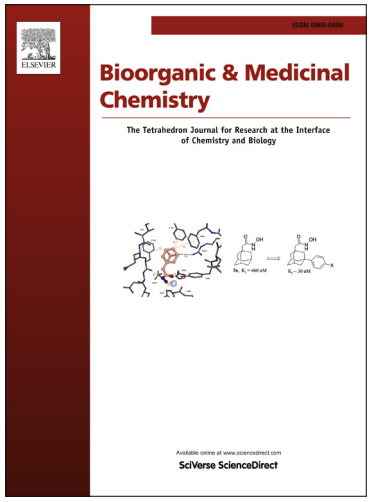

The enzyme 3-hydroxykynurenine transaminase as potential target for 1,2,4-oxadiazoles with larvicide activity against the Dengue vector *Aedes aegypti*

Vanessa S. Oliveira, Cecília Pimenteira, Diana C.B. da Silva-Alves, Laylla L.L. Leal, Ricardo A.W. Neves-Filho, Daniela M.A.F. Navarro, Geanne K.N. Santos, Kamilla A. Dutra, Janaína V. dos Anjos, Thereza A. Soares

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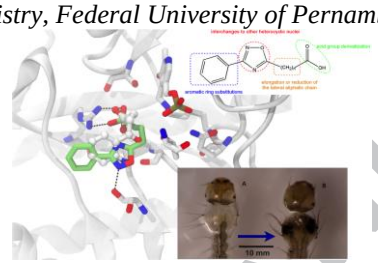

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ABSTRACT

The mosquito *Aedes aegyptii* is the vector agent responsible for the transmission of yellow fever and dengue fever viruses to over eighty million people in tropical and subtropical regions of the world. Exhaustive efforts have lead to a vaccine candidate with only 30% effectiveness against the dengue virus and failure to protect patients against the serotype 2. Hence, vector control remains the most viable route to dengue fever control programs. We have synthesized a class of 1,2,4-oxadiazole derivatives whose most biologically active compounds exhibit potent activity against *Aedes aegyptii* larvae (ca. of 15 ppm) and low toxicity in mammals. Exposure to these larvicides results in larvae pigmentation in a manner correlated with the LC_{50} measurements. Structural comparisons of the 1,2,4-oxadiazole nucleus against known inhibitors of insect enzymes allowed the identification of 3-hydroxykynurenine transaminase as a potential target for these synthetic larvicides. Molecular docking calculations indicate that 1,2,4-oxadiazole compounds can bind to 3-hydroxykynurenine transaminase with similar conformation and binding energies as its crystallographic inhibitor 4-(2-aminophenyl)-4-oxobutanoic acid.

1. Introduction

Aedes aegypti is the vector agent that spreads the yellow fever and the dengue fever viruses, and are endemic in tropical and subtropical regions around the world.[1, 2] Yellow fever affects annually 0.2 million people whereas about 80 million people are infected every year by one of the four dengue virus types. The disease is a serious worldwide public health treat due to the presence of the mosquito in more than 100 countries. Yellow fever vaccination is the most important and effective means to prevent the occurrence of this disease, and carries a low risk of serious adverse events. The live-attenuated 17D vaccine provides protective immunity within one to two weeks in 95% of those vaccinated.[3] However, it has been recently shown that the leading vaccine candidate was only 30% effective against dengue fever in a first large clinical trial[2]. In addition, the vaccine has failed to protect individuals against serotype 2. Currently, the sole effective strategy to control the disease is the vector eradication due to two main factors.[4] First, the sickness incidence is closely related to domiciliary infestation by *Aedes* mosquitoes. Second, there are several reports of vertical transmission of the dengue vírus in *Aedes spp* collected in the field.[5-8] This latter finding implies that adult mosquitoes infected through transovarial transmission do not have to feed in a viremic vertebrate host to infect a naïve host, thus explaining the persistence of the virus in nature in the absence of viremic vertebrate hosts.[9] Therefore, vector control will remain a crucial issue for dengue fever control programs.

One of the major routes of detoxification (removal of reactive oxygen and nitrogen species) in *Aedes aegypti* mosquitoes is the kynurenine pathway. This is the chief catabolic pathway in mosquitoes and the main route of tryptophan metabolism in living organisms.[10, 11] In this biochemical pathway, the most important reaction is the conversion of the harmful metabolite 3-hydroxy-kynurenine (3-HK) into xanthurenic acid (XA), a chemically stable, non-toxic substance by the enzyme 3-hydroxy-kynurenine transaminase (HKT).[12-14] Previous studies have shown that the 3-HK accumulation in adult insects leads to the formation of reactive oxygen species with serious neuronal

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damage and insect death.[14, 15] For this reason, the high conversion efficiency of 3-HK to XA by HKT in mosquito larvae is considered to be a mechanism by which mosquitoes detoxify the chemically reactive 3-HK.[16] Earlier studies have also shown that 3-HK content was quite different in insects at different developmental stages.[12, 17] 3-HK is also the initial precursor for the production of ommochromes, major eye pigments in mosquitoes. Compound eye development and eye pigmentation occur mainly during the pupal and early adult stages.[18-21] For this reason, the 3-HK to XA pathway must be down regulated in pupae to allow for the accumulation and transport of some 3-HK to the compound eyes for pigmentation.[13, 19]

The use of competitive antagonists for HKT can prevent the conversion of the toxic 3-HK to xanthurenic acid in mosquito larvae and adults, causing the accumulation of neurotoxic 3-HK (Fig. 1A).[22-24] As part of a drug discovery effort aiming at the identification of new larvicide candidates, we have found a class of heterocycle derivatives with larvicidal activity against *A. aegypti* (Fig. 1B).[25, 26] Due to the potent activity against the mosquito larvae (ca. of 15 ppm) and low toxicity in mammals, these compounds are the first prototypes of synthetic larvicides containing the 1,2,4-oxadiazole nucleus (Fig. 1B). Despite the potential as anti-dengue larvicide, the biological mechanism of action of this class of compounds has not been elucidated. The identification of a molecular target in *A. aegypti* for 1,2,4-oxadiazole is a pivotal step towards the design and synthesis of novel or modified compounds with improved potency or selectivity. In this work, we assess the prospect of having HKT as a potential inhibitory target for a total of 27 chemical variants of the original scaffold (Table 1). We perform molecular docking calculations to ascertain whether these compounds can bind to the HKT active site in the same conformation observed for the inhibitor 4-(2-aminophenyl)-4-oxobutanoic acid (4-OB) by the means of high-resolution X-ray crystalography.[23] In addition, we have also synthesized six novel oxadiazole compounds and evaluated their larvicide activity against the *A. aegypti* as function of systematic substitution/replacement of chemical functions in specific regions of the 1,2,4-oxadiazole scaffold. Our findings are presented thereafter.

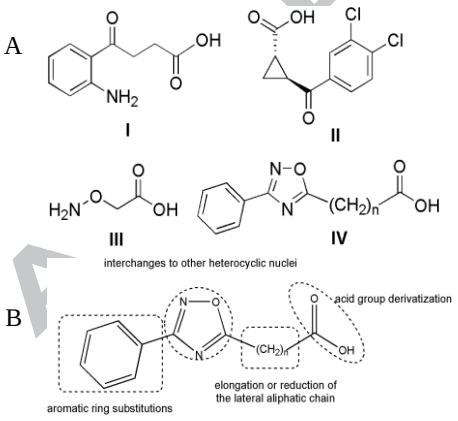


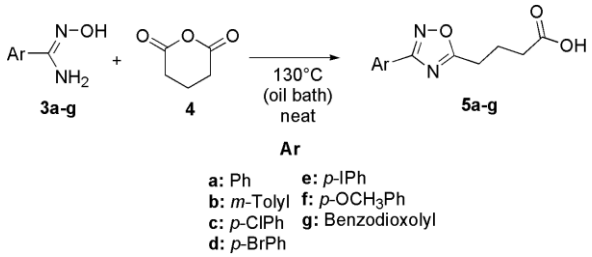
Figure 1. Top panel: antagonists for the HKT enzyme: (I) 4-(2-aminophenyl)-4-oxobutanoic acid, (II) (+)-(1S,2S)-2-(3,4-dichlorobenzoyl)-cyclopropyl-1-carboxilic acid, and (III) amino-oxyacetic acid, (IV) 1,2,4-oxadiazole scaffold. Bottom panel: Chemical modifications performed on the oxadiazole scaffold.

2. Results and Discussion

2.1. Synthesis and Larvicidal Activity of Heterocyclic Compounds

We have previously synthesized and established the larvicidal activity of 3-(3-aryl-1,2,4-oxadiazol-5-yl)propionic acids against *A. aegypti*.[25,26] These compounds promote alterations in the larvae spiracular valves of the siphon and anal papillae, leading to the death of the insect. In this study, we have used 3-(3-aryl-1,2,4-oxadiazol-5-yl)propionic acids as prototypes for chemical modifications aiming at improved larvicide candidates (Table 1). These modifications are depicted in Fig. 1B.

Modifications on the aryl group located at the C-3 position of 1,2,4-oxadiazole resulted in nine bioactive compounds, with the halogen-containing compounds **2f** (chlorine) and **2g (**bromine) exhibiting good larvicidal activities (below 30.0 ppm). On the other hand, the fluoro-compound **2e** had only weak activity (81.2 ppm). These findings indicate that electronic effects are as important as steric effects, since there is a significant difference in the larvicidal profile of the three compounds (Table 1).[25] We have also constructed a homologous series of the prototype 3-(3-phenyl-1,2,4oxadiazol-5-yl)-propionic acid **2a**. Hence we have reduced and elongated, respectively, the aliphatic alkyl chain located at C-5 of 1,2,4-oxadiazole nucleus for compounds **1a-d** and **5a-g**. The 3-(3-aryl-1,2,4-oxadiazol-5-yl)-etanoic acids (**1a-d**) were synthesized according to known literature procedures.[27] The larvicidal activity for this series follows an analogous pattern to **2a-i** with the best results obtained for halogen-containing compounds **1c-d**. The reduction of the aliphatic alkyl chain length led to less potent larvicides (LC_{50} above 45 ppm). In contrast to these findings, the elongation of the alkyl chain, which yielded the 3-(3-aryl-1,2,4-oxadiazol-5-yl)-butanoic acids (compounds **5a-g**) (Scheme 1) provides the best larvicidal candidates (Table 1). Once again, the halogen-containing 1,2,4-oxadiazoles presented the best biological activities, with compound **5d** (bromo-compound) having a LC_{50} value of 10.6 ppm.



Scheme 1. Synthesis of 3-(3-aryl-1,2,4-oxadiazol-5-yl)-butanoic acids (**5a-g**).

We have also changed the central nucleus to an isoxazole moiety. As mentioned earlier, the difference between these two five-membered heterocyclic compounds lies in the replacement of N-4 atom of oxadiazole with a CH group in isoxazole. Compounds **7-9** were synthesized in previous work.[28] A comparison can be made when analysing the LC_{50} values of compounds **2a** and **8** and **9** and **10** (Table 1), where the sole difference is the heterocyclic ring. In both cases, the isoxazole-containing compounds are more potent than the 1,2,4-oxadiazole ones. These results show that the replacement of the 1,2,4-oxadiazole for a isoxazole nucleus results in better larvicidal compounds.

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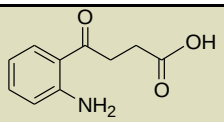
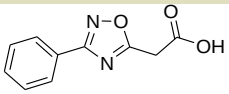
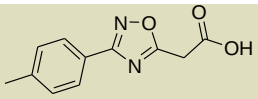
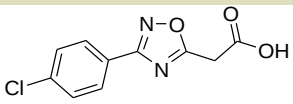
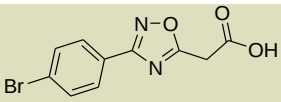
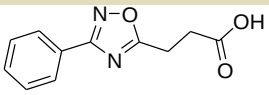
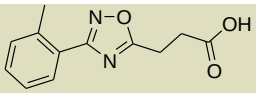
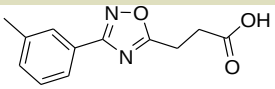
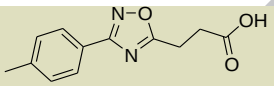
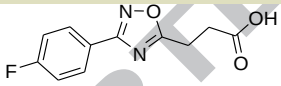
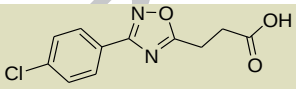
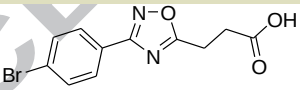
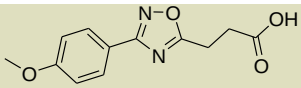
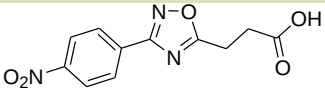
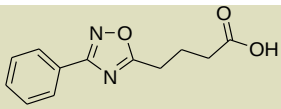
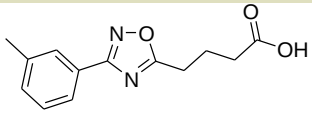
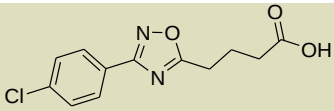
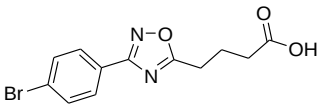
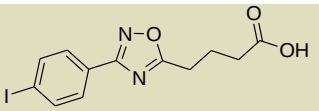
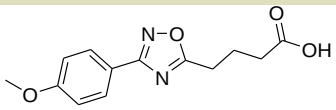
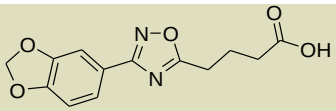
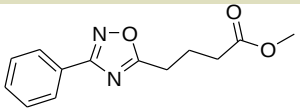
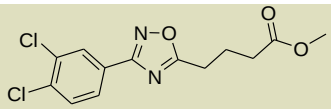
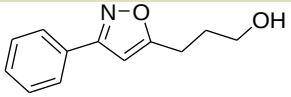
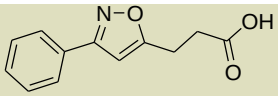
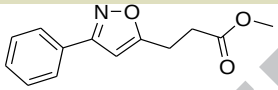
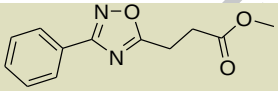
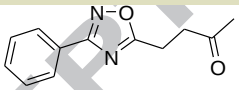
Table 1. 1,2,4-oxadiazole derivatives. Chemical structures, larvicidal activities against the mosquito *Aedes aegypti*, and binding affinities against the enzyme 3-hydroxy-kynurenine transaminase (HKT).

Compound	Chemical structure	LC_{50} (ppm)	Lowest energy conformer	[a]ΔK_i (μM)
4-OB		-	Reference	0
1a		>100	Multiple	36.21
1b		92.3	Multiple	10.14
1c		49.3	Multiple	18.99
1d		67.9	Multiple	13.01
2a		98.6	Multiple	12.98
2b		70.9	Multiple	19.01
2c		63.8	Multiple	6.89
2d		65.8	Multiple	7.76
2e		81.2	Multiple	18.75
2f		28.1	Multiple	8.26
2g		15.2	Multiple	9.48
2h		71.5	Multiple	30.83
2i		50.5	Multiple	6.94
5a		92.7	Inhibitor-like	-1.24
5b		75.9	Inhibitor-like	-2.5
5c		34.9	Inhibitor-like	-2.34

5d		10.6	Inhibitor-like	-2.74
5e		16.2	Inhibitor-like	-3.17
5f		>100	Inhibitor-like*	-1.37
5g		72.9	Inhibitor-like*	-2.53
6a		59.9	Inhibitor-like	-1.45
6b		6.8	Inhibitor-like	-3.2
7		>100	Inhibitor-like**	1.72
8		82.8	Multiple	14.65
9		13.2	Multiple	5.47
10		30.6	Inhibitor-like**	-2.9
11		>100	Inhibitor-like**	2.08

[a] $\Delta K_i = K_i$(oxadiazoles) - K_i(4-OB) where K_i is estimated from the molecular docking calculations. The more negative ΔK_i, the stronger the oxadiazole compound binds to HKT compared to the crystallographic inhibitor 4-OB.

*The inhibitor-like conformation is among the lowest energy conformers, but it is not the lowest energy one.

**Compound binds to HKT in inhibitor-like conformation, but the carbonyl group or hydroxyl interacts preferentially with the side-chain of Gln204 instead of Arg356.

Derivatizations on the carboxylate moiety were made. Esters, an alcohol and a ketone were synthesized to evaluate the the effect of these scaffold modifications on larvicide activity. The transformation of carboxylate moiety into an ester moiety is the most beneficial one. Esters **9** and **10** had better larvicidal activities than their parent acids (**2a** and **8**, respectively).

2.2. Binding of Heterocyclic Derivatives to 3-Hydroxykynurenine Transaminase

The HKT sequence has been isolated from several invertebrate species including *Aedes aegypti*, *Neobelleria bullata*, *Locusta migratoria* and *Anopheles gambiae.*[12, 16] High-resolution X-ray structures are available for HKT from *A. gambiae* at 2.7 Å resolution (PDB IDs 2CH1 and 2CH2).[23] Experimentally determined structures of HKT from other species are currently unavailable in the Protein Database. The HKT sequence of *A. aegypti* (AeHKT) and *A. gambiae* (AgHKT) shares an identity of 73%.[29] Previous studies have also shown that the amino acid sequence in the active site of both enzymes is nearly identical with the triad Ser43-Asn44-Phe45 involved in the substrate recognition process in the two species. Given the high identity between AeHKT and AgHKT primary sequences, we have built a homology-based structural model for AeHKT using the X-ray structure of AeHKT (PDB ID 2CH2) as template. Superposition of the model and template structures showed that all active-site residues within 7 Å cutoff from the atomic coordinates of the 4-OB inhibitor present in the X-ray structure 2CH2 are preserved in the sequence of AeHKT. These residues define the first and second spheres of interaction between inhibitors and the enzyme, and therefore AeHKT and AgHKT can be reliably represented by the same active site conformation, *i.e.* by the AgHKT high-resolution X-ray structure (PDB ID 2CH2). The identical active

site residues in the two enzymes are Met21, Gly23, Pro24, Gly25, Pro26, His79, Leu42, Ser43, Asn44, Phe45, His46, His79, Ile103, Trp104, Ser154, Ser155, Tyr256, His257, His258, His259, Glu342, Val343, Gln344, Gly345, Gly346, Leu347.

In order to assess the ability of our docking procedure to correctly predict the binding modes of heterocyclic derivatives at the AgHKT crystal structure, a validation procedure was devised to demonstrate that the undertaken procedure is able to reproduce the bound conformation of inhibitor 4-OB in the crystallographic structure of the complex. This procedure is described in the computational simulations section. The 4-OB conformations resulting from these simulations were analyzed and compared to its crystallographic conformation bound to the active site of HKT. In all tests, the conformation of the co-crystallized inhibitor in the active site of AgHKT X-ray structure was successfully reproduced with a root-mean-square deviation (RMSD) of less that 0.09 nm (Fig. 3).

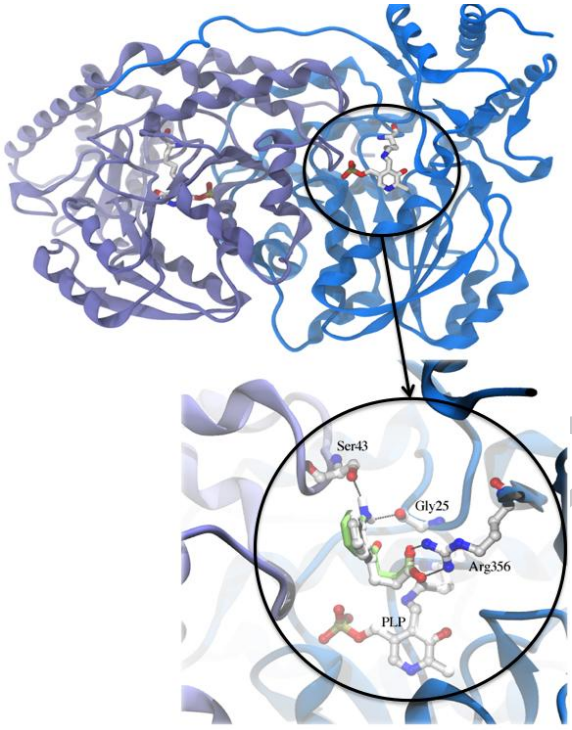


Figure 3. Cartoon representation of the X-ray structure of AgHKT with the co-crystallized inhibitor 4-OB in cpk. The experimental and calculated conformations are show in white and green, respectively. Residues involved in the substrate recognition are also shown in white.

A critical comparison of atomic interactions in different docked complexes against those observed in the 4-OB-AgHKT crystallographic structure was undertaken. A twofold criteria has been used to select potentially effective inhibitors out of the pool of the synthesized compounds. First, potential inhibitors should interact with AgHKT active site analagously to 4-OB. Second, the estimated K_i for these inhibitors should be smaller or equivalent to the value estimated for 4-OB using the same molecular docking procedure (Table 1). The competitive inhibitor 4-OB binds to AgHKT with K_i = 300 μM, and cannot be processed by the enzyme due to the absence of a reactive amino group at the C2 position equivalent to the Cα atom of the natural amino acid substrates.[23] Likewise, reactive amino groups are absent in the heterocyclic compounds.

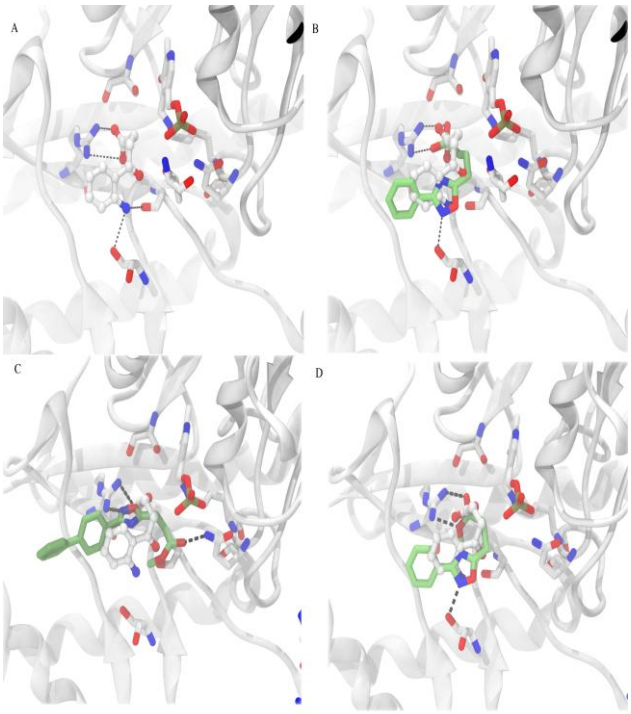


Figure 4. Comparative binding of the co-crystallized inhibitor 4-OB (A) and inhibitor-like conformers of heterocyclic compounds (B-D) to AgHKT. Conformers are representative of the most populated, lowest-energy conformational clusters. Canonical inhibitor-like conformers (B and D), and a variation (C) where Arg356 interacts with the heterocycle atoms.

The 1,2,4-oxadiazole compounds containing a butanoic acid moiety in series **5a-g** bind to AgHKT via a single, predominant conformation much alike the inhibitor 4-OB (Fig. 4B). The K_i values obtained for the lowest energy conformer via molecular docking calculations are in the low micromolar range, similar to the calculated K_i for 4-OB (Table 1). Indeed, all the compounds of this class exhibited calculated K_i values smaller (ΔK_i negative) than that of 4-OB. The replacement of the carboxylate group by an ester in compounds **6a-b** has not altered the interaction mode of the inhibitor with AgHKT. A decrease in the number of carbon atoms in the carboxylate linker of compounds in the series **1a-d** and **2a-i** leads to an increase of the respective K_i values in about one order of magnitude (Table 1). This effect is associated with the occurrence of multiple conformations instead of a single one (Fig. 4C). The chemical substitutions in the aromatic ring do not appear to affect significantly the calculated affinity of the 1,2,4-oxadiazole derivatives for AgHKT. Upon binding to AgHKT, the aromatic ring is loosely positioned in the entry of active site (Fig. 4).

2.3. Larvae Pigmentation upon Exposure to Oxadiazole-based Larvicides

Upon exposition of fourth-instar *A. aegypti* larvae to a solution of compound **2g** at 100 ppm significant alterations in the pigmentation pattern were observed (Fig. 5A and 5B). Likewise, a similar pigmentation pattern is seen when larvae were treated with solutions of isoxazoles **8** and **9 at** 100 ppm (Fig. 5D and 5E). Such pigmentation alterations in *A. aegypti* pattern correlate with the respective LC_{50} measurements, *i.e.* larvae treated with the most potent larvicides (LC_{50} below 20 ppm, **2g** and **9**) exhibited the most intense pigmentation.

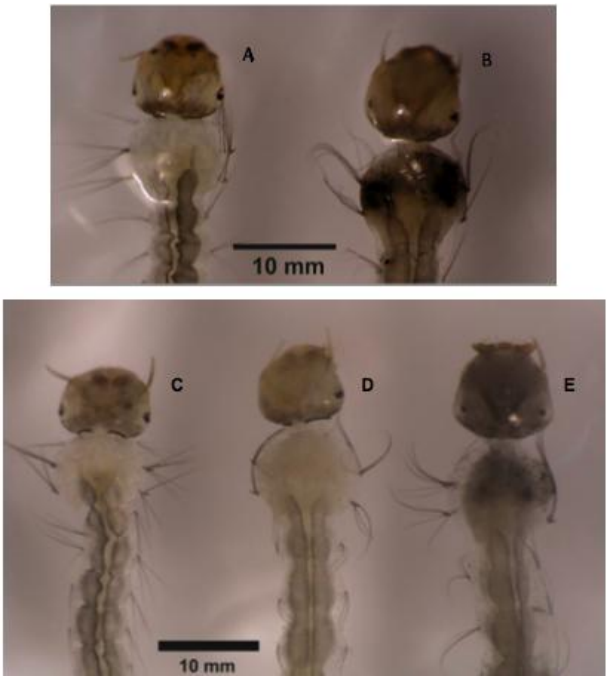


Figure 5. Pigmentation pattern. Optical microscopy images of four-instar larvae of *A. aegypti*. Images made with Olympus SZ 51 stereo microscopes. Top panel: (A) Control and upon 24 h exposure to 100 ppm solution of compound **2g** (B). Bottom panel: (C) Control and upon 24 h exposure to 100 ppm solution of compounds **8** (D) and **9** (E).

3-HK is the initial precursor for the production of the ommochromes responsible for the development of pigmented wings and eyes.[13, 19] During the pupal and early adult stages, the 3-HK to XA pathway must be down-regulated in pupae to allow for the accumulation and transport of some 3-HK to the compound eyes for pigmentation.[13, 19] We hypothesize that larvae pigmentation upon exposure to oxadiazole derivatives may be due to the accumulation and conversion of 3-HK into pigments. In this context, the transaminase HKT is a plausible molecular target for the presented heterocyclic larvicides based on two considerations. First, there is a noticeable chemical similarity between the heterocyclic nucleus and the 4-(2-aminophenyl)-4-oxobutanoic acid (4-OB) antagonist of HKT (Fig. 1A and 1B). Second, our biological activity assays show that upon contact with the synthesized larvicides, the dead larvae exhibit dark pigmentation (Fig. 5). These findings coupled to our molecular docking calculations suggest that 1,2,4-oxadiazole derivatives can rationally designed as inhibitors of HKT, an essential enzyme of kynurenine pathway, the chief catabolic pathway in mosquitoes and the main route of tryptophan metabolism in living organisms.[10, 11].

3. Conclusions

A direct comparison between measured lethal concentration (LC_{50}) and calculated K_i is not possible. LC_{50} values express pharmacodynamical properties, and are not an accurate indicator of binding affinity as is the case for K_i. However, analysis of converging trends between the two quantities can be useful in the deconvolution of various quantities contributing to the LC_{50}. Accordingly, measured LC_{50} values indicate that the transformation of the carboxylate moiety into an ester moiety is advantageous. Esters **9** and **10** display improved larvicidal activities compared to their parent acids (**2a** and **8**, respectively) (Table 1). This pattern is also observed for the calculated K_i which exhibits a decrease from parent to derived compounds. However, compounds **5a-g** exhibit average LC_{50} values which are higher than their cognate esters while binding to HKT with comparable K_i constants (Table 1). This effect may be due to the enhanced lipophilicity of esters, which facilitates the penetration of this class of compounds into the larvae system.[30] Likewise, the addition of halogens (Fluorine, Chlorine, Bromine and Iodine) to the aromatic ring increases the larvicidal activity of the 1,2,4-oxadiazole scaffold without having much effect on the corresponding binding affinities (Table 1). In fact, chemical modifications of the aromatic ring are not expected to impact significantly the binding of 1,2,4-oxadiazole derivatives to HKT because of its location near the entry of the active site (Fig. 4).

Our report suggests that the heterocyclic compounds presented herein have a potent larvicide activity against the mosquito *A. aegypti*. Computational calculations suggest that this heterocyclic scaffold can bind to the enzyme HKT of *A. gambiae* and *A. aegypti* in an analogous conformation to the co-crystallized inhibitor 4-OB, and with comparable binding affinities (Fig. 4, Table 1). HKT has been previously identified as a new target for malaria treatment due to its role in the conversion of the toxic 3-HK into XA.[22-24] The high sequence identity between HKT from *A. gambiae* and *A. aegypti* implies that the enzyme is also a potential new target for dengue fever. Our findings suggest that HKT is a plausible molecular target for inhibition by this type of heterocyclic ring scaffold. Kinetics assays for recombinant HKT and 1,2,4-oxadiazole derivatives are currently under way in order to substantiate these results.

4. Experimental

4.1. Synthesis and Characterization

All commercially available reagents were used without any further purification and the reactions were monitored by TLC analysis with TLC plates containing GF_{254} (E. Merck). Melting points were determined on Electro-thermal (Mel-temp) apparatus and are uncorrected. Column chromatography was performed on Silica Gel 60 (70-230 mesh, E. Merck). NMR spectra were recorded with a Varian UNMRS 400 MHz spectrometer and referenced as follows: ^1H (400 MHz), internal $SiMe_4$ at δ= 0.00 ppm, ^{13}C (100 MHz), internal standard at δ = 77.23 ppm. Elemental analysis was performed with a Carlo Erba instrument model E-1110. Compounds **1a-d** were synthesized and their physical-chemical data were compared to earlier literature.[27] Compounds **2a-i** were synthesized by our group and published elsewhere.[25] Arylamidoximes **3a-g** were prepared according to known literature procedures.[31] Substances **5a**,[32] **5c**,[28] **5d**,[33] **7-9**,[30] **10**,[34] and **11**[35] were synthesized and had their physical-chemical data compared to previous literature.

4.1.1. Synthesis of 3-(3-aryl-1,2,4-oxadiazol-5-yl)-butanoic acids (5a-g)

A suitable arylamidoxime **3a-g** (1.0 mmol) was allowed to react with glutaric anhydride **4** (1.2 mmol) in a round bottom flask, with no solvent, at 130°C (oil bath) for 3-4 hours. When TLC analysis indicated the complete consumption of the starting amidoxime, the reaction was cooled to room temperature, The reaction mixture was then diluted with a concentrated solution of $NaHCO_3$ (10 mL) and AcOEt (10 mL) and this resulting suspension was allowed to stir overnight. The aqueous layer was separated and then a concentrated solution of citric acid was added until total precipitation of the desired compounds. The obtained product was dissolved in chloroform, dried over

anhydrous Na_2SO_4 and re-crystallized from chloroform-hexane to afford the pure products (**5a-g**).

4-(3-*m*-Tolyl-1,2,4-oxadiazol-5-yl)butanoic acid (**5b**): Yield:70%, m.p. (^oC):107-108. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.21 (q, *J* = 7.5 Hz, 2H); 2.41 (s, 3H); 2.55 (t, *J* = 7.5 Hz, 2H); 3.04 (t, *J* = 7.5 Hz, 2H); 7.28 (d, *J* = 7.5 Hz, 2H); 7.95 (d, *J* = 7.5 Hz, 2H) 9.96 (bs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 21.28; 21.47, 25.55, 32.67, 123.70, 127.27, 129.50, 141.48, 178.46, 178.75. Elemental Analysis for $C_{13}H_{14}N_2O_3$: Calc. C, 63.40; H, 5.73; N, 11.38. Found C, 63.45; H, 5.48; N, 11.55.

4-[3-(4-Iodophenyl)-1,2,4-oxadiazol-5-yl]butanoic acid (**5e**): Yield: 55%, m.p. (^oC): 117-118. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.01 (q, *J* = 7.5 Hz, 2H); 2.56 (t, *J* = 7.5 Hz, 2H); 3.05 (t, *J* = 7.5 Hz, 2H); 7.77-7.84 (m, 4H) ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 21.27; 25.99, 32.67, 97.90, 126.15, 128.83, 138.07, 167.70, 167.07, 167.7, 178.32, 179.12. Elemental Analysis for $C_{12}H_{11}IN_2O_3$: Calc. C, 40.24; H, 3.10; N, 7.82. Found C, 40.21; H, 3.32; N, 7.44.

4-[3-(4-Methoxyphenyl)-1,2,4-oxadiazol-5-yl]butanoic acid (**5f**): Yield: 69%, m.p. (^oC): 108-109. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.20 (q, *J* = 7.5 Hz, 2H); 2.55 (t, *J* = 7.5 Hz, 2H); 3.02 (t, *J* = 7.5 Hz, 2H); 3.85 (s, 3H); 6.99 (d, *J* = 8.7 Hz, 2H); 7.99 (d, *J* = 7.5 Hz, 2H); 9.80 (bs, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm): 21.31, 25.56, 32.68, 55.32, 114.19, 119,02, 128.96, 161.84, 167.86, 178.40, 178.63. Elemental Analysis for $C_{13}H_{14}N_2O_4$: Calc. C, 59.54; H, 5.38; N, 10.68. Found C, 60.06; H, 5.12; N, 10.93.

4-[3-(Benzo[*d*][1,3]dioxol-5-yl)-1,2,4-oxadiazol-5-yl]butanoic acid (**5g**): Yield: 60%, m.p. (^oC): 112-113. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.21 (q, *J* = 7.6 Hz, 6.8 Hz, 2H); 2.56 (t, *J* = 7.6 Hz, 2H); 3.03 (t, *J* = 6.8 Hz, 2H); 6.03 (s, 2H); 6.89 (dd; *J* = 8.4 Hz, 1.6Hz, 1H); 7.51 (d, *J* = 1.6 Hz, 1H); 7.63 (dd, *J* = 8.4 Hz, 1H).^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 21.4, 25.6, 32.6, 101.6, 107.5, 108.6, 120.6, 122.3, 148.1, 150.1, 167.9, 177.8, 178.7. Elemental Analysis for $C_{13}H_{12}N_2O_5$: Calc. C, 56.52; H, 4.38; N, 10.14. Found C, 56.70; H, 4.08 ; N, 9.82.

4.1.2. Synthesis of 3-(3-aryl-1,2,4-oxadiazol-5-yl)-butanoic acids methyl esters (6a-b)

To an appropriate solution of 3-(3-aryl-1,2,4-oxadiazol-5-yl)-butanoic acid (5 mmol) in 10 mL of methanol, five drops of concentrated sulfuric acid were added followed by reflux under stirring until TLC analysis indicated the total consumption of the starting acid. Dilution of the contents with water (20 mL) followed by solvent removal under reduced pressure gave the crude product. Extraction of the residue with AcOEt (2 x 20 mL) and drying solvent over anhydrous Na_2SO_4, pursued by solvent removal *in vacuo* furnished the crude product. The residue was chromatographed over silica gel using hexane:EtOAc as eluant (4:1, v/v), crystallized from chloroform and hexane, which after work-up furnished the desired methyl 3-(3-aryl-1,2,4-oxadiazol-5-yl)-butanoates (**6a-b**).

Methyl 4-(3-phenyl-1,2,4-oxadiazol-5-yl)butanoate (**6a**): Yield: 50%, m.p. (^oC): 42-43. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.21 (q, *J* = 7.6 Hz, 7.2 Hz, 2H); 2.50 (t, *J* = 7.2 Hz, 2H); 3.02 (t, *J* = 7.6 Hz, 2H); 3.68 (s, 3H); 7.46 (m, 3H); 8.06 (m, 2H).^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm): 21.4, 25.7, 32.8, 51.7, 126.8, 128.8, 131.1, 168.3, 172.9; 178.9. Elemental Analysis for $C_{13}H_{14}N_2O_3$: Calc. C, 63.40; H, 5.73; N, 11.38. Found C, 63.31; H, 6.06 ; N, 11.33.

Methyl 4-[3-(3,4-dichlorophenyl)-1,2,4-oxadiazol-5-yl]butanoate (**6b**): Yield: 62%, m.p. (^oC): - (oil). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 2.22 (q, *J* = 7.6 Hz, 6.8 Hz, 2H); 2.50 (t, *J* = 6.8 Hz, 2H); 3.03 (t, *J* = 7.6 Hz, 2H); 3.69 (s, 3H); 7.55 (d, *J* = 8.8 Hz, 1H); 7.90 (dd, *J* = 8.8 Hz, 1.6 Hz, 1H); 8.17 (d, *J* = 1.6 Hz, 1H).^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm): 21.6, 25.7, 32.7, 51.8, 126.4, 126.7, 129.3, 130.9, 133.3, 135.4, 166.7, 172.8; 179.5. Elemental Analysis for $C_{13}H_{12}Cl_2N_2O_3$: Calc. C, 49.54; H, 3.84; N, 8.89. Found C, 49.92; H, 4.04 ; N, 8.64.

4.2. Larvicide Activity Measurements

The larvicidal activity of the synthesized drugs was evaluated using an adaptation of the method recommended by the World Health Organization (WHO).[36] A stock solution (100 ppm) was prepared by diluting 0.005 g of test-compounds in 0,7 mL of ethanol, dimethylsulfoxide or acetone (analytical grade) or 2 drops of Tween 80, and completing to a volume of 50 mL with distilled water. In order to test the effect of the compounds on the larvae survival, fourth instar *A. aegypti* was added to the beakers (20 larvae per beaker) containing the synthesized compounds in a range of concentrations obtained by appropriate dilution of the stock solution with distilled water. Four replicate assays were carried out for every sample concentration. A negative control for each assay, using distilled water containing the same amount of co-solvent as the test sample has been used. Mortality of the larvae was determined after 48 h of incubation at 28±2 °C. Larvae were considered dead when no response to stimuli or not rising to the solution surface was observed. Lethal concentration (LC_{50}) values were calculated through probit analysis using the Status Plus 2006 software program.

4.2.1. Computational Calculations

Molecular docking calculations were performed for 22 oxadiazole derivatives against the X-ray structure of AgHKT and the comparative model of AeHKT (Figure 3). The programs AutoGrid v.4.0[37] and AutoDock v.4.0[38] were used during the calculations. Atomic coordinates of AgHKT crystal structure were retrieved from PDB (ID: 2CH2).[23] A dimer containing the cofactor N-pyridoxyl-lysine-5-monophosphate (PLP) linked to Lys205 side chain at the protein binding site was employed after removal of the co-crystallized inhibitor 4-(2-aminophenyl)-4-oxobutanoic acid (4-OB). Polar hydrogens were added to the protein considering amino acid protonation states at pH 7. C-terminal carbonyl oxygens were added when absent. Partial charges for protein atoms were assigned according to AMBER86 force field parameters,[39] while PLP and ligand charges were calculated with the Gasteiger method,[40] ensuring that all residues have presented integer charges. The AMBER86 atom types were assigned to all atoms.[39] In order to assess the predictive quality of our docking procedure, a validation methodology was devised aiming to show that the undertaken procedure is able to reproduce in detail the crystallographic structure of 4-OB in the active site of AgHKT, and generate affinity maps for heterocycle derivatives based on the ones achieved for the crystallographic inhibitor 4-OB. This procedure was performed in three steps: (1) *First simulation*, with all ligand dihedrals fixed, and 10 simulation steps; (2) *Second simulation*, with all ligand dihedrals free, except for the ones formed by ring-amine group and ring-carbonyl, and 10 simulation steps; (3) *Third simulation*, with all ligand dihedrals free (except for the ones formed by ring-amine group and ring-carbonyl), and the ligand being positioned out of the binding site, with 200 simulation steps. Then, ligand conformations resulting from these simulations were analyzed and compared to the crystallographic conformation. The

aforementioned computational procedure was able to successfully reproduce the crystallographic conformation (Fig. 3). A grid with dimensions of 28 Å x 28 Å x 28 Å and spacing of 0.22 Å was centered at the binding site of the enzyme. The binding modes for the oxadiazole derivatives in the active site of AgHKT were predicted based on the same affinity maps generated for compound 4-OB. For atom types in the heterocycle derivatives that were not present in 4-OB, additional affinity maps were calculated using the same settings as for previous affinity maps. During molecular docking calculations, binding energies between the protein and a given ligand configuration were determined based on potential energies stored as affinity maps. Lamarckian genetic algorithm was employed in all molecular dockings with the following parameters:[41, 42] an initial population of 150 individuals/ generation, a maximum number of 27,000 generations, and a total of 2.5×10^7 energy evaluation/generation. Thus, a total of 4.05×10^6 conformations for each of the ligands were generated in each of the 100 steps of the simulation. Mutation and crossover rates corresponded to 0.02 and 0.08, respectively. In the local search, 300 steps were applied with a 0.3 probability of search for one individual. Ligand conformations presenting the most favorable binding energy were selected in each step of the simulation, in such a way that, at the end of calculation, atomic coordinates of the 100 conformers that better fitted the binding site were selected. These conformers were structurally compared through their RMSDs, and clustered into groups of similar conformations. A tolerance of 2 Å for RMSD was employed to assign conformers to the same cluster.

Acknowledgments

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Supplementary Material

Supplementary data associated with this article can be found at http...

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