1. Introduction

Cancer and cardiovascular dysfunction are currently the most diffuse diseases in the developed world, followed by Alzheimer's disease. It is a progressive neurodegenerative disease that leads to gradual memory decline and loss of intellectual abilities, and is associated with behavioural disorders and personality change [1]. Alzheimer's disease is the most common type of dementia and because of the aging population; it has become an ever-increasing social and economic problem. Currently, there are some 46.8 million people around the world who live with Alzheimer’s disease or some other kind of dementia, and this number is expected to double over the next 20 years [2]. Unfortunately, at present there is no treatment that can stop, or preferably reverse, the progression of this neurological disorder. With the current selection of therapeutic agents, it is only possible to slow the progression of the disease, and to reduce the symptomatology.

Patients diagnosed with Alzheimer’s disease show the characteristic neurochemical deficit of the neurotransmitter acetylcholine, especially in the basal forebrain. This is a consequence of enhanced activity of their acetylcholinesterase (AChE; E.C. 3.1.1.7), an enzyme that is mainly located in nerve synapses and...
neuromuscular junctions, where it catalyses cleavage of acetylcholine. As well as decreasing the levels of acetylcholine, AChE is partially responsible for promotion of β-amyloid peptide aggregation, and the consequent formation of amyloid plaques [3,4]. Clinical studies have shown that inhibition of AChE to decrease the rate of acetylcholine hydrolysis is a promising strategy in the therapy of Alzheimer’s disease, as it can reverse the reduced cognition and aberrant behavioural functions associated with Alzheimer’s disease [5]. Indeed, most of the currently available anti-Alzheimer’s drugs are AChE inhibitors, the standard use of which is supported by clinical evidence [6,7]. These inhibitors cannot reduce the rate of decline in the cognitive and functional capacities of patients with Alzheimer’s disease, although over the long term, they can provide meaningful symptomatic benefits.

Alzheimer’s disease is also associated with progressive increase in the activity of the related enzyme butyrylcholinesterase (BChE; E.C. 3.1.1.8). BChE is mainly located in the blood plasma [7], and it is assumed to serve as a ‘back-up’ for when AChE activity is compromised or absent, thus providing further support for and regulation of cholinergic transmission [8]. In this regard, AChE and BChE both represent interesting therapeutic targets for amelioration of the symptoms of Alzheimer’s disease.

The US Food and Drug Administration has approved a number of drugs for the treatment of patients with Alzheimer’s disease, which include the synthetic pharmaceuticals donepezil, rivastigmine, tacrine and galantamine. Galantamine was initially isolated from the common snowdrop, *Galanthus woronowii* [9], and it has contraindications for therapeutic use due to heart problems, epilepsy, and gastrointestinal symptoms. This requires the search for new drugs for the treatment of Alzheimer’s disease that have reduced side effects.

Other important commercially available plant-derived natural AChE inhibitors include physostigmine from the Calabar bean *Physostigma venenosum* [10] and huperzine A from the Chinese club moss *Huperzia serrata* [11]. In recent years, several AChE inhibitors have also been isolated from marine organisms, such as soft corals, molluscs, ascidians, and in particular, sponges (reviewed in Orhan and colleagues [12]). Indeed, nearly half of the marine natural bioactive compounds now derive from sponges, which are considered to be the most bio-prospective [13] and pharmacodiverse marine taxa [14].

Sponges from the genus *Latrunculia* have been recognized as sources of the discorhabdin alkaloids [15–17]. These metabolites are pigments with a very particular structure that is characterised by azacarbocyclic spirocyclohexanone and pyrroloinoquinone units. Over 40 members of the discorhabdin classes have been reported to date, with these defined according to: (i) the presence of sulfur cross-linkage (discorhabdins A, B, D, I, K, L, Q, R, X); (ii) the presence of a methyl sulfide substituent (discorhabdins S, T, U); (iii) the absence of a sulphur atom (discorhabdins C, D, G, E, F, O, P); and (iv) the presence of a rare dimeric structure (*discorhabdin W*). The discorhabdins and discorhabdin-related alkaloids show a plethora of biological effects, which include strong cytotoxic, antimicrobial, antiviral, antimalarial, immunomodulatory, caspase-inhibitory, and feeding-deterrent activities [16]. Due to these biological properties, the isolation, structural determination, reactivity and synthesis of these alkaloids have attracted considerable attention [18].

In 1975, Neeman et al. [19] reported on a structurally unidentiﬁed toxin that they isolated from the Red Sea sponge *Latrunculia magniﬁca* that showed strong BChE inhibition. More recently, Turk et al. [20] evaluated AChE-inhibitory effects of ethanolic extracts obtained from two specimens of Antarctic *Latrunculia* spp. sponges, namely *Latrunculia cf. lendenfeldii* and *Latrunculia cf. bocagei*. These extracts induced 50% inhibition of electric eel AChE (eeAChE) at concentrations of 1.3 ng and 9 ng dried extract/mL, respectively. Thus, the identification of the metabolites responsible for these bioactivities and investigations into their mechanisms of action require further studies.

We initially report here on the isolation of four discorhabdins from methanol extracts of *Latrunculia* spp. sponges that were dredged from the deep Antarctic shelf in the Bransfield Strait near the Antarctic Peninsula in 2013. We further report on the evaluation of these metabolites as AChE inhibitors, with experimental data supported by docking calculations and related to their corresponding anti-BChE activities. One of the most active of these compounds, discorhabdin G, also underwent electrophysiological analysis to define any undesirable effects on neuromuscular transmission.

## 2. Results and discussion

### 2.1. Isolation and structural characterisation of discorhabdin alkaloids 1–4

(+)­Discorhabdin G (1 in Fig. 1) and (−)­3-dihydro-7,8-dehydro discorhabdin C (2 in Fig. 1) were isolated in pure form starting from a crude methanol extract of *L. bocagei*. The first purification step was on a cyanoparticle *L. bocagei* eluting with water/methanol/TF (60:40:1, v/v/v). The fraction collected at 7 min underwent further preparative workup on an RP-18 column with acetonitrile/water/TF (60:40:1, v/v/v), obtaining the fraction eluted at 11 min which was further purified on a cyanoparticle with elution with acetonitrile/water/TF (30:70:1, v/v/v) to give the pure discorhabdin G trisulfate salt (1) as a brown solid. Electrospray ionisation mass spectrometry (ESI-MS) spectra, recorded in positive ion mode by direct infusion from a methanolic solution, showed a double signal at *m/z* 384/386 in a 1:1 ratio, corresponding to the M+ ion, which indicated the presence of one bromine atom. It was confirmed by fragmentation of *m/z* 384 producing a signal at *m/z* 305, due to the loss of the bromine atom. NMR analysis, including 1H NMR spectrum in deuterated methanol (CD3OD) and the connectivity deduced by heteronuclear multiple bond coherence (HMBC) heterocorrelations, allowed the structural assignment, which was in agreement with the reported data for discorhabdin G isolated from *L. apicalis* [21].

The same three-step sequence was required for pure compound 2, obtained evaporating the fraction eluted at 15.2 min in the last step of the work-up for the *L. biformis* extract by cyanoparticle preparative analysis. ESI-MS spectrum recorded in positive ion mode showed signals at: (i) *m/z* 462/464/466, corresponding to the M+ ion and diagnostic for the presence of two bromine atoms, (ii) *m/z* 444/446/448, attributable to the [M–H2O]− ion which supports the presence of a hydroxy group in the structure and (iii) *m/z* 365/367, which are typical of the contribution of two bromine atoms and are due to the [M–H2O–Br]− ion. NMR assignments, supported by long-range 1H,13C correlations by HMBC experiment, were in agreement with previous data reported for 3-dihydro-7,8-dehydro discorhabdin C [22].

The 1H NMR spectrum of the crude methanol extract of *L. bocagei* showed a less complex mixture than that from *L. biformis*, with signals corresponding to two main compounds. These required only a two-step sequence of HPLC purification using a cyanoparticle stationary phase and elution with water/methanol/TF 60:40:1 (v/v/v) to isolate discorhabdin B (3) and discorhabdin L (4) as trifluoroacetate, respectively (Fig. 1). They belong to the discorhabdin subclass that is structurally characterised by an additional sulfur bridge between the C-5 and C-8 positions. (+)-Discorhabdin B was recovered by evaporation of the fraction eluted at the later retention time (15.8 min). The assignment that identified the structure of this monobrominated metabolite was
supported by the isotopic cluster at \( m/z \) 414/416, corresponding to the \( \text{M}^+ \) ion. MS/MS fragmentation, carried out by direct infusion in methanol, indicated that the selected ion at \( m/z \) 414 gave signals at \( m/z \) 334 and \( m/z \) 302, which are attributable to the loss of HBr and HBr plus a S atom, respectively. The \(^1\)H-NMR spectrum recorded in CD\(_3\)OD showed signals that were essentially superimposable on those previously reported for (+)-(6S, 8S)-discorhabdin B, first isolated as its hydrochloride salt from an unclassified \textit{Latrunculia} sp. collected along the New Zealand coast [23], and was characterised by comparison with discorhabdin A, which was established by X-ray diffractometry. The optical activity was also in agreement with that reported for a methanolic solution of (+)-discorhabdin B at a similar concentration. This measurement is particular, because also the enantiomeric (-)-discorhabdin B has been isolated in some cases [16].

(-)-Discorhabdin L (4 in Fig. 1) was recovered by evaporation of the fraction eluted at the earlier retention time (6.5 min) under the same chromatographic conditions for discorhabdin B. Its structural elucidation was obtained on the basis of the \( \text{M}^+ \) signal at \( m/z \) 352 in ESI-MS spectrum and by comparing the \(^1\)H-NMR data with previously reported ones [24]. Some considerations relating to its optical activity are of note. Discorhabdins are pigments with visible activity are of note. Discorhabdins are pigments with visible absorption that can encroach on the sodium line at \( 589 \) nm that is used for optical rotation measurements, so that different values at different concentrations can be obtained, with an \([\delta]_D \) value of zero when a too high concentration is used. This behaviour was observed for discorhabdins bearing the additional ring through the connectivity between N (18) and C (2), as for discorhabdin L [25]. The (-)-(15Z,2R,6S,8R) configuration that was earlier assigned for discorhabdin L through circular dichroism analysis was also in agreement with the isolation of the enantiomeric (+)-discorhabdin L [16].

The purity of the discorhabdins 1–4 used for the biological evaluation was verified by LC–ESI-MS analysis (Figs. S1–S4).

2.2. \textit{In vitro} AChE and BChE inhibitory activities

Among the metabolites purified here, discorhabdin B (3) and 3-dihydro-7,8-dehydrodiscorhabdin C (2) were found to be the most cytotoxic against different murine and human tumour cell lines, with \( IC_{50} \) values around 0.1 \( \mu \)M [22,26,27]. Lam et al. [28] reported that discorhabdins that embody a ring closure between N(18) and C(2), such as discorhabdin L (4), have modest cytotoxic activity, with \( IC_{50} \) values from 1 \( \mu \)M to 15 \( \mu \)M [26,27]. Discorhabdin G (1) was shown to have antibacterial activity and to cause feeding deterrence behaviour in the major Antarctic sponge predator \textit{Perkmaster fuscus} [21].

The cholinesterase inhibitory activities were here determined for the pure discorhabdins 1–4 against eeAChE, recombinant human AChE (hAChE), and horse serum BChE. Each of these metabolites effectively inhibited the cholinesterases in a dose-dependent manner (not shown). The highest inhibitory activities were seen against eeAChE, which probably reflects the ecological role of these sponge metabolites in deterring possible predators. Discorhabdin G (1) and discorhabdin B (3) were the most potent AChE inhibitors of the series, with respective \( IC_{50} \) values of 1.3 \( \mu \)M and 5.7 \( \mu \)M towards eeAChE, and 116 \( \mu \)M and 49.5 \( \mu \)M towards hAChE (Table 1).

The inhibitory constants (\( K_i \)) corresponding to these two most potent AChE inhibitors, 1 and 3, were 1.6 \( \mu \)M and 1.9 \( \mu \)M towards eeAChE, and 56.2 \( \mu \)M and 22.8 \( \mu \)M towards hAChE, respectively (Table 1). These \( IC_{50} \) and \( K_i \) values are within the pharmacologically interesting range when compared with commercial inhibitors used for the treatment of patients with Alzheimer's disease. Physostigmine was used as the positive control and it showed an \( IC_{50} \) of 3 \( \mu \)M towards eeAChE (Table 1), which is comparable to reported value [29], whereas its \( IC_{50} \) obtained for inhibition of hAChE (14.5 \( \mu \)M) was considerably higher than the \( IC_{50} \) reported for AChE isolated from human brain cortex (31 \( \mu \)M) [10]). As an additional reference, the \( IC_{50} \) of galantamine is in the range of 0.25–2.4 \( \mu \)M for eeAChE, and 1.07–8.7 \( \mu \)M for hAChE [29].

For the inhibition of horse serum BChE, discorhabdin G (1) and 3-dihydro-7,8-dehydro discorhabdin C (2) showed the strongest inhibition among the tested compounds, with \( IC_{50} \) of 7 \( \mu \)M and 15.8 \( \mu \)M, respectively (Table 1). In comparison, the \( IC_{50} \) of the reference compound physostigmine salicylate was 28.5 \( \mu \)M (Table 1), while galantamine (1 \( \mu \)M) was reported to inhibit the BChE activity by 80.3\% [30,31]. Discorhabdin G and 3-dihydro-7,8-dehydro discorhabdin C also showed low \( K_i \) at 5.0 \( \mu \)M and 17.5 \( \mu \)M, respectively (Table 1).

The kinetics of the inhibition of these cholinesterases by the tested metabolites, and their analysis using Dixon plots further revealed that discorhabdins 1–3 act as reversible, competitive inhibitors through their binding to the active site of the free AChE or BChE (Fig. 2).

2.3. Molecular docking

In order to understand the interactions of the discorhabdins with the active sites of AChE enzymes and to propose a plausible binding mode that can explain the observed inhibitory activities, docking calculations were carried out using the AutoDock program. Data for eeAChE (1C20) are available by X-ray analysis
with a too low resolution (4.2 Å) to be used in docking calculation, so that *Torpedo californica* (1DX6, 2.3 Å resolution) was adopted as model system.

Preliminary comparison between these two structures was possible using the TM align algorithm, which obtained 59.5% equivalence in amino acid sequence, and very good overlap of their secondary structures. Mostly, these two AChEs showed the same amino acid residues in the active sites, except for phenylalanyl unit F330 in *T. californica* AChE that was replaced by tyrosine Y330 in eeAChE, which indicated very similar active sites for the two AChEs (Fig. S5).

The calculations were performed by Autodock Vina, adopting grid boxes with a spacing of 1.00 Å (details in Materials). The lacking of 3D structure for horse BuChE prevented a similar computational approach.

The docking results for *T. californica* AChE with the tested discorhabdins indicated a behaviour in line with the experimental data. Here, discorhabdin G (1) showed the lowest value of energy and the highest number of hydrophobic interactions, although with no specific H-bonds, which is in agreement with the highest observed inhibitory activity. The experimental trend of 1 > 3 > 2 > 4 is supported by the computational data that take into account the

Table 1: Inhibition of electric eel acetylcholinesterase (eeAChE), human recombinant acetylcholinesterase (hAChE) and equine serum butyrylcholinesterase (BChE) by discorhabdin G (1), 3-dihydro-7,8-dehydrodiscorhabdin C (2), discorhabdin B (3) and discorhabdin L (4).

<table>
<thead>
<tr>
<th>Compound</th>
<th>eeAChE</th>
<th>hAChE</th>
<th>BChE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (µM)</td>
<td>Ki (µM)</td>
<td>IC50 (µM)</td>
</tr>
<tr>
<td>1</td>
<td>1.3 ± 0.2</td>
<td>1.6</td>
<td>116 ± 9</td>
</tr>
<tr>
<td>2</td>
<td>14.5 ± 1.5</td>
<td>3.5</td>
<td>152 ± 12</td>
</tr>
<tr>
<td>3</td>
<td>5.7 ± 0.8</td>
<td>1.9</td>
<td>49.4 ± 7.5</td>
</tr>
<tr>
<td>4</td>
<td>25.7 ± 3.0</td>
<td>15.0</td>
<td>158 ± 15</td>
</tr>
<tr>
<td>Physostigmine</td>
<td>3.0 ± 0.3</td>
<td></td>
<td>14.5 ± 2.0</td>
</tr>
</tbody>
</table>

n.d. not determined due to lack of the substance.

* Discorhabdin as trifluoroacetate (TFA) salt, physostigmine as salicylate salt.
energy, the hydrogen bonds and their distances, and the numbers of hydrophobic interactions (Table 2).

The docking results for each of the tested discorhabdins with hAChE showed higher energy and fewer hydrophobic interactions when compared with inhibition of *T. californica* AChE, which is in line with the experimental behaviour of inhibition towards eeAChE and hAChE tested here. For the discorhabdin G complex with hAChE, the computational data showed lower stability (i.e., corresponding to less negative energy), fewer hydrophobic interactions and a lack of H-bonds, compared to the discorhabdin G complex with *T. californica* AChE. These computational results support the lower inhibition achieved by discorhabdin G with hAChE than with *T. californica* AChE.

These results were in line with the experimental data that showed the trend of discorhabdins $3 > 1 > 2 - 4$. As the most active of this series, discorhabdin B (3) is supported by its three relevant H-bonds, in addition to a series of hydrophobic interactions. A visualisation of ligands 1–4 in the active sites of AChEs from the molecular study is reported in Figs. 3 and 4 and Fig. S6. It is notable that tryptophan W286, valine V294, tyrosine Y341, and phenylalanine F295 are the amino acid residues of hAChE that are involved in each of these complexes between hAChE and discorhabdins.

For discorhabdins G and L, the enolic forms corresponding to the keto groups at the C-3 position are possible through a keto-enolic equilibrium, although their contributions were not evident in the NMR analysis. However, these enolic tautomers were taken into account in the docking calculations, and they demonstrated similar results to the corresponding keto forms. The computational analysis also involved the deprotonated forms of discorhabdins G and B, and 3-dihydro-7,8-dehydro discorhabdin C, as their free imines (1a-3a in Table 2), although these showed no effective changes in comparison with their protonated forms 1–3. This was expected, because N18 position is not involved in any of the interactions of discorhabdins 1–4, and also because at the physiological pH used in the experimental assays, they would have been in their protonated forms. In addition, support comes from the observation that the crude methanol extract of *L. biformis* showed the same inhibitory activity on eeAChE as its protonated form that was obtained after treatment with the same water/methanol/TFA eluent used in the
Table 2
Data from docking calculation of discorhabdins 1–4 with the indicated AChEs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Torpedo californica AChE (1DX6)</th>
<th>Homo sapiens AChE (4MOE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔE kcal/mol</td>
<td>H-bond</td>
</tr>
<tr>
<td>1</td>
<td>-12.0</td>
<td>-</td>
</tr>
<tr>
<td>1a</td>
<td>-11.5</td>
<td>2.83</td>
</tr>
<tr>
<td>1 enol form</td>
<td>-11.2</td>
<td>-</td>
</tr>
<tr>
<td>1a enol form</td>
<td>-10.9</td>
<td>2.84</td>
</tr>
<tr>
<td>2</td>
<td>-10.6</td>
<td>3.31</td>
</tr>
<tr>
<td>4 enol form</td>
<td>-11.1</td>
<td>2.85</td>
</tr>
<tr>
<td>Physostigmine</td>
<td>-8.9</td>
<td>3.08</td>
</tr>
</tbody>
</table>

* a 1a, 2a and 3a as deprotonated form.
* b Calculated docking energy in Kcal/mol.
* c Numbering for discorhabdin structures as reported in Fig. 1.
* d H-bond distance in Å are reported in brackets.

chromatographic isolation (IC50 = 1.6 μg dried extract/mL).

Taking into account the interactions with hAChE that are more relevant for further biological applications, a qualitative correlation between structures and bioactivities indicated that the HN9/OC11/HN13 moiety is involved in H-bonds for the most active discorhabdin B, as well as for the other metabolites, although through hydrophobic interactions. This result can be used for the development of synthetic analogues with simpler structures.

2.4. Electrophysiological study of discorhabdin G

The use of AChE inhibitors can produce unwanted side effects, like single twitch potentiation in muscle, and the inability of skeletal muscle to sustain tetanic contraction [32–34]. Therefore, additional electrophysiological investigations were carried out with one of the most active of these discorhabdins, discorhabdin G. These were designed to determine its effects on nerve-evoked and directly elicited single muscle twitches and on tetanic contractions of the isolated mouse hemidiaphragm with the corresponding nerve.

While 8.43 μM discorhabdin G reduced the eeAChE activity in vitro by 80%, it appeared to have no effects on directly and indirectly evoked muscle twitch amplitudes, nor on the amplitudes of directly and indirectly evoked tetanic muscle contraction (Fig. S7 and S8).

Inhibition of the AChE of the neuromuscular junction is associated with the inability to sustain a tetanic contraction under repetitive high frequency stimulation of the motor nerve [35]. The effects of discorhabdin G on the maximal amplitude of nerve-evoked sustained contraction was thus also investigated. There were no observable effects of discorhabdin G on the indirectly evoked tetanic contraction (Fig. 5). To determine possible inhibitory effects on AChE in the neuromuscular junction, electrophysiological recordings from superficial muscle fibres were performed in the same neuromuscular preparation exposed to 2 μM conotoxin GIIIb, which effectively blocks NaV1.4 sodium channels in vertebrate skeletal muscle, to thus prevent muscle fibre contraction. Discorhabdin G did not increase the amplitude of the EPPs or the EPP half decay after either 30 min or 60 min exposure of the neuromuscular preparation to this discorhabdin (Fig. 6).

3. Conclusions

For many years, the Latrunculia spp. sponges have been the focus of studies, as they represent a source of a wide series of discorhabdin alkaloids that show interesting cytotoxic, antimalarial, antimicrobial and anti-inflammatory activities. To date, cholinesterase inhibitory activities have been assigned only to crude
extracts from some species of tropical and Antarctic *Latrunculia* spp. sponges, without associating them to any identified pure metabolites in these extracts. Based on the data reported here, these discorhabdin metabolites appear to be responsible for the previously described cholinesterase inhibitory activities of such extracts. The evaluation of isolated discorhabdins G (1), B (3) and L (4), and 3-dihydro-7,8-dehydro discorhabdin C (2) against both eeAChE and hAChE, and also horse serum BChE, demonstrated a competitive reversible mechanism of inhibition with discorhabdin G as the most potent inhibitor of eeAChE and horse serum BChE.
and discorhabdin B as the most potent inhibitor of hAChE. Docking calculations for different AChEs defined the interactions involved between these discorhabdin ligands and the active sites of the AChEs, which provided further support for the experimental data. Electrophysiological experiments with discorhabdin G showed that it has no undesirable effects on neuromuscular transmission and skeletal muscle function in this setting, thus favouring its use in the therapy of cholinesterase inhibition, as this appears to avoid the side effects produced by some of the known cholinesterase inhibitors. Thus, based on the scaffold of discorhabdin metabolites, these findings are promising as a guide towards the synthesis of potential agents for the treatment of Alzheimer’s disease.

4. Materials and methods

4.1. Chemistry

All of the solvents used were from Riedel-de Haën—Sigma Chemical Co., and were of HPLC grade. Evaporation was carried out at 25 °C and reduced pressure. Thin layer chromatography was carried out on Merck RP-18 F254, Merck CN F254 or Kieselgel 60 0.2-mm plates, with visualisation using UV light or marking of visible spots. NMR spectra were recorded on a spectrometer Bruker-Avance 400 using a double-resonance broadband inverse (BBI) probe for 1H at 400 MHz and a broadband observe (BBO) probe for 13C at 100 MHz in CD3OD, relative to the solvent residual signals, with δH = 3.31 ppm and δC = 49.00 ppm, respectively. Structural assignments were supported by HMBC experiments comparison with reported data. Polarimetric data were obtained with an apparatus (Jasco-DP-181) equipped with a sodium lamp at 589 nm (line D) or a mercury lamp at 577 nm, with [α]D reported as deg mL g⁻¹. Infrared spectra were recorded using a spectrometer (FT-IR Tensor 27; Bruker) equipped with an attenuated transmitter reflection device at 1 cm⁻¹ resolution in the absorption region 4000 cm⁻¹ to 1000 cm⁻¹. A thin solid layer was obtained by evaporation of the methanolic solution of the samples. The instrument was purged with a constant dry nitrogen flow. Spectra processing was carried out with the Opus software package. ESI-MS mass spectra were recorded using a Bruker Esquire-LC spectrometer equipped with an ion trap analyser. The atmospheric pressure electrospray ion source was used in positive ion mode, under the following conditions: source temperature, 300 °C; drying gas, N2; flow rate, 4 L/min; and scan range, 100–1000 m/z. The mass spectra were analysed using Data Analysis, version 3.0 (Bruker Daltonics GmbH). Online liquid chromatography–ESI MS analysis was performed using liquid chromatograph (Hewlett-Packard) with a photo diode array detector (1100 Series Agilent). The HPLC conditions included: Lichrospher 100 CN column (250 mm × 4 mm; 5 μm); flow rate, 1 mL/min; detector, 254 nm; gradient mobile phase, water/methanol/TFA, from 80:20:1 (v/v/v) to 50:50:1 (v/v/v), over 20 min, and then to 0:100:1 (v/v/v) over 30 min.

4.2. Sponge collection

Two specimens of cold-water sponges that belong to the genus *Latrunculia* were obtained from the Senckenberg Research Institute and Natural History Museum, Germany. These sponges had been dredged from coastal shelf environments around the Antarctic Peninsula (60°–70° S; 8°–61° W) during the deployment of an Agassiz trawl, at depths between 200 m and 600 m, during the ANT XXIX/3 expedition of the German Research Vessel “Polarstern” in 2013 (for environmental and faunistic information, see Ref. [36]). The sponge samples were frozen at −20 °C immediately after collection, and lyophilised prior to extraction. One specimen (SMF11203) was collected at 330 m in depth and was identified as *Latrunculia biformis*, and the other specimen (SMF11172) was collected at 430 m in depth and was identified as *Latrunculia bocagei*. The sponge samples have been entered in the Senckenberg Sammlungs management database (http://sesam.senckenberg.de/).

4.3. Isolation and structural characterisation of discorhabdin alkaloids 1–4

For each sponge specimen, 0.5 g freeze-dried sponge material was extracted ultrasonically (3 ×) using methanol, for 1 h at 25 °C. After each extraction step, the mixture was centrifuged to remove solid particles, and then the extract was collected and evaporated to dryness on a rotatory vacuum evaporator at 25 °C. These extracts were dark brown in colour, and constituted the crude organic extracts (*L. biformis*: sample v171, 180 mg; *L. bocagei*: sample v105, 157.9 mg).
The methanol extract from *L. biformis* was dissolved in water/methanol and subjected to HPLC purification on a preparative CN column (Merck Lichrosphere 100, 250 mm × 4.0 mm, 5 μm) eluting with water/methanol/TFA (60:40:1, v/v/v), at a flow rate of 5 mL/min and under UV detection at 254 nm. The fraction collected at 7 min was evaporated and injected again onto an RP-18 HPLC column (Merck Lichrosphere) and eluted with acetonitrile/water/TFA (60:40:1, v/v/v). The residue obtained was subjected to preparative HPLC under the same conditions as the first step adopted for purification of discorhabdins 1 and 2. The fraction collected at 6.5 min was evaporated to dryness and the residue was further purified on the same column, by elution with water/methanol/TFA (60:40:1, v/v/v). The peak obtained at 15.8 min gave (+)-discorhabdin B trifluoroacetate salt (3 in Fig. 1. 3.2 mg, 0.002% in the crude extract). The fraction collected at 4.2 min in the first HPLC step was resuspended in water/methanol/TFA (60:40:1, v/v/v) and eluting with water/methanol/TFA (60:40:1, v/v/v), to obtain (+)-discorhabdin L trifluoroacetate salt (4) from the fraction eluted at 7.0 min (1.9 mg, 0.0012% in the crude extract).

4.3.1. Data for (+)-discorhabdin C (1)

Isolated as trifluoroacetate salt (MW = 498.25 g/mol), [x]D = +28° (c 1.3 mg/mL, MeOH).

1H NMR (400 MHz, CD3OD): δ 7.65 (1H, s, H-1), 7.16 (1H, s, H-14), 6.38 (1H, d, J = 7.8 Hz, H-8), 5.40 (1H, d, J = 7.8 Hz, H-7), 3.89 (2H, t, J = 7.8 Hz, 2H-17), 2.98 (3H, m, H-4a and 2H-16), 2.70 (2H, t, J = 3.0 Hz, 2H-17), 2.57 (1H, d, J = 11.6, 3.6 Hz, 1H-7), 2.30 (series of m, H-4b, and 2H-16), 1.92 (1H, s, H-1), 1.24 (1H, s, 1H-14), 1.12 (1H, s, 1H-8), 5.40 (1H, d, J = 7.8 Hz, H-8), 3.90 and 3.83 (2H, two m, 2H-17), 2.92 (2H, dd, J = 12.0, 3.6 Hz, 2H-17), 2.57 (1H, d, J = 12.0 Hz, H-7).

13C NMR (100 MHz, CD3OD): δ 167.2 (C11), 148.4 (C10), 127.0 (C14), 118.9 (C15), 114.1 (C4), 120.2 (C20), 68.5 (C21), 68.7 (C26) and 67.3 (C8), 51.5 (C17), 37.9 (C7), 20.1 (C16); from HMBC correlations: δ 184.7 (C3), 171.4 (C5), 125.6 (C12), ESI(+) MS m/z 352 [M]+, ESI(−) MS/MS (352); m/z 335, 324, 281, 250.

4.3.2. Data for (+)-discorhabdin L (4)

Isolated as trifluoroacetate salt (MW = 465.40 g/mol), dark-green solid: [x]D28 = +407° (c 0.8 g/mL, MeOH).

1H NMR (400 MHz, CD3OD): δ 7.11 (1H, s, H-14), 6.14 (1H, s, H-4), 5.58 (1H, brd, J = 3.6 Hz, H-8), 4.63 (1H, d, J = 3.6 Hz, H-1), 4.15 (d, J = 3.6 Hz, H-2), 4.01 (1H, dd, J = 14.2, 7.5, 3.0 Hz, 1H-17), 3.90 (1H, ddd, J = 14.2, 7.5, 3.0 Hz, 1H-17), 3.18 and 3.06 (2H, two m, 2H-16), 2.95 (1H, dd, J = 12.0, 3.6 Hz, 1H-7), 2.57 (1H, d, J = 12.0 Hz, H-7).

13C NMR (100 MHz, CD3OD): δ 167.2 (C11), 148.4 (C10), 127.0 (C14), 118.9 (C15), 114.1 (C4), 120.2 (C20), 68.5 (C21) and 68.7 (C26) and 67.3 (C8), 51.5 (C17), 37.9 (C7), 20.1 (C16); from HMBC correlations: δ 184.7 (C3), 171.4 (C5), 125.6 (C12), ESI(+) MS m/z 352 [M]+, ESI(−) MS/MS (352); m/z 335, 324, 281, 250.

4.3.3. Protonation of methanol extract from *Latrunculia biformis*

The residue from crude methanol extract (5 mg) was suspended in 1 mL of methanol/water/TFA as used for the HPLC purification of discorhabdin metabolites. This solution was stirred at room temperature for 15 min, and then evaporated to dryness, to give the residue used in the further biological assays.

4.4. Cholinesterase inhibition

The cholinesterase activities were measured by the Ellman method [37], using acetylthiocholine chloride (0.25, 0.5 or 1.0 mM; Sigma, USA) as substrate in 100 mM potassium phosphate buffer, pH 7.4, at 25 °C. Three cholinesterases were used as the source of enzyme, at the final concentration of 0.0075 U/mL: eeAChE (from *Electrophorus electricus*), hAChE, and horse serum BChE (all Sigma, USA). The hydrolysis of acetylthiocholine chloride was followed in a microplate reader (Kinetic; Dynex Technologies, USA) at 405 nm. Stock solutions of the discorhabdins (2 mg/mL) were prepared in ethanol/deionised water (1:9, v/v). Cholinesterase inhibition was monitored over 5 min from the addition of the discorhabdins (progressively diluted in 100 mM potassium phosphate buffer, pH 7.4). Phystostigmine salicilate (Sigma, USA) was dissolved in 100% ethanol and used as the positive control. All readings were corrected according to the appropriate blanks. Blank reactions without the inhibitors were run in the presence of the appropriate ethanol/water dilutions (1:9; v/v) for the discorhabdins, or in the presence of pure ethanol for phystostigmine. Each measurement was repeated at least three times. The inhibitory constants (Ki) for discorhabdin L towards hAChE and horse serum BChE were not determined, due to lack of the isolated discorhabdin L.

4.5. Muscle contraction

Male Balb/c mice (2–3 months old) were obtained from the Animal Breeding Facility in the Veterinary Faculty, University of Ljubljana. The study on isolated neuromuscular preparations was carried out in strict accordance with Slovenian legislation, as harmonised with the European Communities Council Guidelines (Directive 86/609/EGS of 24 November 1986; Directive 2010/63/EU of 22 September 2010). All in-vitro experiments on isolated organs were approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary and Plant Protection (permit no. 34401–12/2012/2). The mice were sacrificed by cervical dislocation, followed by immediate exsanguination. The diaphragm with corresponding phrenic nerves was dissected out and used. A hemidiaphragm was tightly pinned to the Rhodorsil-coated organ bath containing oxygenated standard Krebs-Ringer solution (154 mM NaCl, 2 mM CaCl2, 5 mM KCl, 1 mM MgCl2, 5 mM HEPES, 11 mM D-glucose, pH 7.4), at 22 °C–24 °C. The tendinous side of the hemidiaphragm was attached with a steel hook via silk thread to an isometric force displacement transducer (FT 03; Grass Instruments, USA).
MEPP recordings were digitised (Digidata 1440A), with the pClamp intracellular borosilicate microelectrodes pulled with a micropipette. Nerve-evoked single isometric twitches were recorded as follows: the motor nerve of the isolated neuromuscular preparation was stimulated with a square-pulse stimulator (S-48; Grass Instruments, West Warwick, RI, USA) via a suction electrode, with pulses of 0.1 ms duration, 0.1 Hz stimulation rate, and supramaximal voltage typically 6 V–8 V. Directly evoked single isometric twitches were evoked by stimulating the hemidiaphragm preparation with a platinum electrode assembly placed along the organ bath, with pulses of 0.1 ms duration, 0.1 Hz stimulation rate, and supramaximal voltage of 60 V–80 V. Direct or nerve-evoked tetanic muscle contraction recordings were obtained by stimulating the hemidiaphragm with trains of pulses (1000 ms duration, at 80 Hz). The electrical signals from muscle contraction experiments were amplified using a strain gage amplifier (P122; Grass Instruments, West Warwick, RI, USA), and then digitised at a sampling rate of 1 kHz using a data acquisition system (Digidata 1440A; Molecular Devices, Sunnyvale, CA, USA). The effects of discorhabdin G on the muscle contraction of the hemidiaphragm preparation were measured over 60 min of exposure.

4.5.1. Muscle fibre membrane potentials

For the muscle fibre membrane potentials, the experiments were performed at room temperature (22–24 °C) on the mouse oxygenated hemidiaphragm preparations, pretreated for 30 min with 2 μM µ-conotoxin GIIIB, an inhibitor of muscle sodium channels, to prevent muscle contraction and to record full-sized end-plate potentials. The resting membrane potential, endplate potentials and miniature endplate potentials (MEPPs) were recorded from endplate regions in superpotentials (EPPs) and miniature endplate potentials. The resting membrane potential, endplate potentials. The resting membrane potential, endplate potentials were evoked by stimulating the hemidiaphragm preparation with a platinum electrode assembly placed along the organ bath, with pulses of 0.1 ms duration, 0.1 Hz stimulation rate, and supramaximal voltage of 60 V–80 V. Direct or nerve-evoked tetanic muscle contraction recordings were obtained by stimulating the hemidiaphragm with trains of pulses (1000 ms duration, at 80 Hz). The electrical signals from muscle contraction experiments were amplified using a strain gage amplifier (P122; Grass Instruments, West Warwick, RI, USA), and then digitised at a sampling rate of 1 kHz using a data acquisition system (Digidata 1440A; Molecular Devices, Sunnyvale, CA, USA). The effects of discorhabdin G on the muscle contraction of the hemidiaphragm preparation were measured over 60 min of exposure.

Vc = V0 × (−70)/E

where Vc is the normalised amplitude of the EPPs or MEPPs, V0 is the recorded amplitude, and E is the resting membrane potential.

4.5.2. Data analysis and statistics

The statistical analysis of the data was carried out using SigmaPlot for Windows 12.5 (Systat Software Inc., Germany). The data are presented as means ± SEM. They were first tested for normality (Shapiro–Wilks) and equal variance for assignment to parametric or non-parametric analysis. For the membrane potential data, two-tailed Student t-tests were used, and P ≤ 0.05 was considered to be statistically significant.

4.6. Computational details

Calculations were carried out on a PC running at 3.4 GHz on an Intel i7 2600 quad core processor with 8 GB RAM and 1 TB hard disk, with Windows 7 Home Premium 64-bit SP1 as the operating system. The ligands were build using PC Model version 6.0 (Serena Software, Bloomington, IN 47402–3076) and minimised using the force field MMX. The minimised molecules were saved in pdb extension. The AutoDock Tools (ADT) package version 1.5.6rc3 [38] was used to generate the docking input files and to analyse the docking results, with Autodock Vina 1.1.2 [39] used for the docking calculations. The different crystallographic structures of AChE were from the Protein Data Bank (PDB; http://www.pdb.org/). The structure of hAChE in complex with dihydrotanshinone (4MOE) and the structure of the Torpedo AChE complexed with (−)-galantamine (1DX6) were determined by X-ray crystallography, with a resolution of 2.0 Å and 2.3 Å, respectively [40,41]. The structures were modified as follows: the ligand and all of the crystallisation water molecules were removed, with the file saved in pdb extension. All hydrogen atoms were added using AutoDock Tools (ADT), and the Gasteiger–Marsili charges were calculated, with the resulting file saved in pdbqt extension. Rotatable bonds were defined for each minimised ligand molecule. For the docking calculation, for 4MOE, a grid box of 14 × 14 × 14 Å in the x, y and z directions was created, with spacing of 1.00 Å and centred at x = −17.171, y = −42.304, z = 25.612, and for 1DX6, a grid box of 16 × 16 × 16 Å in the x, y and z directions was created, with spacing of 1.00 Å and centred at x = 3.627, y = 64.986, z = 64.364. Vina parameters were set as follows: exhaustiveness of the local search, 100; number of conformations to calculate, 10. To validate the goodness of the calculation, the original ligand was re-docked and visual inspection of the data showed very tight overlap. The results are expressed as the energy associated to each ligand–enzyme complex in terms of the Gibbs free energy values. The visual ligand–enzyme interactions were displayed using LigPlot [42].

The structural comparisons of AChE 1DX6 from Torpedo californica used in the docking calculations with AChE used in the biological assays (see AChE 1C2O) and Homo sapiens hAChE (4MOE) were obtained by applying the TM-align server (version 20160521) [43].

Author contributions

T.B. purified the natural products from the crude extracts and wrote the manuscript; A.D. carried out molecular docking studies; P.Z. purified natural products and carried out part of the enzyme inhibition experiments, M.C.Z. and R.F. carried out the electrophysiological experiments and wrote the manuscript; D.J. and D.K. collected the sponges during an Antarctic expedition, identified them, and prepared the crude extracts; Z.K. wrote the manuscript; I.M. supervised the chromatographic purification of the metabolites, characterised their structures, and wrote the manuscript; K.S. planned, supervised, and carried out part of enzyme inhibition experiments, and wrote the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest regarding this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmec.2017.05.019.

References