column chromatography on silica gel to give the desired product. For the spectral data of the compounds synthesized *e.g.* **8**, **9**, **10** *etc*., see ESI.†

*Preparation of (4-(*tert*-butyldimethylsilyloxy)-5-methoxy-1-(phenylamino)-2,3-dihydro-1*H*-inden-2-yl)-2-hydroxyacetate (**11**).* To a solution of (3*R*,3a*R*,8b*R*)-ethyl-5-(*tert*-butyldimethylsilyloxy)-6-methoxy-1-phenyl-3,3a,4,8b-tetrahydro-1*H*-indeno[1,2-*c*]isoxazole-3-carboxylate (**8f**) (0.470 g, 1.0 mmol) in MeOH (5 mL) was added 5% Pd/C (47 mg) under balloon pressure of H_2. TLC shows completion of reaction after 6 h. The Pd was filtered through celite, and the filtrate was concentrated under high vacuum. The residue was purified by column chromatography with EtOAc–hexane (2 : 8) as an eluent to yield the desired product; yield (80%). R_f = 0.4 (2 : 8 EtOAc–Hex); IR (cm^{-1}): 3340, 3079, 2930, 2847, 1681, 1650, 1585; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18 (t, J = 7.93 Hz, 2H), 6.82–6.65 (m, 5H), 5.15 (d, J = 7.3 Hz, 1H), 4.34 (d, J = 4.7 Hz, 1H), 3.96 (q, J = 10.7, 7.2 Hz, 2H), 3.77 (s, 3H), 3.63 (dd, J = 10.7, 7.1 Hz, 1H), 3.28 (bs, 1H), 3.26–2.96 (m, 2H), 1.11 (t, J = 7.1 Hz, 3H), 1.01 (s, 9H), 0.17 (d, J = 6.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7, 150.8, 141.2, 136.6, 135.9, 132.7, 128.8 (2C), 126.7, 125.3 (2C), 118.7, 109.9 (2C), 71.1, 65.6, 55.1, 42.7, 28.2, 25.9 (3C), 18.6, 14.4, −4.1 (2C); Mass (ES): *m*/*z* 494.23 (M + Na, 100%), 379.19 (100%); HRMS (ESI): calcd for $C_{26}H_{37}NO_5SiNa$ $(M + Na)^+$ 494.2338 found 494.2341.

*Preparation of 5-(*tert*-butyldimethylsilyloxy)-3-hydroxy-6-methoxy-1-phenyl-1,3a,4,8*b*-tetrahydroindeno[1,2-*b*]pyrrol-2(3*H*)-one (**12**).* To a solution of **8f** (0.470 g, 1.0 mmol) in AcOH–THF–H_2O (2 : 1 : 1, 40 mL) was added Zn dust (0.4 g, 6.1 mmol) at 60 °C. The reaction mixture was stirred for 5 h. After completion of the reaction the mixture was cooled to room temperature and Zn was filtered off. The filtrate was concentrated to remove THF, and neutralized with saturated $NaHCO_3$ solution. The reaction mixture was extracted with CH_2Cl_2 (2 × 20 mL). The combined organic layer was washed with brine (5 mL), dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography using EtOAc–hexane (3 : 7) as an eluent to yield a colorless solid (0.255 g, 60%); mp: 160–163 °C R_f = 0.4 (3 : 7 EtOAc–Hex); IR (cm^{-1}): 3450, 3088, 2965, 2874, 1700, 1630, 1565; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40–7.33 (m, 2H), 7.26–7.20 (m, 3H), 6.46 (q, J = 8.2 Hz, 2H), 5.30 (d, J = 6.30 Hz, 1H), 4.73 (d, J = 8.3 Hz, 1H), 3.70 (s, 3H), 3.59–3.50 (m, 1H), 3.30 (s, 1H), 3.24–3.03 (m, 2H), 1.00 (s, 9H), 0.15 (d, J = 1.5 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.3, 150.7, 141.2, 136.6, 132.8, 128.8 (2C), 126.7, 125.4 (2C), 118.7, 109.9, 71.0, 65.6, 55.1, 42.6, 28.3, 26.0 (3C), 18.6, −4.04, −4.07; mass (ES): *m*/*z* 448.19 (M + Na, 20%), 371.23 (40%), 313.19 (100%); HRMS (ESI): calcd for $C_{24}H_{31}NO_4SiNa$ $(M + Na)^+$ 448.1920, found 448.1909.

*Preparation of (4-(*tert*-butyldimethylsilyloxy)-5-methoxy-1-(phenylamino)-2,3-dihydro-1*H*-inden-2-yl)methanol (**13**).* The title compound was synthesized from **8a** by using a procedure similar to the synthesis of **11**; brown viscous oil, yield (88%), R_f = 0.4 (1 : 9 EtOAc–Hex); IR (cm^{-1}): 3373, 3079, 2930, 2854, 1600, 1494, 1443; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27–7.22 (m, 2H), 6.89–6.80 (m, 3H), 6.73 (q, J = 8.1 Hz, 2H), 5.06 (d, J = 6.6 Hz, 1H), 3.84–3.77 (m, 4H), 3.72 (dd, J = 11.2, 4.6 Hz, 1H), 3.40–3.27 (bs, 1H), 3.05–2.86 (m, 3H), 1.02 (s, 9H), 0.19 (d, J = 12.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 150.1, 148.0, 141.1, 137.3, 133.1, 129.4 (2C), 118.1, 116.1, 113.6 (2C), 110.8, 63.4, 61.0, 55.3, 44.5, 31.1, 25.9 (3C), 18.6, −4.0, −4.2; mass (ES): *m*/*z* 422.21 (M + H, 40%), 400.23 (35%), 307.17 (100%); HRMS (ESI): calcd for $C_{23}H_{34}NO_3Si$ $(M + H)^+$ 400.2307 found 400.2313.

*Preparation of 7-(*tert*-butyldimethylsilyloxy)-8-methoxy-1-phenyl-5,5a,6,10b-tetrahydro-1*H*-indeno[1,2-*e*][1,4]oxazepin-2(3*H*)-one (**14**).* To a solution of (4-(*tert*-butyldimethylsilyloxy)-5-methoxy-1-(phenylamino)-2,3-dihydro-1*H*-inden-2-yl)methanol (**13**) (0.422 g, 1.0 mmol) in dry DMF (10 mL) were added ethylbromo acetate (0.2 g, 1.2 mmol) and oven dried K_2CO_3 (0.548 g, 4.0 mmol). The mixture was refluxed for 36 h. After completion of the reaction, the mixture was diluted with saturated NH_4Cl solution (20 mL) and extracted with CH_2Cl_2 (2 × 25 mL). The combined organic layer was collected, washed with 2 N HCl and water, then dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by column chromatography on silica gel using EtOAc–hexane (3 : 7) as an eluent to afford the desired product as a brown solid (0.228 g, 60%); mp: 280–282 °C; yield (60%), R_f = 0.4 (3 : 7 EtOAc–Hex);

IR (cm^{-1}): 3021, 2973, 2877, 1720, 1615, 1585; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (t, J = 7.7 Hz, 2H), 7.34 (d, J = 7.3 Hz, 2H), 7.31–7.23 (m, 1H), 6.92 (d, J = 8.0 Hz, 1H), 6.75 (d, J = 8.0 Hz, 1H), 5.30 (d, J = 7.1 Hz, 1H), 4.34 (d, J = 15.5 Hz, 1H), 4.11 (dd, J = 12.6, 3.9 Hz, 1H), 3.91 (d, J = 15.5 Hz, 1H), 3.79 (s, 3H), 3.72 (dd, J = 12.7, 4.8 Hz, 1H), 2.94 (dd, J = 20.0, 13.3 Hz, 3H), 1.00 (s, 9H), 0.20 (s, 3H), 0.13 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) 172.6, 150.2, 144.5, 141.3, 134.8, 131.8, 129.2 (2C), 126.9, 126.5 (2C), 114.7, 110.5, 72.5, 71.7, 68.8, 55.2, 43.5, 32.0, 25.8 (3C), 18.5, −4.2, −4.4; mass (ES): m/z 462.20 (M + Na, 100%), 440.22 (M + H, 10%), 289.16 (80%); HRMS (ESI): calcd for $C_{25}H_{33}NO_4$ NaSi $(M + Na)^+$ 462.2076, found 462.2060.

A typical procedure for the removal of tert-*butyldimethylsilyloxy group of compound* ***8c****.* To a solution of 4-(5-(*tert*-butyldimethylsilyloxy)-6-methoxy-3,3a,4,8b-tetrahydro-1*H*-indeno[1,2-*c*]isoxazol-1-yl) benzonitrile (**8c**) (1 mmol) in dry THF (5 mL) was added *tetra*-butyl ammonium iodide (1.5 mmol) at 0 °C. The reaction mixture was stirred at the same temperature for 0.5 h, and THF was removed under high vacuum followed by extraction with DCM (2 × 10 mL). The combined DCM layer was collected, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified using column chromatography over silica gel to give the product 4-(5-hydroxy-6-methoxy-3,3a,4,8b-tetrahydro-1*H*-indeno[1,2-*c*]isoxazol-1-yl)benzonitrile (**15**) as a white solid; mp 180 °C; yield (88%), R_f = 0.2 (1 : 9 EtOAc–Hex); IR (cm^{-1}): 3430, 3256, 3060, 2973, 2868, 2205, 1620, 1555, 1486, 1276; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (d, J = 8.9 Hz, 2H), 7.19 (d, J = 8.9 Hz, 2H), 6.93 (d, J = 8.1 Hz, 1H), 6.84 (d, J = 8.2 Hz, 1H), 5.67 (s, 1H), 5.35 (d, J = 7.6 Hz, 1H), 4.10–4.05 (m, 1H), 3.98–3.85 (m, 4H), 3.56–3.48 (m, 1H), 3.30–3.22 (m, 1H), 3.03 (dd, J = 16.9, 3.1 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.0, 146.6, 141.4, 134.5 (2C), 133.4, 128.6, 119.5, 116.1, 114.0 (2C), 110.7, 103.8, 75.0, 74.6, 56.4, 46.7, 34.2, 29.7; Mass (ES): m/z 309.13 (M + H 100%).

For the spectral data of other compounds synthesized *e.g.* **16–17** and **19–28**, see ESI.†

Biology

Evaluation of test compounds in the zebrafish model of anxiety

Materials and methods. Wild type zebrafish (*Danio rerio*) were maintained as per the procedure reported earlier.[16] The studies on anxiety assessment using the light/dark box paradigm were conducted based on the parameters of percentage time spent in light, duration of erratic movements and the number of erratic movements from the published protocol.[14] Drug administration was carried out by a procedure reported earlier.[17] Clonidine, an anxiolytic agent, was used as a positive control to ascertain the validity of the experiments. Evaluation was conducted in three parts: (a) screening of capsaicin for assessment of its anxiogenic activity in adult zebrafish, (b) screening of test compounds at a single dose to identify the most potent anxiogenic agent, and (c) multi-dose studies on the most potent agent to verify anxiogenic activity.

Statistical analysis. Statistical analysis was performed using GraphPad Prism software. Data were represented using mean and standard error of the mean (±SEM). Data were analysed using analysis of variance (ANOVA) followed by Tukey's multiple comparative test. Statistical significance was set at the $p < 0.05$ level.

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