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1 **In Silico Chemical Library Screening and Experimental Validation**
2 **of Novel Compounds with Potential Varroacide Activities.**

3

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23 **Abstract**

24 The mite *Varroa destructor* is an ectoparasite and has been identified as a major cause of
25 worldwide honey bee colony losses. The use of yearly treatments for the control of varroosis is
26 the most common answer to prevent collapses of honey bee colonies due to the mite. However,
27 the number of effective acaricides is small and the mite tends to become resistant to these few
28 active molecules. In this study, we have been looking for a new original varroacide treatment
29 inhibiting selectively *Varroa destructor* AChE (*vdAChE*) with respect to *Apis mellifera* AChE
30 (*amAChE*). To do this an original drug design methodology was used applying virtual
31 screening of the CERMN chemolibrary, starting from a *vdAChE* homology sequence model.
32 By combining the *in silico* screening with *in vitro* experiments, two promising compounds were
33 found. *In vitro* tests of AChE inhibition for both species have confirmed good selectivity
34 toward the mite *vdAChE*. Moreover, an *in vivo* protocol was performed and highlighted a
35 varroacide activity without acute consequences on honey bee survival. The two compounds
36 discovered have the potential to become new drug leads for the development of new treatments
37 against the mite varroa. The method described here clearly shows the potential of a drug-design
38 approach to develop new solutions to safeguard honey bee health.

39

40

41 **Keywords:** varroa;

42 Acetylcholinesterase;

43 acaricide;

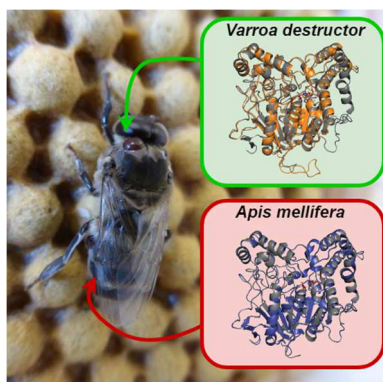
44 *in silico* screening,

45 docking;

46 honey bees.

47

48 **Graphical abstract**



49

50

51 **Highlights**

- 52 • This is the first study using a drug design approach to search for new compounds against
- 53 the mite *Varroa destructor*.
- 54 • A homology model of *vdAChE* was used to perform virtual docking screening.
- 55 • Inhibitory activities on honey bee and on varroa mite AChE enzymes were investigated.
- 56 • Varroacidal hits were confirmed through *in vivo* experiments.

57

58

59 **1. Introduction**

60 *Varroa destructor* (Acari: Varroidae) is a hemotophagous honey bee mite. It likely shifted from
61 its original host *Apis cerana* to the Western honey bee *Apis mellifera* in the first half of the
62 twentieth century [1]. Since then, varroa has spread worldwide, Australia remaining the only
63 continent free from this mite.

64 Varroa is known to be one of the main culprits for the disastrous colony losses that have been
65 reported in Europe and North America [2],[3]. The varroa mite has damaging physical and
66 physiological effects and may weaken bees' immunity, allowing pathogens, especially viruses,
67 to multiply unchecked [3]. In temperate climates, most of the colonies of Western honey bees
68 will be damaged or even collapse within a few years if no control or inappropriate control
69 methods are used [4]. Nowadays, beekeepers utilize a wide range of different chemical
70 substances, application techniques and methods to keep mite populations under control [5].
71 Chemical control with synthetic acaricides remains a crucial component of an integrated pest
72 management (IPM) of the mite varroa, but only a small number of compounds are suitable for
73 mite control. Synthetic acaricides such as fluvalinate, flumetrine, coumaphos, cymiazole and
74 amitraz have been successfully used to control varroa. Nevertheless, it has been observed that
75 organisms can develop resistance via behavioural changes (e.g. avoiding the pesticide), reduced
76 penetration, expression of certain detoxification enzymes, or target site desensitivation by
77 modifications of the active site [6]. The ability to develop resistance to a wide range of
78 pesticides is a widespread phenomenon among mites [7] and it is almost inevitable that varroa
79 becomes resistant against commonly used synthetic acaricides. It may in fact already be the
80 case as an efficacy decrease to several active ingredients used for its control has been reported
81 [8]. A way to limit this tolerance is to diversify the active molecules. Moreover, despite recent
82 results [9],[10], no new active compounds have been registered for more than 25 years [11].
83 Therefore, there is an urgent need to find new acaricide compounds.

84 According to the IRAC (Insecticide Resistance Action Committee) mode of action
85 classification scheme (www.iraac-online.org [12]), currently available insecticides and
86 acaricides can affect physiological functions such as nerve and muscle, growth and respiration.
87 Most current chemical compounds act on nerve and muscle targets such as sodium channel,
88 nicotinic acetylcholine receptors, octopamine receptors or acetylcholinesterase. The latter is the
89 target we focused upon in this study.

90 **Acetylcholinesterase (AChE, EC 3.1.1.7) plays a key role in cholinergic synapses in the insect**
91 **central nervous system. AChE rapidly hydrolyses the neurotransmitter acetylcholine in the**
92 **cholinergic system and terminates nerve impulses. Blockage of AChE leads to increased**
93 **acetylcholine levels, causing a continuous stimulation resulting in muscular dysfunction,**
94 **paralysis, and death. Due to its crucial role, AChE has been exploited as the target of**
95 **organophosphate and carbamate pesticides to control many arthropod pests [13],[14],[12]. The**
96 **overall structure of AChE is ellipsoidal and it belongs to the class of α/β proteins. It consists of**
97 **a 12-stranded central mixed β -sheet surrounded by 14 α -helices. The 3D structure of AChE has**
98 **revealed that, like other serine hydrolases, it contains a catalytic triad His, Ser, Glu [15]. This**
99 **triad is located at the bottom of a 20 Å deep cavity, named the "aromatic gorge" as about 40%**
100 **of its lining is composed of aromatic rings.**

101 In this study, we have been looking for a new original varroacide treatment inhibiting
102 selectively the *Varroa destructor* AChE (*vdAChE*) with respect to *Apis mellifera* AChE
103 (*amAChE*). **To do this, a structure-based virtual screening for drug discovery approach,**
104 **currently used in human health, was applied (see supporting information Figure 1). A virtual**
105 **screening of the CERMN chemolibrary, starting from *vdAChE* homology sequence model, was**
106 **carried out. This screening was based on the active site topology, using the docking approach.**
107 The varroacide ability of selected compounds from virtual screening was validated by *in vitro*
108 and *in vivo* biological tests.

109

110

111

112 **2. Material and methods**

113 **2.1. Library**

114 The chemical library (CERMN database, <http://www.cermn.unicaen.fr/>) contained at the date
115 of screening 9788 compounds resulting from different research programs carried out by the
116 CERMN laboratory in the field of medicinal chemistry. Standard tools of the ChemAxon
117 Package were used (<http://www.chemaxon.com/>) to generate a 3D structure for each putative
118 ligand as well as their ionization state at pH=7.4.

119

120 **2.2. Docking Screening**

121 In this study, the 3D model of *vdAChE* was generated using the homology sequence approach
122 as by Dulin *et al.* [9]. **To build this model, *vdAChE* sequence from the published genome
123 sequences of *Varroa destructor* was used (accession number BRL_Vdes_1.0 (genome size
124 294.13 Mb)) [16]. The 3D model was built using the crystal structure (PDB: 1DX4) of
125 *Drosophila melanogaster* AChE complex with tacrine [17]. **The first sequence alignment (28%
126 of sequence identity) was produced by the @TOME server [18] and then it was manually
127 optimized (Figure 1). The homology model was generated with the Modeller software [19].
128 The folding quality of the *vdAChE* model was estimated using Verify3D [20] and Eval23D
129 [21]. Qualitative Model Energy Analysis (QMEAN) score was also determined to estimate the
130 quality of the homology model [22]. QMEAN scoring function is based on the linear
131 combination of six structural descriptors and reflects the predicted global model reliability
132 ranging from 0 to 1.****

133 The model was next optimized by energy minimization and a quick molecular dynamic
134 simulation using CHARMM software version c40b2 [23] using all-atoms CHARMM 36 force
135 field [24]. The system was surrounded by a rectangular box of TIP3P water molecules [25] and
136 neutralized with 0.15 M KCl using CHARMMGUI solvator [26]. Periodic boundary conditions
137 were applied to the system using the IMAGE algorithm. Van der Waals interactions were
138 truncated using a switching function between 10 and 12 Å with a cut-off distance of 16 Å and
139 long-range electrostatic interactions were calculated with the particle-mesh Ewald (PME)
140 method [27]. The vacuum dielectric was constant. After an energy minimization, dynamics
141 simulation was performed using the Leapfrog Verlet algorithm with a 1 fs step. Systems were
142 gradually heated until 300 K, by 10 K jumps during 20 ps and then the dynamic was
143 temperature-equilibrated during 20 ps via heating reassignment under constant volume
144 conditions. Finally, the system ran freely for 6 ns under NVT ensemble using Hoover
145 thermostat. An average structure was generated from the trajectory and resubmitted to a quick
146 energy minimization to optimize its geometry. This minimized average structure was used in
147 the docking screening.

148 The Gold program v5.3 [28, 29] was employed to generate a docked conformation for each
149 library compound and calculate the fitness score to estimate its binding efficiency. This
150 program applies a genetic algorithm to explore conformational spaces and ligand binding
151 modes. Generally, the higher the fitness value calculated by the fitness function, the higher the
152 predicted affinity should be. Four different fitness functions are available in the program v5.3
153 and after the docking tests on *vdAChE*, GoldScore fitness function was selected for our
154 screening study and the docking was carried out using the default parameters. All compounds
155 were docked within a sphere of 6 Å radius around the previously positioned ligand, pirimicarb.
156 During the docking procedure side chains of three amino acids in the binding site were kept

157 flexible: Trp₁₁₅, Gln₁₉₁, Lys₂₇₇. For each ligand the best scoring position was selected for
158 analysis.

159

160 ***2.3. Ligand position optimization by Molecular dynamics simulations.***

161 To optimise the ligand position in the *vdAChE* binding site a molecular dynamic simulation
162 was carried out using NAMD 2.12 [30] with the all-atom CHARMM 36 forcefield for proteins
163 [24] and CGENFF for the synthetic ligands [31, 32]. To simulate aqueous solvent environment,
164 the ligand/*vdAChE* complex was surrounded by a rectangular box of TIP3P water molecules
165 [25] and 0.15 M of KCl as in model optimisation [26]. The chosen box size ensured, for each
166 complex, that the simulated complex was at a minimum distance of 10 Å from the edge.
167 Periodic boundary conditions were applied to the systems using the IMAGE algorithm. Van der
168 Waals interactions were truncated using a force switching function between 10 and 12 Å and
169 the PME approximation [27] was used to calculate long-range electrostatic interactions. The
170 SHAKE algorithm was applied to restrain all bonds involving hydrogen atoms [33] and the
171 vacuum dielectric constant was used during all calculations. The systems first underwent an
172 energy minimization in 10000 steps. Then the minimized systems were heated to 303.15 K and
173 the dynamics were temperature-equilibrated during 50 ps via heating reassignment under
174 constant volume conditions. Finally, the systems ran freely for 20 ns under NPT conditions.
175 Langevin dynamics with a damping coefficient of 1 ps⁻¹ was used to maintain the system
176 temperature and Nosé-Hover Langevin piston method to control the pressure at 1 atm. The
177 trajectories generated were taken for subsequent analysis using CHARMM program version
178 c40b2 [23].

179

180 ***2.4. In vitro tests of varroa and bee AChE biological activity***

181 Inhibitory capacity of selected compounds on *vdAChE* and *amAChE* enzymatic activity was
182 evaluated using an adapted spectrometric method of Ellman [34] in a 96 well plate.
183 Acetylthiocholine iodide and 5,5-dithiobis- (2-nitrobenzoic) acid (DTNB) were purchased from
184 Sigma Aldrich. Heads of bees or whole varroas were homogenized in Tris buffer (10 mM,
185 pH=7.0) containing 1 M of NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% (w/v)
186 Triton X-100.

187 In our procedure, in each well, 100 μ L of 0.3 mM DTNB dissolved in phosphate buffer pH 7.4
188 were added followed by 50 μ L of test compound solution (or 50 μ L of phosphate buffer for
189 control) and 50 μ L of enzyme solution. Initial absorbance was measured at 412 nm and used to
190 correct the final absorbance. After 5 min of preincubation, the reaction was initiated by the
191 injection of 50 μ L of 10 mM acetylthiocholine iodide solution. The hydrolysis of
192 acetylthiocholine was monitored by the formation of a yellow 5-thio-2-nitrobenzoate anion as
193 the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of
194 acetylthiocholine, at a wavelength of 412 nm every minute for 30 min using a 96-well
195 microplate plate reader (TECAN Infinite M200, Lyon, France). Test compounds were
196 dissolved in analytical grade DMSO. Absorbance at 30 minutes (or at 10 minutes for honey bee
197 samples) was used to evaluate the inhibition activity.

198 First screening of AChE activity was carried out at a 10^{-4} M concentration of selected
199 compounds, with carbamate formetanate hydrochloride (10^{-4} M) used as positive control [9].
200 For compounds with significant inhibition at 10^{-4} M (> 50%), IC_{50} (concentration required to
201 inhibit 50% of AChE activity) absorbance values were determined for a range of 10^{-3} - 10^{-10} M
202 concentrations. IC_{50} values were calculated by fitting the response points to nonlinear fit-log
203 inhibitor concentration curve using GraphPad Prism 7.0 Software (GraphPad Software, San
204 Diego, CA, USA).

205

206

207 **2.5. Laboratory bioassays**

208 *In vivo* tests were carried out in France (46°26'13.3"N 1°19'37.1"E). Introduction of honey bees
209 and varroa mites in cages allowed us to evaluate the acute toxicity of the compounds
210 simultaneously for both species. Phoretic varroas were collected the day of the experiment,
211 using a modified icing sugar-shake method [35]. About 200 to 300 honey bees were collected
212 using a varroa-counter jar (Swienty A/S). Approximately 30 g of powdered sugar (CristalCo,
213 Daddy, France) was added and the jar gently rolled, to distribute the sugar, and then inverted
214 and shaken for 1 to 3 minutes. This sugar shake method allows to separate varroas from honey
215 bees without harming bees or mites. In parallel, workers bees of unknown age were collected
216 using a ventilated transparent container, in colonies free from clinical symptoms of diseases
217 and without any varroacide treatment for six months.

218 Immediately upon arrival from the field, within one hour after collection, varroas were gently
219 brushed away from the sugar and kept on moist paper at room temperature until use. Inactive or
220 moribund mites were discarded.

221 Prior to treatment, bees were anesthetized with carbon dioxide. A 3 L per hour carbon dioxide
222 flow for 20 seconds, followed by 2 minutes in a hermetic container, allowed sufficient
223 anaesthesia of honey bees. After anesthetization, each honey bee was topically exposed to the
224 studied molecules by depositing 1 μ L drop of selected compound dissolved in acetone. To
225 solubilize both sr2091 ((3aR)-4-(dimethylamino)-3,3a-dihydro-1H-thiazolo[4,3-
226 c][1,4]benzodiazepine-10-thione, 98% purity) and sr2093 (4-[2-(dimethylamino)ethylamino]-
227 3,3a-dihydro-1H-thiazolo[4,3-c][1,4]benzodiazepine-10-thione, 80% purity), the addition of
228 HCl was necessary (1 mole per mole of active substance). Solutions were vortexed vigorously
229 before use. Before honey bees became active again, 10 varroa were deposited on their
230 cephalothorax, then bees and varroa were immediately transferred into cages. The insects were

231 given approximately 15min to recover from the carbon dioxide treatment, and any dead bees
232 were removed from the experiment. Solvent controls received 1 μ l of acetone only, negative
233 controls received no treatment and as positive control we applied a widely used varroacide
234 molecule amitraz, at a concentration of 0.1 μ g per honey bee. Amitraz targets octopamine
235 receptors, not AChE, but it is effective on varroa mites without causing acute damage to honey
236 bees. Any experiment with negative control mortality higher than 30% was discarded from the
237 analysis.

238 Single-use cages consisted of 1 oz cups made of food grade and clear plastic. Approximately 60
239 holes were made into the side and lid of the plastic cups. Bees were provided ad libitum a 50%
240 (weight/volume) sucrose solution made by dissolving refined sugar in water. The syrup was
241 distributed through a pierced 1.5 mL Eppendorf tube, with one tube fixed in each cage lid.

242 Cages were maintained in darkness at room temperature, and mortality of honeybees and
243 varroas was assessed 24h after treatments. At the end of these experiments, all cages were
244 placed at -20 °C to kill honeybees, to assess the exact number of varroas in each cage and take
245 in account any natural varroa infestation in the calculation of the mite's mortality rate.

246 A generalized linear model [36] with binomial distribution was used to compare the mortality
247 of each group with the negative control group mortality. Post hoc comparison was conducted
248 using the function *glht*, from the package "multcomp" to test for differences between all pairs
249 of treatments. Analyses were performed using the statistical software R version 3.3.2 (R Core
250 Team 2016).

251

252 **3. Results**

253 **3.1. CERMN chemolibrary screening**

254 In order to find new and original selective inhibitors of *vdAChE*, virtual screening of the
255 CERMN chemolibrary was carried out. Structure-based screening using a docking approach
256 was applied to the *vdAChE* homology model built using the *vdAChE* sequence from the varroa

257 genome [16] on a *dmAChE* template. The insertions/deletions (1 to 16 amino acids, Figure 1)
258 were identified in the sequence alignment. These were however located in neighboring surface
259 loops and have no effects on the secondary structure elements. The *vdAChE* homology model
260 generated presented a root-mean-square (RMS) difference of 1.012Å (C α atoms) from the
261 *dmAChE* X-ray structure, taking into account all intracellular and extracellular loops. A
262 QMEAN score of 0.62 (>0.50) for our model suggested that the homology model built was
263 reliable.

264 Before docking, the *vdAChE* homology model was submitted to energy minimization and
265 quick molecular dynamics simulation at 300 K (6 ns) under NVT conditions. An average
266 structure was generated from the trajectory and resubmitted to a quick energy minimization to
267 optimize side chain geometry.

268 Docking screening was carried out using the GOLD (Genetic Optimisation for Ligand
269 Docking, [29]) program on this optimized average structure. GOLD is an automated ligand
270 docking program that uses a genetic algorithm. **GOLD's evolutionary algorithm modifies the**
271 **position, orientation and conformation of a ligand to fit into one or more low energy states of**
272 **the protein active site. It maps ligand geometry parameters onto populations of chromosomes**
273 **and then runs evolutionary rounds of mutation, crossover, scoring and selection to optimise**
274 **protein-ligand interactions. For solution selection, we applied the GoldScore score function and**
275 **we kept the three binding site residues flexible, Trp₁₁₅, Gln₁₉₁, Lys₂₇₇.** The value of the
276 GoldScore gives a guide as to how good the pose is: the higher the score, the better the docking
277 result is likely to be. **The 3D structure for each compound from the CERMN chemical library**
278 **(9788 compounds) was generated at its ionization state at pH=7.4 and docked into the *vdAChE***
279 **binding site. For each compound the best pose, with the highest score, was retained. The 1000**
280 **compounds representing the highest GoldScore values (varying from 99.77 to 66.69) from the**
281 **total of 9788 in the CERMN chemolibrary were selected for subsequent analysis. We have**

282 observed that the elongated shape of the AChE binding site causes the GOLD software to
283 overestimate the Goldscore value for compounds with high molecular weights. The molecular
284 weights among the 1000 selected compounds varied from 175.23 to 830.84 g·mol⁻¹. In order to
285 **focus** on moderate molecular weight compounds, which are easier to pharmacomodulate, a
286 threshold of 350 g·mol⁻¹ was applied on the selected 1000 compounds. We chose this value
287 with reference to the molecular weight of pirimicarb (238.29 g·mol⁻¹), a selective carbamate
288 insecticide which has shown good affinity and selectivity for *vdAChE* in our previous studies
289 [9, 37]. Among the 1000 compounds, 215 had a smaller molecular weight than 350 g·mol⁻¹. To
290 take into account the coherence between GoldScore and molecular weight, the GoldScore value
291 was divided by the molecular weight [38, 39] and the 53 compounds with the highest
292 GoldScore/Molecular Weight ratio were selected for biological evaluation. From the 53
293 selected compounds, 46 were evaluated at 1·10⁻⁴ M ligand concentration by *in vitro* biological
294 tests with the aim to detect *vdAChE* inhibition potency. Among the tested compounds, four had
295 an inhibition activity higher than 20% and one close to 60% (see Table 1). As the goal of this
296 screening was to find compounds able to inhibit *vdAChE* and not *amAChE*, not to compromise
297 the health of bees, inhibition activity of *amAChE* was also evaluated. Among the three
298 compounds with lesser inhibition potency (about 20%), sr2114 and sr2112 inhibited *amAChE*
299 in the same way, while sr2090 inhibited *amAChE* very weakly. Compound sr2091 [40], which
300 had the highest *vdAChE* inhibitor potency (59%) among the screened compounds, had at the
301 same time a weak *amAChE* inhibition activity as desired (Table 1). Therefore, this compound
302 presents the desired properties for a potentially new acaricide.

303 As the four compounds emerged from our screening have a tricyclic structure including a
304 benzodiazepine ring, we assessed a larger part of the chemical space by *in vitro* tests on the
305 whole chemical family related to these compounds [40], to ensure better understanding of the
306 structure activity relationship (SAR). The *vdAChE* inhibition potency of 25 additional

307 compounds of this chemical family was measured (Table 2). Three additional compounds with
308 *vdAChE* inhibition potency greater than 20% were discovered, of which one had inhibition
309 activity of 53% (sr2093). All additional compounds tested presented small *amAChE* inhibition.
310 Therefore, among the tested compounds, in addition to sr2091, sr2093 has also come out as a
311 potential acaricide (Table 2). For both compounds, the IC_{50} for *vdAChE* inhibition was
312 determined (see Table 3). The IC_{50} values obtained for sr2091 and sr2093 were respectively 16
313 $\pm 12 \mu\text{M}$ and $11 \pm 6 \mu\text{M}$, and they are of the same order as the IC_{50} of pirimicarb ($IC_{50}=6.6$
314 $\pm 0.8 \mu\text{M}$), another potential varroacide compound targeting *vdAChE* [9].

315

316 **3.2. Laboratory bioassays**

317 To confirm the *in silico* and *in vitro* hypotheses, the toxicity of sr2091 and sr2093 was assessed
318 using cage tests. This device allows the evaluation of acute toxicity of compounds
319 simultaneously for both species. Observed mean mortalities (\pm se) for both species are shown in
320 Table 4. There was no honey bee mortality detected for any tested conditions. The varroa mean
321 mortality in the negative control group was 8.6%. A solvent-control test was also carried out
322 and no statistical difference with the negative control mortality was observed. The compounds
323 sr2091 and sr2093 were effective at 87% ($p<0.001$) and 82% ($p<0.001$) respectively at an
324 application of 10 μg per bee. However, there was no significant mortality of varroa mites when
325 the deposit was reduced to 1 μg per bee ($p=0.88$ and $p=0.50$ for sr2091 and sr2093,
326 respectively). In conclusion, a significant mortality of the mites was observed when 10 μg of
327 both sr compounds was applied, and no effect was observed on honey bee survival.

328

329 **3.3. sr2091/*vdAChE* and sr2093/*vdAChE* complexes optimized by Molecular Dynamic** 330 **Simulation**

331 To analyze in detail the fixation mode of sr2091 and sr2093 in the binding site of *vdAChE*, the
332 initial result from screening was optimized by a molecular dynamic simulation. The interaction
333 energy sr2091/*vdAChE* and sr2093/*vdAChE* along the trajectory was calculated and for each
334 complex studied the one with the strongest interaction energy was saved for subsequent
335 analysis (Figure 2). To investigate the sr2091 interaction mode in the *vdAChE* binding cavity,
336 the interaction energies atom per atom with whole *vdAChE* were calculated (Figure 3A, 3B).
337 Analyses highlighted as crucial atoms for compound binding: the sulphur and one nitrogen
338 atom of the thiazolodiazepine ring (S4 and N21) and the sulphur of the thiolactame group
339 (S10). Sulphur atoms interact principally through van der Walls interactions, while the
340 interaction of N21 with *vdAChE* binding site is of an electrostatic type.

341 In parallel, interaction energies per *vdAChE* residue with whole sr2091 were calculated and
342 they highlighted as key residues for sr2091 fixation: Lys277, Ala112, Gly111, Trp115, Arg62
343 and His432 (see Figure 2C). Visualization of the sr2091/*vdAChE* complex showed that Lys277
344 and His432 side chains are close to nitrogen atom N21 of the thiazolodiazepine ring, N21
345 distance from Lys277 N ζ is about 3.7 Å and from His432 N δ 1 about 3.7 Å (Figure 2). The
346 Lys277 N ζ is also spatially close to sulphur atom S10 of the thiolactame group, $d_{S10-N\zeta}=3.5$ Å
347 (Figure 2). The thiazolo diazepine ring sulphur atom S4 is situated in the proximity of Arg62
348 side chain, the distance between S4 and Arg N ϵ atom is about 3.4 Å. The hydrophobic residues
349 Gly111, Ala112 and Trp115 were also suggested in our analysis as important for sr2091
350 fixation and their interactions are principally of the van der Walls type.

351

352

353 **4. Discussion**

354 In this study, we carried out a virtual screening of the CERMN chemolibrary, containing 9788
355 compounds, on the 3D homology sequence model of *vdAChE* with the aim of selecting a new

356 potential effective varroacide. A post processing treatment was applied on the thousands of
357 selected potential *vdAChE* inhibitors by docking screening. The ACh binding cavity in
358 *vdAChE* is elongated, with a second cavity entrance, and therefore the docking screening
359 selected mainly rather bulky compounds (MW ranging from 175.23 to 830.84 g·mol⁻¹). In order
360 to focus our screening on moderate molecular weight compounds we have carried out post-
361 processing based on the molecular weight of the compound as discussed previously by Pan *et*
362 *al.* [38] and by Jhoti and Leach[39] and we have limited our selection to 53 compounds (MW ~
363 175.23 - 300.9 g·mol⁻¹).

364 Among the 46 compounds evaluated *in vitro* from the 53 virtually selected, four have shown an
365 inhibition activity toward *vdAChE* higher than 20% at 10⁻⁴ M ligand concentration. As these
366 four molecules were structurally related, all members of this compound family have also been
367 tested *in vitro*. From the *in vitro* screening, sr2091 and sr2093 came out as potential
368 varroacides, with IC₅₀ for *vdAChE* at the ten micromolar range and they have presented only
369 weak *amAChE* inhibition. As the purpose of new varroacide discovery is to find compounds
370 inhibiting selectively *vdAChE* without altering honey bee health, the compounds highlighted
371 during this screening were interesting and their acute toxicity for both species was studied.

372 During the laboratory acute toxicity biassays, a significant mortality of the mites, but not of
373 honey bees, was observed when 10 µg of each sr compound was applied. Unlike amitraz, they
374 were not effective when applied at 1 µg per bee. Differences in efficacy have already been
375 observed by Papachristoforou *et al.* for different treatments against varroa [41]. A topical
376 application of 1.81 µg/mite of amitraz completely inhibited a varroa mite's gravitational reflex,
377 while the volatile acaricides formic acid, thymol crystals, and Apiguard® eliminated this
378 reflex for doses of 13.83, 250 and 1000 mg/mite, respectively. Therefore, in our experiment,
379 the gap between effective doses should not be considered as an eliminatory criterion. It was for
380 instance proposed that acaricide treatments, that cause more than 70% mite mortality, with less

381 than 30% bee kill, should be considered as mite selective and acceptable for mite management
382 [42]. Virtual screening of the CERMN chemolibrary has identified a new compound family
383 inhibiting *vdAChE* at micromole concentrations and inhibiting *amAChE* only weakly. This new
384 compound family is structurally different compared to other acaricides available on the market
385 which could delay the appearance of resistances. Furthermore the sr family members are
386 reversible AChE inhibitors which should guarantee fewer side effects.

387 Even if the sr family varroacide activity is rather moderate, the sr compounds discovered can
388 serve as a lead for future optimization programs to generate new original varroacide
389 compounds without effects on the honey bee. The *in vitro* screening on the whole chemical
390 family allowed the determination of chemical groups responsible for *vdAChE* inhibition
391 activity (Table 1 and Table 2). Firstly, both the sulphur atoms of the thiazolodiazepine ring and
392 the thiolactame function are necessary for *vdAChE* inhibition. We observed that the
393 replacement of the thiolactame function by a lactame function (entry 4 of Table 1 compared
394 with entry 20 of Table 2) led to a loss of *vdAChE* inhibition and that the replacement of the
395 sulphur atom of the thiazolodiazepine ring by a carbon atom decreases drastically the inhibition
396 activity on *vdAChE*, from 60% to 20% (Table 1, entries 3 and 4) and from 63% to 0% (Table 2
397 entry 13 and 21). Our *in vitro* structure-activity analysis on the sr family did not allow us to
398 evaluate the importance of the two nitrogen atoms in the tricycle, since in all tested compounds
399 these two nitrogen atoms were present. Nevertheless, we could evaluate the influence of the
400 nature of the substituent on the nitrogen atom in the aliphatic chain anchored on the tricyclic
401 core on *vdAChE* inhibition: the replacement of the aliphatic chain by cyclic or aromatic
402 substituents led to inactive, or less active, compounds (Table 1 entry 4 compared with Table 2
403 entries 10 and 22). The presence of a basic centre in the aliphatic chain led generally to a better
404 inhibition potency, and the best results were obtained with compounds possessing an aliphatic
405 chain with a basic centre (Table 1, entry 4 and Table 2, entry 21). In summary, the presence of

406 sulphur atom on the thiazolodiazepine ring and on the thiolactame function is necessary for
407 *vdAChE* inhibition and the presence of nitrogen in the aliphatic chain is desirable.

408 To obtain a better understanding of the fixation mode of sr2091 and sr2093, molecular
409 dynamics simulations were carried out on the complexes with *vdAChE* built by docking. The
410 calculated interaction energy per atom for sr2091 highlighted the crucial role of three atoms in
411 sr binding to *vdAChE*, two sulphur atoms and nitrogen N21 in the thiazolodiazepine ring.
412 Molecular dynamics results have shown that sr compounds reach the bottom of the *vdAChE*
413 binding groove and interact directly through nitrogen N21 of the thiazolodiazepine ring with
414 His432 a residue of the catalytic triad. The sr compounds also established electrostatic
415 interactions with two polar residues, Lys277 (the highest contribution) and Arg62. The Lys227
416 nitrogen is spatially close to nitrogen N21 of the thiazolodiazepine ring and sulphur S10 of
417 thiolactame, while Arg62 interacts with sulphur S4 of the thiazolodiazepine ring (Figure 2).
418 This result is in agreement with our observations on structure activity relation from *in vitro*
419 tests on the sr family. We concluded from *in vitro* test results that the presence of both sulphur
420 atoms is necessary for good *vdAChE* inhibition activity. Nevertheless, *in vitro* tests did not
421 allow us to evaluate experimentally the importance of both nitrogen atoms in the thiazolo
422 diazepine ring, as in all tested compounds these two nitrogen atoms were present. From the
423 modelling results, nitrogen in position N21 plays a more important role for sr2091 binding than
424 nitrogen in position N8, as it mainly participates in the electrostatic contacts with Lys277.
425 Interestingly, sequence alignments of *vdAChE* with *amAChE* (Supporting Information, Figure
426 S2) showed that these residues are not conserved in *amAChE*. They are substituted by two
427 hydrophobic residues in *amAChE*, a Leu and a Trp, respectively, which could explain the poor
428 *amAChE* inhibition activity of sr compounds compared to *vdAChE*.

429

430

431 **5. Conclusion**

432 With the aim to improve the diversity of varroa treatment, we have performed a computer aided
433 drug-design approach, currently used for human health. This is the first time that this approach
434 is developed for honeybee health.

435 The virtual screening of a chemolibrary, using a protein–ligand docking protocol, carried out
436 on the *vdAChE* homology model, has identified a new, chemically original, compound family
437 inhibiting *vdAChE*, with the best inhibition activity at a micromole concentration. Importantly,
438 the discovered family inhibits *amAChE* only weakly. The structure-activity and molecular
439 modelling analysis showed that the presence of both sulphur atoms and of two nitrogen atoms
440 is crucial for sr compounds fixation. The modelling results suggested that sr compounds reach
441 the bottom of the binding groove and interact directly with the histidine of the catalytic triad.
442 From modelling results, two polar residues, Lys277 and Arg62, appeared overriding for sr
443 compound binding to *vdAChE*. These two residues are replaced by hydrophobic ones in
444 *amAChE*, which could explain the selectivity of sr compounds toward the mite. Biologically,
445 these two compounds have the desired properties for a potentially new acaricide and they can
446 serve as a lead for future optimization programs to generate new varroacid compounds without
447 effects on the honey bee. Our results represent the first step towards the development of a new
448 veterinary product. More field tests are still required to assess, inter alia, any sublethal and long-
449 term side effects on adult bees and honey bee brood, and confirm the varroacide efficiency.
450 However, the method described here clearly shows the potential of a drug-design approach to
451 develop new solutions to safeguard honey bee health.

452

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460

461 **Compliance with Ethical Standards**

462 C. Riva has been co-funded by Normandy County Council and Vétô-pharma.

463 The authors declare that they have no conflict of interest.

464 All applicable international, national, and/or institutional guidelines for the care and use of
465 animals were followed.

466

467

References

- [1] Oldroyd BP. Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honeybees. *Trends Ecol Evol.* 1999;14:312-5.
- [2] Dahle B. The role of *Varroa destructor* for honey bee colony losses in Norway. *J Apic Res.* 2010;49:124–5.
- [3] De Guzman LI, Rinderer TE. Identification and comparison of *Varroa* species infesting honey bees. *Apidologie.* 1999;30:85–96.
- [4] Boecking O, Genersch E. Varroosis—the ongoing crisis in bee keeping. *J Für Verbraucherschutz Leb.* 2008;3:221–8.
- [5] Rosenkranz P, Aumeier P, Ziegelmann B. Biology and control of *Varroa destructor*. *J Invertebr Pathol.* 2010;103:S96–S119.
- [6] Sammataro D, Untalan P, Guerrero F, Finley J. The resistance of varroa mites (Acari: Varroidae) to acaricides and the presence of esterase. *Int J Acarol.* 2005;31:67–74.
- [7] Croft BA, Van de Baan HE. Ecological and genetic factors influencing evolution of pesticide resistance in tetranychid and phytoseiid mites. *Exp Appl Acarol.* 1988;4:277–300.
- [8] Milani N. The resistance of *Varroa jacobsoni* Oud. to acaricides. 1999.
- [9] Dulin F, Zatylny-Gaudin C, Ballandonne C, Guillet B, Bonafos R, Bureau R, et al. Protecting honey bees: identification of a new varroicide by in silico, in vitro, and in vivo studies. *Paras Res.* 2014;113:4601-10.
- [10] Ziegelmann B, Abele E, Hannus S, Beitzinger M, Berg S, Rosenkranz P. Lithium chloride effectively kills the honey bee parasite *Varroa destructor* by a systemic mode of action. *Scientific Reports* 2018;8:683 doi:10.1038/s41598-017-19137-5.
- [11] Mutinelli F. Veterinary medicinal products to control *Varroa destructor* in honey bee colonies (*Apis mellifera*) and related EU legislation-An update. *J Apicult Res.* 2016;55:78–88.
- [12] Sparks TC, Nauen R. IRAC: Mode of action classification and insecticide resistance management. *Pesticide Biochemistry and Physiology.* 2015;121 122-8.
- [13] Casida JE, Durkin KA. Anticholinesterase insecticide retrospective. *Chem Biol Interact.* 2013;203:221–5.
- [14] Van Leeuwen T, Tirry L, Yamamoto A, Nauen R, Dermauw W. The economic importance of acaricides in the control of phytophagous mites and an update on recent acaricide mode of action research. *Pestic Biochem Physiol.* 2015;121:12–21.
- [15] Sussman JL, Harel M. Atomic structure of acetylcholinesterase from *Torpedo californica*: a prototypic acetylcholine-binding protein. *Science.* 1991;253:872.
- [16] Cornman SR, Schatz MC, Johnston SJ, Chen Y-P, Pettis J, Hunt G, et al. Genomic survey of the ectoparasitic mite *Varroa destructor*, a major pest of the honey bee *Apis mellifera*. *BMC Genomics.* 2010;11:602:602.
- [17] Harel M, Kryger G, Rosenberry TL, Mallender WD, Lewis T, Fletcher RJ, et al. Three-dimensional structures of *Drosophila melanogaster* acetylcholinesterase and of its complexes with two potent inhibitors. *Protein Sci.* 2000;9:1063–72.
- [18] Pons JL, Labesse G. @TOME-2: a new pipeline for comparative modeling of protein-ligand complexes. *Nucleic Acids Res* 2009;37:W485–W91.
- [19] Eswar N, Eramian D, Webb B, Shen MY, Sali A. Protein structure modeling with MODELLER. *Methods Mol Biol.* 2008;426:145–59.
- [20] Eisenberg D, Luthy R, Bowie JU. VERIFY3D: assessment of protein models with three-dimensional profiles. *Methods Enzymol.* 1997;277 396–404.

- [21] Gracy J, Chiche L, Sallantin J, (1993). Improved alignment of weakly homologous protein sequences using structural information *Protein Eng.* 1993;6:821–9.
- [22] Benkert P, Tosatto SCE, Schomburg D. QMEAN: a comprehensive scoring function for model quality assessment *Proteins.* 2008;71:261–77.
- [23] Brooks BR, Brooks CL, MacKerell AD, Nilsson L, Petrella RJ, Roux B, et al. CHARMM: The Biomolecular Simulation Program. *J Comput Chem.* 2009;30:1545-614.
- [24] Best RB, Zhu X, Shim J, Lopes PEM, Mittal J, Feig M, et al. Optimization of the Additive CHARMM All-Atom Protein Force Field Targeting Improved Sampling of the Backbone ϕ , ψ and Side-Chain χ_1 and χ_2 Dihedral Angles. *J Chem Theory Comput.* 2012;8:3257-73.
- [25] Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. *J Chem Phys.* 1983;79:926-35.
- [26] Jo S, Kim T, Iyer VG, Im W. CHARMM-GUI: a web-based graphical user interface for CHARMM. *J Comput Chem* 2008;29:1859-65.
- [27] Darden T, York DM, Pedersen LG. *J Chem Phys.* 1993;98:10089.
- [28] Jones G, Willett P, Glen RC. Molecular recognition of receptor sites using a genetic algorithm with a description of desolvation. *J Mol Biol.* 1995;245:43-53.
- [29] Jones G, Willett P, Glen RC, Leach AR, Taylor R. Development and Validation of a Genetic Algorithm for Flexible Docking. *J Mol Biol.* 1997;267:727-48.
- [30] Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, Villa E, et al. Scalable Molecular Dynamics with NAMD. *J Comput Chem* 2005;26:1781-802.
- [31] Vanommeslaeghe K, Hatcher E, Acharya C, Kundu S, Zhong S, Shim J, et al. CHARMM General Force Field: A Force field for Drug-Like Molecules Compatible with the CHARMM All-Atom Additive Biological Force Field. *J Comput Chem.* 2010;31:671-90.
- [32] Yu W, He X, Vanommeslaeghe K, MacKerell Jr AD. Extension of the CHARMM General Force Field to Sulfonyl-Containing Compounds and Its Utility in Biomolecular Simulations. *J Comput Chem* 2012;33:2451-68.
- [33] Ryckaert JP, Ciccotti G, Berendsen HJC. Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes, *J Comput Phys.* 1977;23:327-34.
- [34] Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol.* 1961;8:88-90 IN1, 1–5.
- [35] Fakhimzadeh K. Effectiveness of confectioner sugar dusting to knock down Varroa destructor from adult honey bees in laboratory trials. *Apidologie.* 2001;32:139–48.
- [36] McCullagh P, Nelder JA. *Generalized Linear Models.* 2nd ed. ed: London: Chapman and Hall; 1989.
- [37] Riva C, Sokolowski M, Normand J, Sopkova-de Oliveira Santos J, Halm-Lemeille MP. Effect of oral exposure to the acaricide pirimicarb, a new varroacide candidate, on *Apis mellifera* feeding rate. *Pest Manag Sci.* 2018;doi: 10.1002/ps.4876.
- [38] Pan YP, Huang N, Cho S, MacKerell AD, MacKerell ADJ. Consideration of molecular weight during compound selection in virtual target-based database screening. *J Chem Inf Comput Sci.* 2003;43.
- [39] Jhoti H, Leach A. *Structure-based drug discovery:* Springer; 2007.
- [40] Gillard A-C, Rault S, Boulouard M, Robba M. Thiazolo[4,3-c] [1,4]Benzodiazepines. I. Synthesis of Amidine Derivatives. *J Heterocyclic Chem.* 1995;32:1741-5.
- [41] Papachristoforou A, Papaefthimiou C, Zafeiridou G, Goundy V, Watkins M, Theophilidis G. Monitoring the gravitational reflex of the ectoparasitic mite *Varroa Destructor*. A novel bioassay for assessing toxic effects of acaricides. *Pest Biochem and Physiol.* 2011;101:109-17.

[42] Lindberg CM, Melathopoulos AP, Winston ML. Laboratory evaluation of miticides to control *Varroa jacobsoni* (acari: Varroidae), a honey bee (hymenoptera: Apidae) parasite. J Econ Entomol 2000;93:189–98.

Figure captions

Figure 1. Sequences alignment between *dmAChE* and *vdAChE* used for the model building. β -strands are represented by orange arrows and α -helix by green ones. Amino acids of the catalytic triad are marked by an asterisk.

Figure 2. Sr2091 (A) and sr2093 (B) position in *vdAChE* binding sites after the optimization by molecular dynamic simulation. The compounds and the selected side chains of the binding site residues are in stick and the protein in ribbon representation. This figure was made with PYMOL (DeLano Scientific, 2002, San Carlo, USA).

Figure 3. (A) sr2091 compound formula (B) Calculated interaction energy per sr2091 atom with *vdAChE* (C) Calculated interaction energy per residue of *vdAChE* with sr2091.

Table 1. Best Compounds from the Docking Virtual Screenings

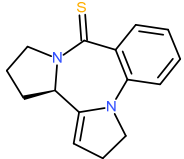
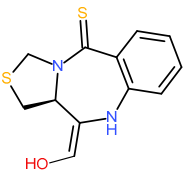
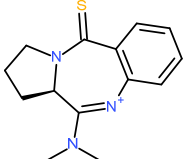
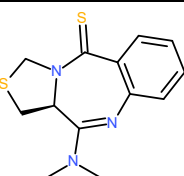
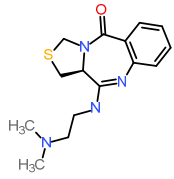
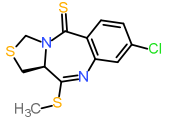
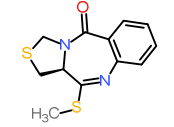
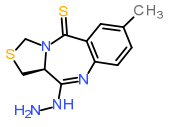
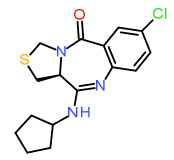
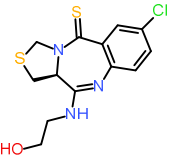
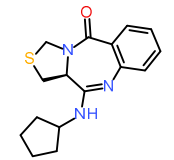
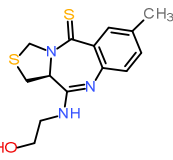
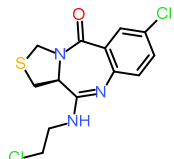
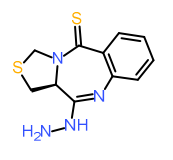
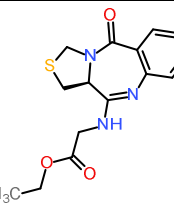
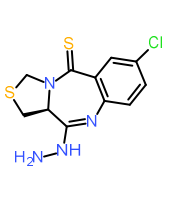
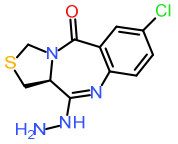
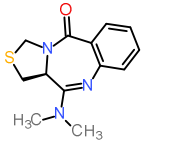
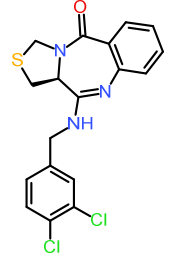
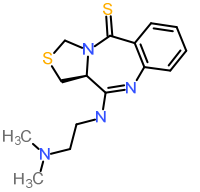
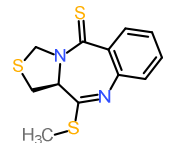
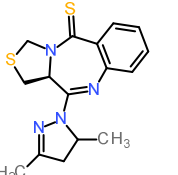
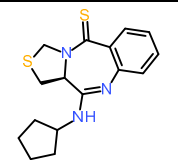
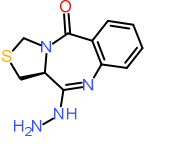
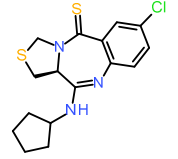
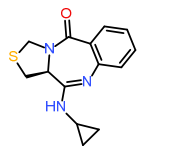
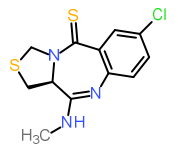
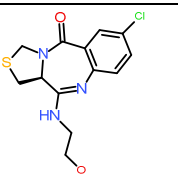
	Compound Name	Compound diagramme	Molecular Weight [g/mol]	Gold Score Fit	Gold Score/MW	vdAChE inhibition	amAChE inhibition
1	sr2114		257.35	71.67	0.2785	29%	18%
2	sr2090		265.35	73.37	0.2765	21%	5%
3	sr2112		260.38	71.92	0.2762	22%	25%
4	sr2091		277.41	73.44	0.2647	59%	21%

Table 2. Results of *in vitro* screening on sr2091 chemical family

	Compound Name	Compound diagramme	vdAChE inhibition	amAChE inhibition		Compound Name	Compound diagramme	vdAChE inhibition	amAChE inhibition
1	mr19199		5%	0%	14	sr2021		5%	0%
2	sr1975		5%	0%	15	sr2083		3%	0%
3	sr1982		4%	0%	16	sr2085		13%	0%
4	sr1984		3%	0%	17	sr2086		19%	1%
5	sr1986		4%	0%	18	sr2088		5%	0%
6	sr1989		1%	0%	19	sr2089		4%	0%

7	sr1990		3%	4%	20	sr2092		3%	0%
8	sr1993		12%	0%	21	sr2093		63%	15%
9	sr2020		20%	0%	22	sr2097		5%	0%
10	sr2099		6%	0%	23	mr17862		2%	0%
11	sr2100		4%	0%	24	mr17861		3%	0%
12	sr2102		5%	0%	25	mr19924		2%	0%

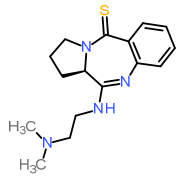
13	sr2109	 <chem>CN(C)CCN1C(=N2C(=S1)C=CC=C2)C3CCCN3</chem>	0%	5%
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Table 3. Inhibition percentage (\pm standard error) and IC₅₀ (\pm standard error) values for two best compounds from the screenings (n=3)

		amAChE	vdAChE
sr2091	Inhibition (10 ⁻⁴ M)	21% (\pm 0.06)	59% (\pm 0.07)
	IC ₅₀ (μ M)	ND	16 (\pm 12)
sr2093	Inhibition (10 ⁻⁴ M)	15% (\pm 0.02)	63% (\pm 0.06)
	IC ₅₀ (μ M)	ND	11 (\pm 6)
Formetanate (control)	Inhibition (10 ⁻⁴ M)	97% (\pm 0.005)	84% (\pm 0.04)

Table 4. Results of *in vivo* tests. Data correspond to six replicates, each carried out with 10 honey bees and 10 varroas.

Compound		Mortality^a	
		Honey bee	Varroa
sr2091	10 μ g	0%	86.7% (\pm 3.3)*
	1 μ g	0%	14.2% (\pm 1.1)
sr2093	10 μ g	0%	81.7% (\pm 2.1)*
	1 μ g	0%	19.1% (\pm 1.0)
Acetone control		0%	9.6% (\pm 0.9)
Amitraze control	1 μ g	0%	100%*

^a percent mean mortality (\pm standard error) * indicate significant difference determined by post-hoc test (* p-value < 0.01)

FIGURE 1.

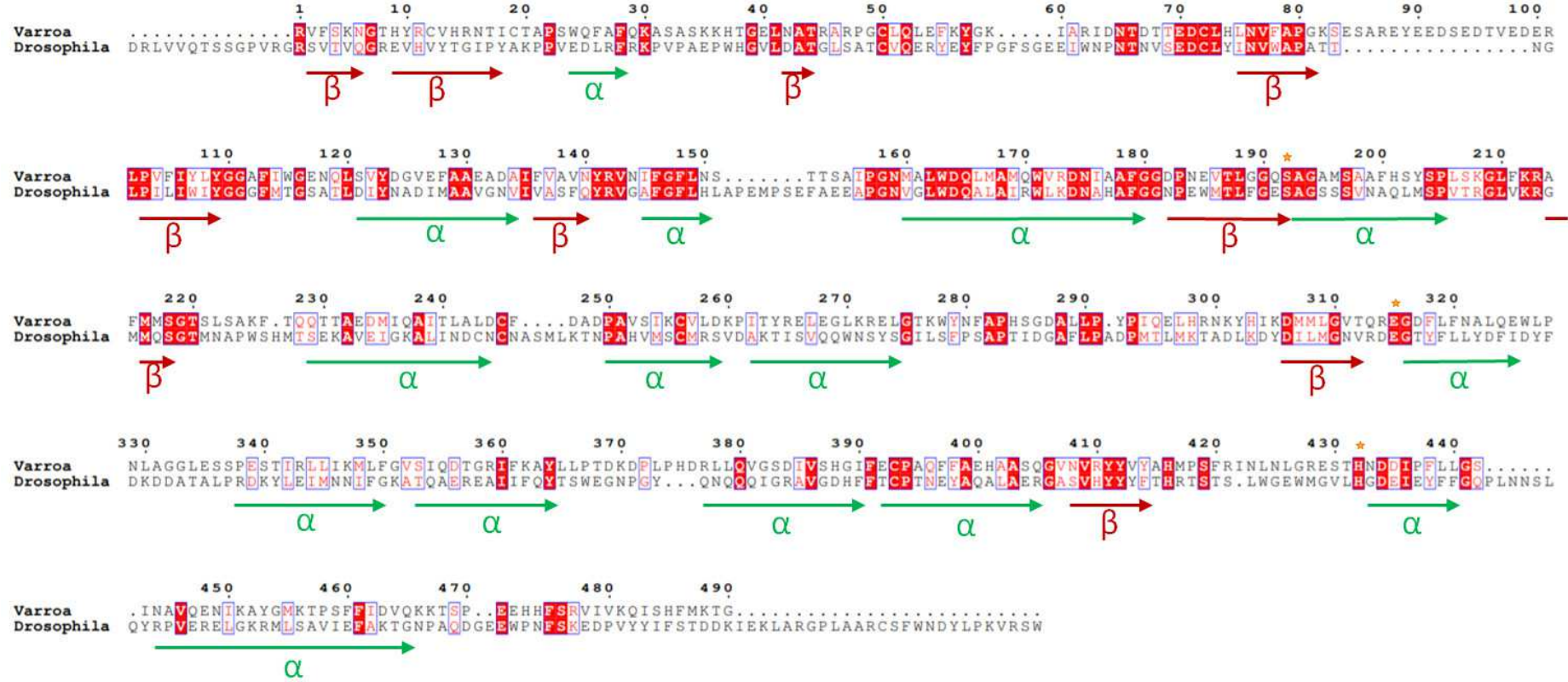


FIGURE 2.

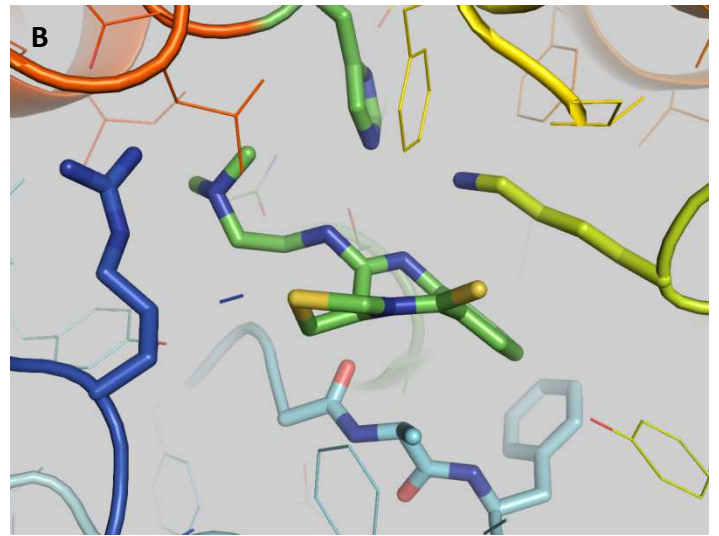
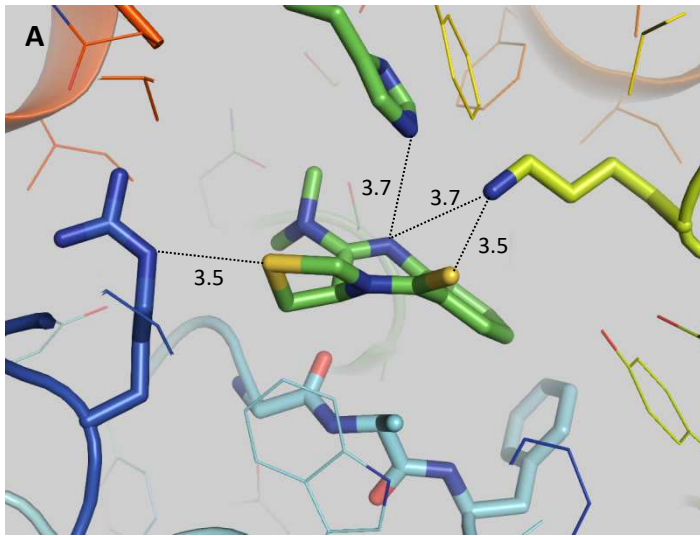
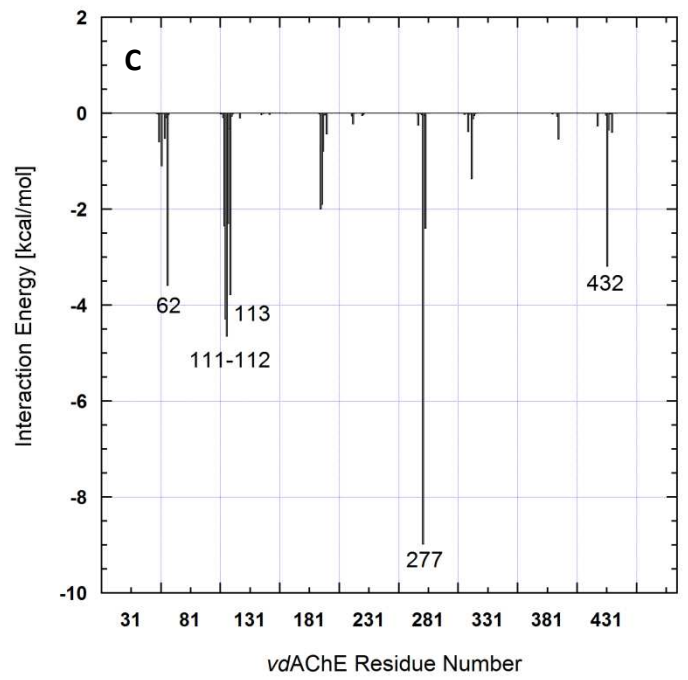
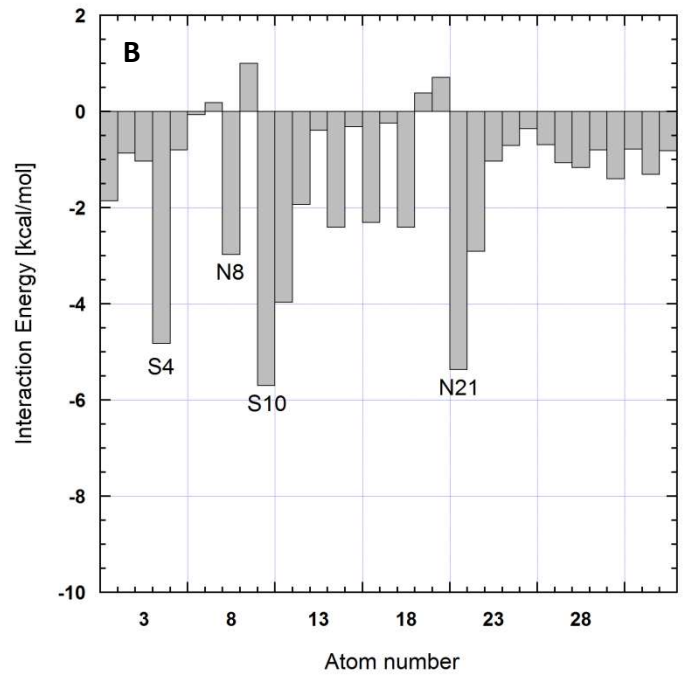
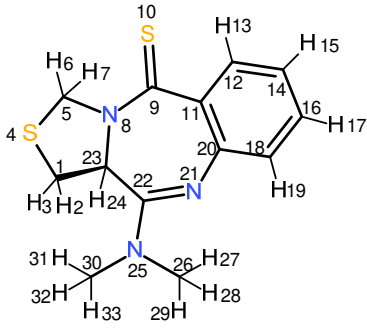


FIGURE 3.

A





Varroa +
destructor

