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Paper

$Ce(SO_4)_2$-Catalysed the Highly Diastereoselective Synthesis of Tetrahydroquinolines Via imino Diels Alder ABB' Type Reaction and their *In Vivo* Toxicity and Imaging in Zebrafish Embryos

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An efficient and practical approach has been developed for the synthesis of *N*-(tetrahydroquinolinyl-4) amides **3a-l** with good yields and high diastereoselectivity. The strategy comprises the domino type ABB' imino Diels Alder reaction catalysed by a cerium(IV) salt between anilines and *N*-vinyl amides for the preparation of a 12-membered library of tetrahydroquinolines that were tested for their in vivo toxicities against zebrafish embryos, determining their LC_{50} values where *N*-(8-methoxy-2-methyl-tetrahydroquinolinyl-4) acetamide **3k** was identified as the most toxic derivative with a LC_{50} below 95 µM (24 mg/L). Finally, the phenotypes induced, at concentrations below their LC_{50}, were analyzed at 48, 72 and 96 hours post fertilization where the embryos treated manifested diverse visual phenotypes such as big yolk sacs (**3b**, **3h**, **3j**), pericaldial edemas (**3a**, **3i**) and red blood cells in the liver region (**3b**, **3l**) in comparison to the morphology of the control embryos, phenotypes that could be associated with specific biological targets.

Introduction

The 1,2,3,4-tetrahydroquinoline core (THQ) is a privileged class of *N*-heterocycle present in a number of natural and synthetic products with biological activity, playing an important role in medicinal chemistry.[1,2] Particularly, substituted 2-methyl-THQs derivatives have found many applications in pharmaceutical industries such as antibiotics,[3,4] inhibitors of the P-glycoprotein in multidrug resistant cancer cells[5] and antagonists of the prostaglandin D2 receptors.[6] They have also shown a control in the expression of the Ecdysone Receptor (EcR) in the *Aedes aegypti* model for agrochemical purposes[7] and have been employed as chiral ligands for transition-metal catalysts in asymmetric organic synthesis.[8]

Due to their biological and chemical properties, the development of newer and efficient methodologies for the heterocyclic ring construction of 2-methyl-THQs derivatives is of current interest and is still in demand. During the last decade, one-pot and multicomponent reactions have allowed the direct synthesis of these molecules from simple substrates in a highly efficient manner through the Povarov reaction.[9] Several Lewis acids ($BF_3{\cdot}OEt_3$, $InCl_3$, $Ln(OTf)_3$) and Brønsted acids (HSO_3Cl, TFA, TsOH) have been found to be an excellent catalysts for the three-component Povarov reaction (ABC) in the construction of a highly substituted libraries of THQs,[10] however these conditions have not been so efficient for the synthesis of 2-methyl-THQs derivatives.[11]

Since 2011, our group has explored an extended version of multicomponent reactions, designated as ABB' type reactions, in which the component B is incorporated in two distinct manners (B and B') into the component A, ensuring the complexity and functional diversity of the final product.[12] As a result, we developed a novel approach for the synthesis of 2-methyl-THQs via Povarov reaction catalysed by phthalic acid.[13] Although the good yields in which the new THQs derivatives were obtained, this method was recently improved by the replacement of the solvent medium from acetonitrile to SDS as a micellar aqueous medium, generating a new library of amidyl-2-methyl-THQs in high yields and diastereoselectivity.[14]

Nowadays, preliminary *in vivo* toxicological tests of organic Small Molecules (SMs) are consider as one of the main and necessary steps during the discovery and development of future drugs. Among the different models for *in vivo* bioprospection of novel SMs, the zebrafish embryo model provides an inexpensive, reliable and efficient first-level screening model for testing toxicity, efficacy, and tissue-targeting for a large number of these SMs because of the close homology between the zebrafish and human genome.[15]

Furthermore, huge amounts of zebrafish embryos can be generated, developed quite rapidly and synchronously with well-defined developmental stages, where its transparency at embryonic and larval stages facilitate the direct visual observation of the toxic and phenotypic effects of SMs *in vivo*.[16,17]

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† Electronic Supplementary Information (ESI) available: Detailed experimental procedures, characterization data, ^1H NMR, ^{13}C NMR, DEPT-135, COSY, HMBC and HSQC NMR charts and all photographic records of the phenotypic changes exhibit during the zebrafish embryo development. See DOI: 10.1039/x0xx00000x

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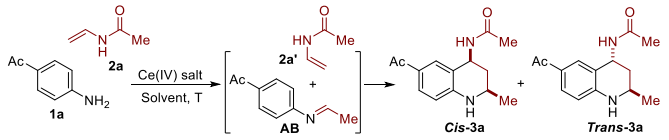
According to the statements described above and with the current need to conduct studies related with the synthesis of 2-methyl-THQs that could be tested in the zebrafish model in order to reveal their toxicological and phenotypic profile, our research was focused on: *i*) based on the previous reports where cerium salts, specially Cerium Ammonium Nitrate (CAN), have catalysed the formation of THQs and quinolines from vinyl ethers, [18] establish the optimal conditions for the reaction of anilines and *N*-vinyl amides catalysed by Ce(IV) salts according to the variables: solvent, reaction times, temperature, loading catalyst and the yield in which the desired product is obtained; *ii*) with the standardized conditions, preparing a 12-membered library of 2-methyl-THQs through the imino Diels Alder (Povarov) ABB' type reaction; *iii*) determine the toxicity (LC_{50}) of the prepared 2-methyl-THQs in the zebrafish embryos and *iv*) analysed the phenotypes induced by these SMs at concentrations below their LC_{50}, in the zebrafish embryos at 24, 48, 72 hours post chemical exposure. All this, in order to contribute in future SAR studies of this interesting class of molecules.

Results and Discussion

We initiated our study by testing CAN, a well-known promoter of *Single-Electron Transfer Reactions* (SETR) via cation radical-mediated chain mechanism,[19] as catalyst in the model reaction between 4-aminoacetofenone **1a** and *N*-vinyl acetamide **2a**. The first experiments were carried out in two different solvents (MeOH and MeCN) and using CAN (10 mol %) as a catalyst, resulting in the formation of the respective 6-acetyl-2-methyl-4-acetamido-1,2,3,4-tetrahydroquinoline **3a** after 30 minutes with 92 % yield when MeCN was used as a solvent at room temperature (Table 1, entry 2).

Table 1 Synthesis of 2-methyl-THQ **3a** catalysed by Ce(IV) salts. Screening of Ce(IV) salts, solvent systems and temperature range.[a]



Entry	Ce(IV) salt (mol %)	Solvent	Time (h)	Yield (%)[b]	d.r. (*cis*/*trans*)[c]
1	CAN (10)	MeOH	2	56	87/13
2	CAN (10)	MeCN	0.5	92	87/13
3	$Ce(SO_4)_2$ (30)	MeCN	5	78	97/3
4	$Ce(SO_4)_2$ (15)	MeCN	6	89	97/3
5	$Ce(SO_4)_2$ (15)	MeCN	6	67[d]	95/5

[a] Reaction performed on a 1 mmol scale using **1a** (2 mmol), **2a** (4.2 mmol), catalyst (10-30 mol %) and in the respective solvent 20 mL at room temperature. [b] Isolated yield after chromatographic purification. [c] Diastereomeric ratio of **3a** was determined by ^{1}H NMR analysis. [d] Reaction performed at 60 °C.

Encouraged by the promising results obtained with CAN, but being aware of the tedious work-up operations during the extraction and purification process, and knowing the oxidizing properties of CAN that may cause the oxidation of THQ core to the corresponding quinoline,[20] we were forced to find and evaluate alternative cerium (IV) salts as a possible catalyst for this ABB' type reaction. With that knowledge in mind, our attention was drawn to explore the use of $Ce(SO_4)_2$ due to its low toxicity, solubility in many organic solvents, low cost, stability to air moisture and commercial availability in comparison to CAN. Additionally, this catalyst has been used as a mild and efficient oxidant catalyst for various organic transformations.[21-23] In that order, we performed the model reaction in the presence of $Ce(SO_4)_2$, studying the effect of reducing the catalyst loading from 30 to 15 mol %, in MeCN at room temperature, obtaining the desired **3a** compound in good to excellent yields, 78-89 % (Table 1, entries 3-5).

Although the use of CAN as a catalyst provided the 2-methyl-THQs **3a** in shorter times (0.5-2 h) and in good yields (56-92 %) than the experiments performed with $Ce(SO_4)_2$, the selection of the better reaction conditions were done based on the best yield and diastereomeric ratio in which **3a** was isolated (Table 1). Thus, we found that at higher loading catalyst, 30 mol %, the product **3a** was obtained in 78 % yield and with a *cis*-diastereoselectivity (*cis*/*trans* 97:3) when the reaction was performed at room temperature (entry 3).

Interestingly, when the loading catalyst was decreased to 15 mol % the best results in terms of yield and diastereoselectivity were obtained for **3a** (89 %, *cis*/*trans* = 97/3) after 6 hours (entry 4). Finally, increasing the reaction temperature to 60 °C significantly decrease the yield and the diastereoisomeric ratio of the Povarov reaction, suggesting a negative influence in the chemical transformation associated with the equilibrium of the reacting species (entry 5).

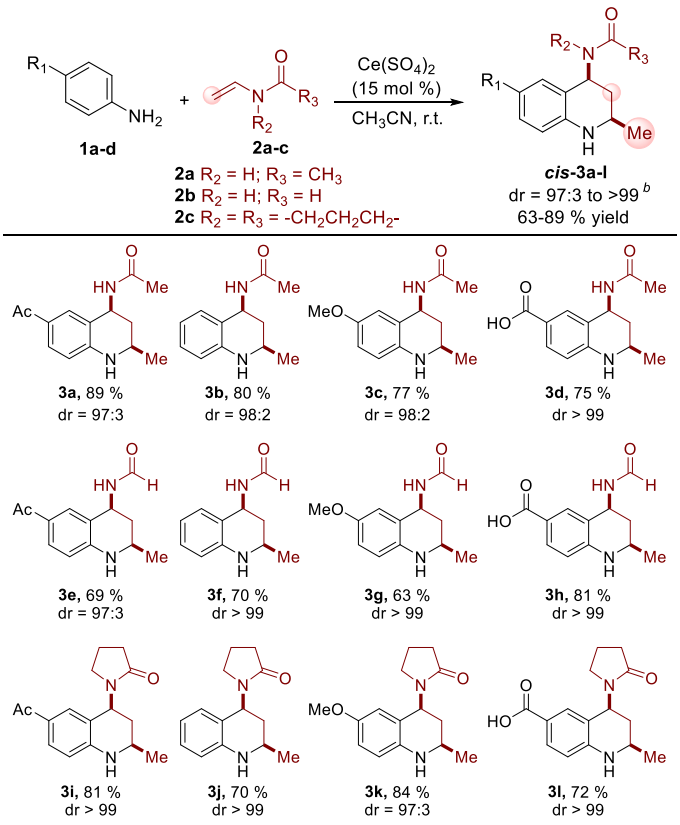
Further studies by ^{1}H NMR and COSY experiments led to establish the complete stereochemistry of *cis*-**3a** through the *trans*-diaxial relationship between protons H_{4ax} and H_{3ax} (J = 11.5 Hz) and H_{2ax} and H_{3ax} (J = 11.5 Hz), which means that *cis*-THQ **3a** was formed selectively. This finding shows the high diasteroselectivity of the imino Diels Alder ABB' type reaction catalysed by $Ce(SO_4)_2$, improving the results previously reported by Menéndez *et. al.*[18] where under the catalysis of CAN the respective THQs were obtained in a diastereoisomeric ratio of 90:10 (*cis*/*trans*). A possible explanation for the high diastereoselectivity displayed by this reaction catalysed by $Ce(SO_4)_2$, could be related with the Molecular Orbital (MO) interactions, where an *endo* approach is often preferred between the diene **AB** and the dienophile **2a'**,[24] and with the nature of ligands bonded to the cerium(IV) ion. In the case of $Ce(SO_4)_2$, the SO_4^{2-} ligands adopt a tetrahedrical disposition that promote the coordination between the metal and the nitrogen present in the diene **AB** (N···Ce(IV)), as well with the oxygen of the dienophile **2a'** (Ce(IV)···O). Thus, the proximity and strength of these interactions increase the diastereoselectivity of the reaction in contrast with CAN, where the NO_3^- ligands form a bulky complex with a cuboctahedron geometry that reduce the force of these interactions and lower the diastereoselectivity.[25]

Having the optimized reaction conditions in hand, the versatility, substrate scope and limitations of our protocol for the Povarov reaction were broadened to other anilines and diverse enamides with different functional groups, where the reaction proceeded smoothly and afforded the corresponding

2-methyl-THQs **3a-l** in good to excellent yields (63-89 %) with a diastereoselectivity > 97 in all cases (Table 2).

The substituents influence at the aniline ring was first investigated finding that the reaction could proceed well using diverse *para*-substituted anilines **1a-d** (H, OMe, Ac, COOH) giving the corresponding products **3** without affecting the yield or diastereoselectivity of the THQ product.

Table 2 Substrate scope for the Povarov ABB´ type reaction catalyzed by $Ce(SO_4)_2$. Synthesis of 2-methyl-THQs under the optimized reaction conditions.[a]



[a] Reaction performed on a 2 mmol scale using **1a** (2 mmol), **2a** (4,2 mmol), catalyst (15 mol %) in MeCN (20 mL) at room temperature. [b] Isolated yield after SiO_2 column chromatography. Diastereomeric ratio of **3a-l** was determined by ^1H NMR analysis.

However, the chemical structure of the enamides **2a-c** influenced negatively the yields in which the product **3** was isolated, suggesting that the reactivity of enamides follows an apparent trend of **2a** ≥ **2c** > **2b**. The structure of the 2-methyl-THQs **3a-l** was elucidated through ^1H, ^{13}C and 2D NMR experiments, where the HMBC and NOESY experiments were relevant to prove the relative stereochemistry of C-2 and C-4 and the diastereoselectivity of the Povarov ABB´ type reaction (Figure 1).

For the case of **3i**, the stereochemistry of methyl group the stereochemistry of C-2, where the methyl group locates in an equatorial position. Finally, the key NOESY correlation of protons H_{ax} at C-2 and $H_{ax'}$ at C-4 indicates that they are on the same plane, establishing the stereochemistry of C-4, confirming the *cis*- configuration of groups bonded to C-2 and C-4.

Previous debates and discussions have suggested that the reaction mechanism for the formation of THQs under the

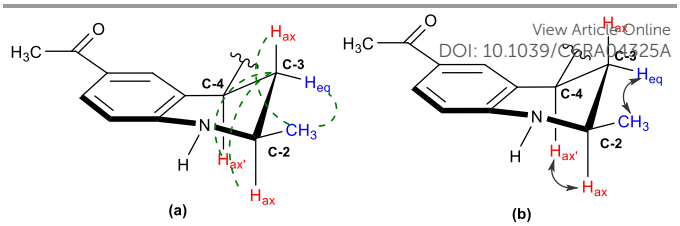
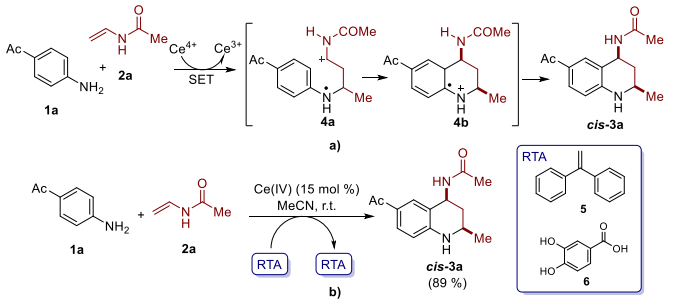


Fig. 1 Selected (**a**) HMBC (----) and (**b**) NOESY (↔) correlations for compound **3i**.

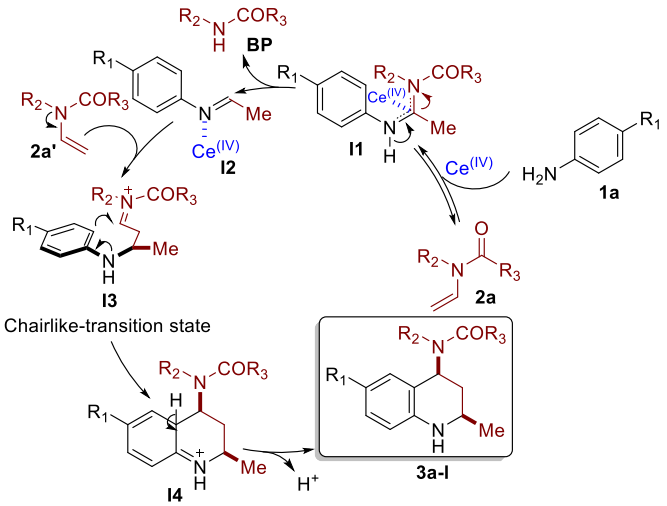
catalysis of Ce(IV) salts like CAN would take place through a concerted asynchronous transition states through the species **4a-b** generated by a SETR (Scheme 1a).[26-28] In order to prove this hypothesis, our first attempt was aimed to the addition of some Radical Trapping Agents (RTAs) to the reaction mixture with the idea that these will react with the intermediates **4a-b**, diverting in that way the course of the reaction.[29] However, when 1,1-diphenylethylene **5** or 3,4-dihydroxybenzoic acid **6**, two well-known RTAs, were introduced in excess to our model reaction between **1a** and **2a**, using $Ce(SO_4)_2$ or CAN, the reaction proceeded normally and afforded the corresponding 2-methyl-THQ **3a** in 89 % yield, indicating that expected radical intermediates **4a-b** were not formed (Scheme 1b).



Scheme 1 a) Proposed intermediates **4a-b** in a radical mechanism **b)** Control experiment performed for the model reaction with the addition of radical traps (RTAs).

Another debated mechanistic proposal has suggested a stepwise mechanism via ionic intermediates and with the purpose of confirming this hypothesis we used two nucleophiles such as ethanol and *n*-butanol to trap the iminium cation **I3** in the model reaction in presence of $Ce(SO_4)_2$ (Scheme 2).[30] We observed that during the course of the reaction the GC/MS analysis revealed that the addition products, which would incorporate a nucleophile molecule, were not observed under equimolar amounts respect to **1a**. According to these results and the reported background on mechanistic aspects of imino DA reactions,[31] a possible mechanism now can be propose. First, the key intermediate **I2** is formed *in situ* when the aniline **1a** reacts with the enamide **2a** obtaining the intermediate **I1**, which is stabilized by Ce(IV) that also promote the liberation of an amide as a by-product **BP**. The second role of Ce(IV) salt is to stabilize the imine **I2** and make it act as an more electron-poor diene to undergo the Povarov reaction catalysed by $Ce(SO_4)_2$ with the other enamide molecule in a stepwise manner. This leads to the formation of intermediate **I3** which during the last cyclization step will adopt a chair-like transition state, explaining the preference for an equatorial arrangement of the

methyl (C-2) and amido (C-4) groups and the high *cis* stereochemistry observed for the intermediate **I4**, that finally suffers a deprotonation to give **3a-l**.



Scheme 2 Proposed mechanism for the reaction of arylamines and *N*-vinyl amides under $Ce(SO_4)_2$ catalysis.

Finally, having this chemical toolbox available, we focussed our efforts in exploring their biological value in order to search for functional SMs in two zebrafish screens. These are (*i*) embryo toxicity testing, and (*ii*) phenotypic analysis of images of zebrafish embryos treated with the 2-methyl-THQs **3a-l**, divided into three groups, 2-methyl-4-acetamido-THQs **3a-d**, 2-methyl-4-formyl-THQ **3e-h** and 2-methyl-4-pyrrolidin-2-one-THQs **3i-l**.[17]

Determination of zebrafish embryo LC_{50}

After the zebrafish embryos were established as a potential alternative for the acute toxicity test of SMs, Ali *et al*. described the standard protocol for the rapid toxicity assessment[32] that we adapted to determine the toxicity of the 2-methyl-THQs **3a-l** library in zebrafish embryos.

Zebrafish embryos were exposed to 2-methyl-THQs **3a-l** to determine their level of toxicity. The SMs were introduced into the E3 medium after dechorionation at 24 hours post fertilization (hpf) and the exposed embryos were incubated until 96 hpf, point that corresponds to the final stage of embryogenesis. The exposed embryos were incubated at 28 ± 2 °C and were examined at 24, 48 and 72 hours of chemical exposure (96 hpf) using a light-dissecting stereomicroscope. Once the embryos were classified as dead according to the established endpoints,[33] the data collected from three independent exposures were analysed statistically and the determined LC_{50} values, at 96 hpf expressed in µmol/L, are shown in Table 3.

Comparing the results depicted in Table 3, where the LC_{50} is expressed in mg/L, with the acute toxicity rating scale established the by the Fish and Wildlife service (FSW), we found that almost all the 2-methyl-THQs **3a-l** can be classified as a practically nontoxic or slightly toxic agents possessing a LC_{50} between 24 to 116 mg/L.[34]

Table 3 Zebrafish embryo LC_{50} values found for the 2-methyl-tetrahydroquinoline-based small molecule library.

Comp.	Zebrafish LC_{50}[a]		Aquatic animal acute toxicity[b]
	µmol/L ± SEM	mg/L ± SEM	
3a	351.2 ± 5.7	86.5 ± 1.4	ST
3b	223.8 ± 7.1	45.7 ± 1.5	ST
3c	400.4 ± 7.2	93.8 ± 1.7	ST
3d	429.7 ± 0.3	106.7 ± 0.1	PN
3e	429.3 ± 0.4	99.7 ± 0.1	ST
3f	216.0 ± 7.1	41.1 ± 1.4	ST
3g	435.9 ± 3.7	95.9 ± 0.8	ST
3h	449.2 ± 10.2	105.2 ± 2.4	PN
3i	426.3 ± 7.0	116.1 ± 1.9	PN
3j	330.7 ± 13.7	76.2 ± 3.2	ST
3k	94.9 ± 3.7	24.7 ± 1.0	ST
3l	358.4 ± 9.0	98.3 ± 2.5	ST

[a] LC_{50} values are the mean ± SEM of three different experiments in triplicate. [b] Toxicity scale (mg/L) = highly toxic 0.1–1 (HT), moderately toxic 1–10 (MT), slightly toxic 10–100 (ST), practically nontoxic 100–1000 (PN) and relatively harmless >1000 (RH).

We also found that the THQ bearing a methoxy group in position C-6 and the pyrrolidin-2-one core at C-4 **3k** resulted to be the most lethal compound (LC_{50} = 25 mg/L). This may be due to the synergism of these two organic functions since compounds **3c** and **3g**, where the methoxy group is present with the acetamido and formyl moiety, and compounds **3i**, **3j** and **3l**, where the pyrrolidin-2-one core is present without the methoxyl group, exhibit a LC_{50} above 77 mg/L.

Regarding to the less toxic compounds, we observed that the compounds substituted with the carboxyl group at C-6 **3d**, **3h** and **3l** resulted to be practically nontoxic (LC_{50} = 98.3-105.2 mg/L), similar to the toxicity of aspirin (LC_{50} 101 mg/L) in zebrafish.[35]

In general, taking into account that the toxicity of unsubstituted THQs in C-6 (**3b**, **3f**, **3j**) resulted to be independent of the group present in C-4 (pyrrolidin-2-one, acetamide or formyl moieties), the toxicity of the 2-methyl-THQs **3a-l** is strongly correlated with the nature of the substituent in C-6, fact that needs to be proved in further studies with di and tri substituted THQs in order to select novel nontoxic agents that could be used in advance biological studies.

In vivo zebrafish phenotyping

Once the LC_{50} for compounds **3a-l** was determined, a range of concentrations were established in order to screen our molecules using the zebrafish embryos and study how the activity and normal expression of endogenous genes and proteins are subtly manipulated by the exogenous molecules 2-methyl-THQs **3a-l**.[36]

Table 4 shows a summary of the morphological defects observed from the Prim-5 stage (24 hpf) to 96 hpf after the chemical exposure with the twelve selected 2-methyl-THQs **3a-l** at concentrations below their LC_{50}.

Compounds **3c**, **3e** and **3g** induced a developmental delay in the treated embryos, exhibiting a delayed hatching after 96 hpf that did not allow the observation of phenotypes around the

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spinal cord, yolk sac or heart cavity at these concentrations and two or three concentrations below the LC_{50} value.

Table 4 Summary of effects of 2-methyl-THQs **3a-l** on zebrafish embryos after 96 hpf

Comp. (μM)	Morphological defects[a]				
	Curved bodies	Delayed hatching	Yolk sac edema	Mild intestine	Pericardial edema
3a (100)	+	-	++	++	+++
3b (150)	++	-	+++	+++	+
3c (200)	-	++++	-		-
3d (200)	+	-	++		+++
3e (125)	-	++++	-		-
3f (150)	+/-	-	+		+/-
3g (250)	-	++++	-		-
3h (200)	+/-	-	++++	+++	++
3i (100)	+	-	+/-		++++
3j (200)	+++	-	+++	+	+
3k (25)	++	-	+/-		+/-
3l (200)	++	-	++		+
Control[b]	-	-	-		-
Blank[c]	-	-	-		-

[a] ++++ = very severe effect (75–100%); +++ = severe effect (50–75%); ++ = moderate effect (25–50%); + = minimal effect (5–25%); +/− = either minimal or no effect (0–5%); − = no effect (0%). [b] E3 medium + DMSO (2 %). [c] E3 medium without DMSO.

In general, all the embryos treated with compounds **3a-l**, at three or four concentrations below the LC_{50} of each derivative, did not manifest any visual phenotype and those embryos reached their corresponding development stage after 96 hpf, without any visual evidence that might indicate that the morphology of these treated embryos differed from the control embryo morphology (see ESI†).

However, at one or two concentrations below the LC_{50} of each compound, the treated embryos exhibit big yolk sac edemas (YS), mild intestine (MI), curved bodies (CB), and pericardial edemas (PE) around the heart cavity compared to the control embryo morphology, phenotypes that were identified as the major visual morphological defects observed for rest of the series of 2-methyl-THQs **3a-l** (Table 3).

The CB abnormalities exhibited by almost all the THQs **3a-l**, in low or high degree, were concentration-dependent of the administered compound and cannot be associated with any gene, enzyme or protein at first sight because the additional observation of the yolk sac and pericardial edemas in these treated embryos could be come together with the CB. If the larvae is unable to absorb the nutrients, minerals, phospholipids, triaglycerols and vitamins from the intestinal tract, edemas will be formed in the heart cavity deriving in heart failure and slow the heart rate. The distribution of those endogenous reserves from the YS and oxygen into the bloodstream will eventually delay the development of the treated embryos, resulting in CB.[37]

Compounds **3d**, **3h** and **3j** showed the most severe edemas around the yolk sac, accompanied by disorders or abnormalities in the middle intestine, at concentrations below their LC_{50}, but the PE showed by these embryos was not too severe. This could suggest that these compounds will not reach higher concentrations in the cardiovascular system, maybe because they have affected the liver during their transformation, damage that disturbs the subsequent metabolism of the lipids contained in the yolk sac during the early stages of development, exhibiting wider YS than those of the control (Figure 2).[38]

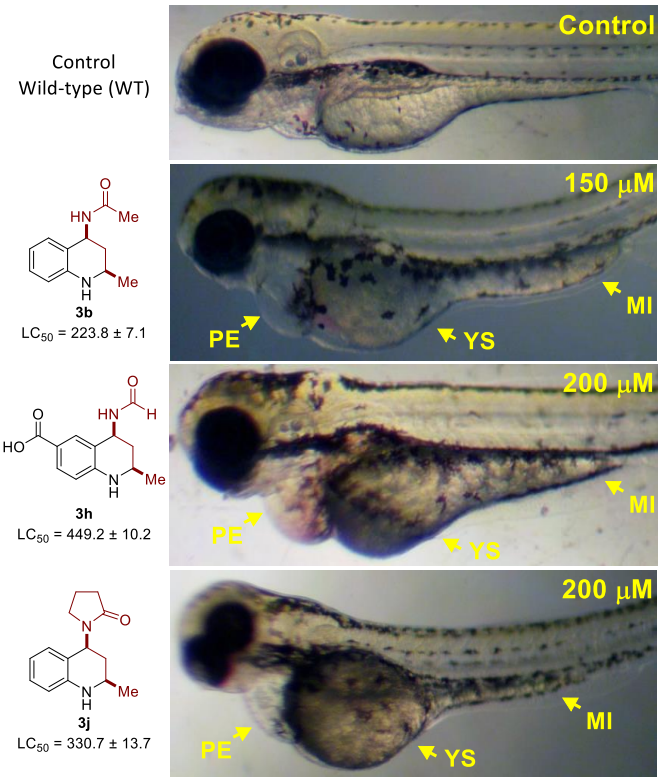


Fig. 2 Microscope photographs at 96 hpf of the treated embryos with compounds **3b** (150 μM), **3h** (200 μM) and **3j** (200 μM); main phenotypes identified: severe yolk sac edema (YS); abnormalities across the middle intestine (MI) and mild pericardial edemas (PE).

We evidenced that these phenotypes were not dependent on the LC_{50} of each derivative, since the YS, MI or PE phenotype did not result to be lethal to the treated embryos; for example, compound **3h**, one of the less lethal compound, displayed several YS and MI and mild MI phenotypes at concentrations much lower than the LC_{50}. Additionally, the degree in which the phenotypes YS, MI and PE are expressed for compounds **3b** and **3j** can be correlated with the substituent in C-4, where we found that the *N*-acetyl group resulted to be more toxic than the pyrrolidin-2-one moiety for the unsubstituted THQs **3b** and **3j**, specially for the gut, mid intestine and heart cavity.

Pericardial inflammation of the heart cavity was observed for compounds **3a**, **3d**, **3i** and **3l**, accompanied with CB, YS and MI, malformations that were identified as the main phenotypes observed in these embryos when they were compared to the control embryos. Although the embryos treated with compound **3e** did not break their membrane, showing a delayed hatching after 96 hpf that did not allowed the identification of other phenotypes. The other compounds substituted with the acetyl group at C-6 (**3a** and **3i**) exhibit the most large PEs among the series of compounds **3a-l**. In comparison with the control, where the looping process places the ventricle (**V**) and atrium

(**A**) side by side, so that the two chambers largely overlap each other and have a small size that make them indistinguishable by the lateral view. The embryos treated with compounds **3a** and **3i** showed looping defects characterized by the abnormal morphology of these heart chambers, resulting in a linear heart with a stretched string-like atrium, positioned the **V** anterior to the **A** where they can be easily distinguished without overlap into the prominent bulbs around the heart cavity (Figure 3).

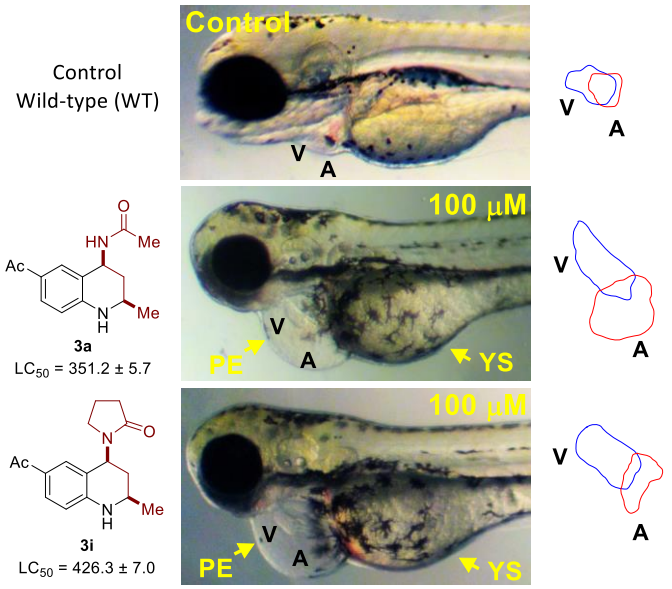


Fig. 3 Microscope photographs at 96 hpf of the treated embryos with compounds **3a** (100 µM) and **3i** (100 µM); Heart looping defects of the treated embryos, arrowhead indicates a large pericardial edemas (PE) and yolk sac edemas (YS). Blue and red indicate the ventricle (**V**) and atrium (**A**), respectively.

We also noted that the morphology of **V** and **A** in the treated embryos became abnormal after 72 hpf when the observed looping disturbance creates an alteration such that the ventricle was positioned anterior to the atrium. These phenotypes, that clearly involves a severe cardiac insufficiency, can be correlated with the Leucine-rich Repeat Containing protein 10 (Lrrc10), a cardiac-specific factor that could be perturbed by the exogenous agents **3a** and **3i**, disturbing the normal cardiac development that promotes the heart's looping and compaction within the pericardium.[39]

In addition to defective hearts and PE phenotypes, the treatment of embryos with THQs **3a-l**, especially the molecules **3d** and **3l** substituted with the carboxylic acid group at C-6, allowed the visualization of an accumulation of Red Blood Cells (RBCs) in the liver region that suggests some kind of liver damage after the THQs exposure. This agglomeration perhaps will be the responsibly of the circulation defects, and consequently leads to malformations in the heart development, since it has been established the functional relationship between the cardiovascular and digestive system in zebrafish (Figure 4).[40]

With the evidence of the PE, YS, MI and RBCs phenotypes, our findings indicate that THQs exposure (24-96 hpf) cause liver and heart failure that may lead to embryonic death. A possible

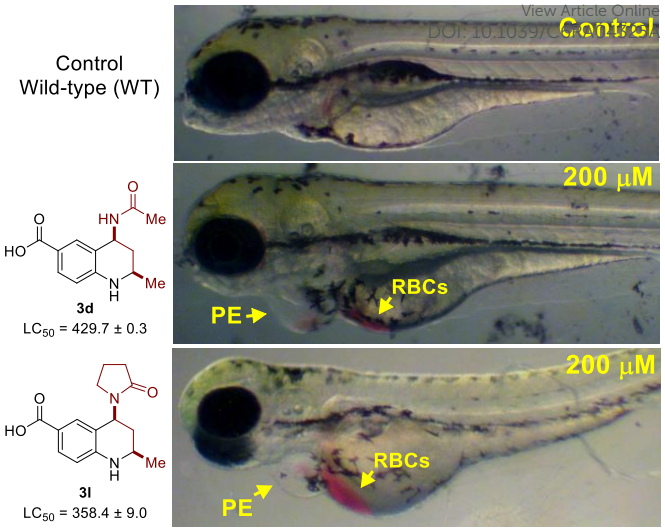
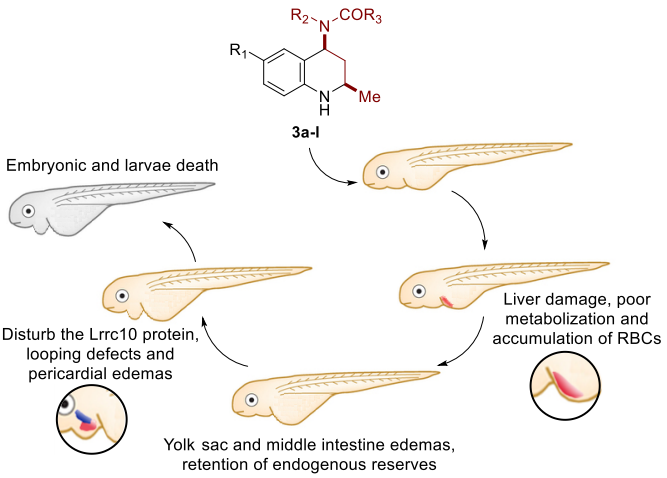


Fig. 4 Microscope photographs at 96 hpf of the treated embryos with compounds **3d** (200 µM) and **3l** (200 µM); Embryos showed mil pericardial edemas (PE) and an accumulation of red blood cells (RBCs) inside the yolk sac in the liver region.

mechanism that explains its toxicological process is defined herein (Scheme 3).



Scheme 3 Proposed toxicological and phenotypic profile of the THQs **3a-l**.

First, the absorption of the THQs by the digestive system induces a liver injury when these molecules are trying to be metabolize by this organ. Once the liver is damaged, blood cells accumulate around this tissue and the larvae is unable to absorb the nutrients, minerals and vitamins from the intestinal tract into the bloodstream, resulting in developmental delays and edemas in the YS and the MI. Since the blood circulation has been perturbed, the insufficient blood flow that contains low to middle concentrations of THQs and their metabolites will cause defects during the heart development after the Lrrc10 protein is disturbed, resulting in the abnormal morphology of the heart chambers and in PEs.

Therefore, if the liver and heart are damaged, the cessation of blood circulation and the lack of nutrients distribution will finally induce the loss of renal function and the eventual death

of the embryo, however additional studies are needed to prove this hypothesis.

Conclusions

We developed a mild and efficient protocol for the diastereoselective synthesis of 4-amidyl-2-methyl-THQ based on the imino Diels Alder (Povarov) ABB´ type reaction catalysed by $Ce(SO_4)_2$, in which the yields and the diastereoselectivity of the obtained products were improved in comparison to previous reports. This strategy, which can be adapted to the synthesis of a range of other natural and synthetic tetrahydroquinolines, lead to the synthesis of a diverse library of THQs **3a-l** from readily available starting materials and tolerance of a variety of substituents under the reaction conditions, providing the desired tetrahydroquinoline derivatives in modest to high yields and with excellent diastereoselective ratio.

Finally, when this library was tested on the zebrafish screen, we found that the molecule **3k** was the most toxic derivative with a LC_{50} below 95 µM, where the pyrrolidine ring at position C-4 and the methoxy group at position C-6 of the THQ core were identified as the main toxicophores. Notwithstanding that to understand the precise mode of action of these compounds that were active in this phenotypic screen much work would be needed. The selection of the molecules that induced the discussed visual phenotypes (**3a**, **3b**, **3d**, **3h**, **3i**, **3j** and **3l**) will reduce the time and cost of further biological assays that pretend to use these structures as a start point in the discovery and development of new bioactive agents.

Experimental

Chemistry

Infrared (FT-IR) spectra were recorded on a Lumex Infralum FT-02 spectrometer, ν_{max} in cm^{-1}. Bands are characterized according to the functional group. ^1H NMR spectra were recorded on a Bruker Avance-400 (400 MHz) spectrometer. Chemical shifts are reported in ppm with the solvent resonance as the internal standard ($CDCl_3$: δ 7.26 ppm; DMSO-d_6: δ 2.50 ppm). Data were reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublets, br = broad, m = multiplet), coupling constants (Hz) and integration(see ESI†). ^{13}C NMR spectra were recorded on a Bruker Avance-400 (400 MHz) spectrometer with complete proton decoupling. Chemical shifts are reported in ppm from solvent resonance as the internal standard ($CDCl_3$: δ 77.00 ppm; DMSO-d_6: δ 40.45 ppm). On DEPT-135 spectra, the signals of CH_3 and CH carbons are shown as positive (+) and CH_2 carbons are shown negative (-). Quaternary carbons are not shown. A Hewlett Packard 5890a Series II Gas Chromatograph interfaced to an HP 5972 Mass Selective Detector (MSD) with an HP MS ChemStation Data system was used for MS identification at 70 eV using a 60 m capillary column coated with HP-5 [5%-phenylpoly (dimethylsiloxane)]. Accurate mass data were obtained on Micromass Q-TOF by electrospray ionisation (ESI). Melting points were measured on a Fisher Johns melting point apparatus and are uncorrected.

Unless otherwise noted, all reactions have been carried out with distilled and dried solvents and under atmosphere pressure. All work-up and purification procedures were carried out with reagent grade solvents (purchased from Aldrich and Merck) in air. Thin-layer chromatography (TLC) was performed using Merck silica gel 60 F254 precoated plates (0.25 mm). Column chromatography was performed using silicagel 60 (0.063 - 0.200 mm) 70-230 mesh.

*General procedure for the synthesis of the 2-methyl-THQs **3a-l***

To a stirred MeCN solution (20 mL) of $Ce(SO_4)_2$ (15% mol), the starting aniline **1a-d** was added (2 mmol), after its dissolution, the corresponding *N*-vinyl amide **2a-c** (4.2 mmol) was added to the reaction mixture and it was stirred at room temperature for the indicate time. After completion of the reaction as indicated by TLC, the mixture was treated with a saturated aqueous solution of $NaHCO_3$ and extracted with ethyl acetate (3 × 30 mL), dried over anhydrous Na_2SO_4 and evaporated. Crude product was purified by column chromatography on silica gel using a mixture of petroleum ether and ethyl acetate as eluent to give the THQ derivatives. Compounds that resulted to be poorly soluble in common solvents (CH_2Cl_2, $CHCl_3$ and EtOAc) and very soluble in DMSO, MeCN or MeOH, solvents used for their synthesis, NMR experiments and biological studies.

*Toxicity testing and phenotypic screening of 2-methyl-THQs **3a-l** using the zebrafish embryo model*

Wild-type adult zebrafish of both sexes were separated in two tanks (30 L each), according to their gender, at 26 ± 2 °C under natural light-dark photoperiods. The fishes were feed twice daily and the water quality was recorded weekly, in order to acclimate the fishes for at least two weeks before experiments begin. For the reproduction of the adult fishes, small breeding tanks were set up in the evening previous to experiment, each containing three males and one female specimen. The tanks were isolated until next morning when the lights switch on and the natural mating occurs, without any perturbation.

The adult fishes were returned to their corresponding tank and the embryos were collected, pooled and washed with E3 medium and transferred into a 92 mm glass Petri dish. Further, dead, delayed, malformed and unfertilized embryos were identified under a dissecting microscope and removed by select aspiration with a pipette. This last procedure was repeated at 12 and 20 hpf in order to remove the unfit embryos. Throughout this period of time, the embryos were kept at 28 ± 2 °C in an incubator under natural light-dark photoperiods.

The selected embryos of 24 hpf from the Petri dish were gently distributed into 96-well plates, placing a single embryo and 200 µL of E3 medium per well.

Adult zebrafish were care and used according to the Guide of the National Institute of Health for Care and Use of Laboratory Animals, keep them healthy and free of any signs of disease. The Ethics and Research Committee of the Heart Institute of Bucaramanga approved the protocol under the Acta Number 050 of May 26 of 2012.

Determination of zebrafish embryo LC_{50}

For this experiment, in total 72 embryos were required per sample in order to run three independent experiments in three different plates, and each compound was evaluated three times in the same plate, allowing the evaluation of four samples peer plate. Compounds **3a-l** were diluted into the E3 screening medium with 2% V/V of DMSO and aliquots of 200 µL were prepared at concentrations starting from 12.5 and finishing in 1250 µM (geometric series). The LC_{50} determination (expressed in µmol of compound/L of solution) was based on the cumulative mortality after 72 hours of chemical exposure (96 hpf). Each embryo was examined under a dissecting microscope and the statistical analysis was made using Regression Probit analysis with *SPSS* for windows version 19.0. Data are expressed as the standard error of the mean (SEM) of three different experiments in triplicate.

Phenotypic screening using the zebrafish embryo model

Compounds **3a-l** were diluted into the E3 screening medium with 2% V/V of DMSO and aliquots of 200 µL were prepared at concentrations from 5-400 µM, depending on the LC_{50} of each THQ. The surrounding medium (200 µL) was carefully removed from the embryonic plates using an 8-multichannel pipette and then the appropriate chemical aliquot of each compound (200 µL), previously prepared, were added into the corresponding well of the embryonic plate. Eight controls wells were used peer plate, each containing E3 medium with 2% V/V of DMSO. The embryonic plates were incubated at 28 °C and examined at 48, 72 and 96 hours post-fertilization (hpf) using an OPTIKA zoom stereo microscope (trinocular version of model SZM-1).

Acknowledgements

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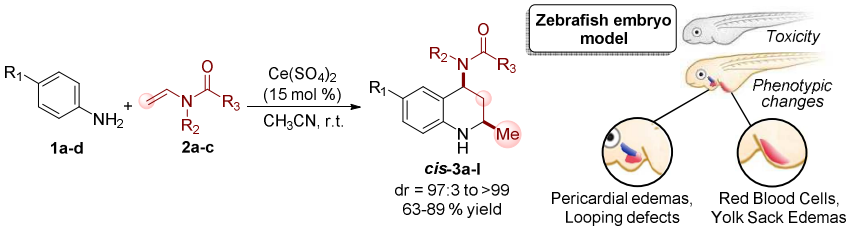
Table of contents/Graphical abstract



The synthesis of tetrahydroquinolines via Povarov reaction has been developed using $Ce(SO_2)_4$ as a catalyst, this efficient protocol allowed the toxicity and phenotypic study of these products using the zebrafish embryo model