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Hesperine, a new imidazole alkaloid and α-synuclein binding activity of 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine from the marine sponge *Clathria (Thalysias) cf. hesperia*

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ABSTRACT

During a high-throughput screen of 300 Australian marine invertebrate extracts, the extract of the marine sponge *Clathria (Thalysias) cf. hesperia* was identified with α-synuclein binding activity. The bioassay-guided purification of this extract resulted in the isolation of 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) as the α-syn binder along with one new compound, hesperine (**1**), and five known compounds, indole-3-carboxaldehyde (**3**), (*Z*)-2′-demethylaplysinopsin (**4**), 2-amino-4′-hydroxyacetophenone (**5**), 4-hydroxybenzoic acid (**6**) and 4-hydroxybenzaldehyde (**7**). Herein, we report the structure elucidation of hesperine (**1**) and α-syn binding activity of 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**)

Introduction

Accumulated deposits of amyloid protein is a pathological hallmark of diseases like Parkinson's disease (α-synuclein) [1], Alzheimer's disease (amyloid beta) [2,3] and type 2 diabetes mellitus (Islet amyloid polypeptide) [4]. The aggregation of one such protein, α-synuclein (α-syn), can be triggered in experimental cellular and animal models, when short misfolded α-syn fibrils are introduced to the extracellular environment of cells like neurons [5,6]. The aggregation mechanism is a template-dependent cascade that results in the deposition of β-sheet-rich amyloid positive aggregates [1,7]. In disease conditions, the initiation of these critical misfolding events are not well understood, with several studies suggesting environmental factors, such as pesticides, may play a part [8]. Multiple studies have shown that the initiation of the amyloid aggregation cascade leads to cellular toxicity resulting in various metabolic dysfunctions and death of affected cells [9–11]. As a result, the discovery of therapeutics that can slow or halt this process has become the focus of many researchers. Direct inhibition of the amyloid aggregation cascade by small molecules is a therapeutic approach that has become more prevalent over the last decade, with the potential to significantly modify the pathological characteristics of diseases with protein misfolding and β-sheet-rich amyloid deposits [12–14]. Several high-throughput assays have been developed to detect small molecules that show activity towards amyloid proteins. This includes mass spectrometry (MS) based amyloid binding assays [15] and thioflavin T (ThT) amyloid dye aggregation assays [16]. There are also medium- to high-throughput cell-based assays to test these candidate molecules against fibril induced intracellular aggregates of α-syn [17]. Such techniques have facilitated the discovery and validation of inhibitors against amyloid protein aggregations *in vitro* and *in vivo*.

Marine sponges and their microbe symbionts have proven to be a rich source of bioactive secondary metabolites. The chemical investigation of these unique organisms has yielded a large number of diverse molecules with a range of therapeutic applications [18]. This paired with the abundant and diverse assortment of marine sponge species, makes them the ideal source for extracts to be used during high-throughput biological screening applications [19]. In a recent biodiscovery assay, a collection of extracts from 300 marine invertebrate species was screened

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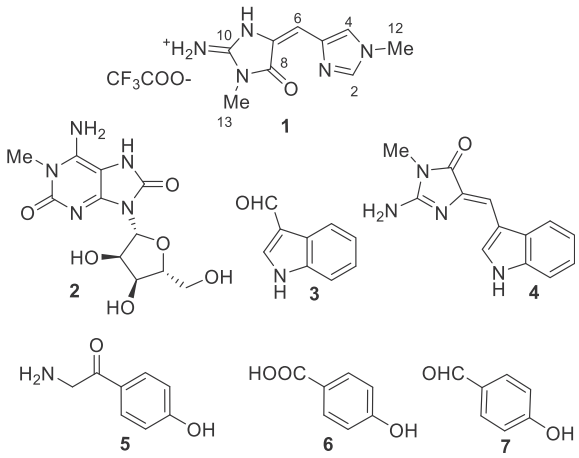

Fig. 1. Compounds isolated from *Clathria (Thalysias) cf. hesperia,* Hesperine (**1**); 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**): indole-3-carboxaldehyde (**3**); (*Z*)-2′-demethylaplysinopsin (**4**); 2-amino-4′-hydroxyacetophenone (**5**); 4-hydroxybenzoic acid (**6**) and 4-hydroxybenzaldehyde (**7**).

Table 1
^{1}H NMR (500 and 800 MHz) and ^{13}C NMR (125.7 MHz) Spectroscopic Data for Hesperine (**1**) in DMSO-d_6.

Position	δ_C, Type	δ_H (*J* in Hz)
2	140.1 CH	8.06 s
4	126.0 CH	7.71 s
5	134.8C	–
6	107.9 CH	6.91 s
7	126.0C	–
8	162.6C	–
10	155.2C	–
11	NH	10.07 bs
12	33.6 CH_3	3.74 s
13	26.0 CH_3	3.14 s
14	NH_2	9.60 bs
		8.93 bs

for binding activity towards the α-syn protein. This resulted in the extract of the marine sponge *Clathria (Thalysias) cf. hesperia* (QM G335574) displaying binding activity. The bioassay-guided purification of this extract resulted in the isolation of one new compound, hesperine (**1**) and six known compounds including 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) as the α-syn binder.

Results and discussion

The freeze-dried sponge material was exhaustively extracted using MeOH. Binding activity was evaluated using an *in vitro* MS binding assay that provided the active compound's mass (313.1 Da). The extract was then separated using a C_{18} silica gel HPLC column, eluted with H_2O to MeOH. Bioassay and MS (*m/z* 314.1) guided purification of the resulting fractions yielded the known natural product 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) [20], which was responsible for the binding activity observed from this extract. The separation process also led to the isolation of one new natural product, hesperine (**1**) and five known natural products: indole-3-carboxaldehyde (**3**) [21]; (*Z*)-2′-demethylaplysinopsin ((*Z*)-4-((1*H*-indol-3-yl)methylene)-2-amino-1-methyl-1*H*-imidazol-5(4*H*)-one) (**4**) [22]; 2-amino-4′-hydroxyacetophenone (**5**) [23]; 4-hydroxybenzoic acid (**6**) [24] and 4-hydroxybenzaldehyde (**7**) [25] (Fig. 1). The known compounds (**2**–**7**) were identified by analysis of MS and 1D and 2D NMR data and compared to the literature.

Hesperine (**1**) was purified by C_{18} HPLC using MeOH/H_2O containing TFA as a facilitator to sharpen peaks for basic compounds and therefore it was isolated as its TFA salt as a light brown amorphous solid.

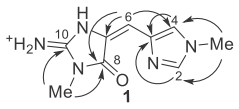

Fig. 2. Select HMBC correlations for hesperine (**1**).

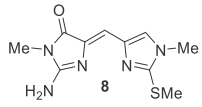

Fig. 3. Corallistine (**8**) isolated from the marine sponge *Corallistes fulvodesmus*.

It possessed a $[M]^+$ ion at *m/z* 206.1040 in the HRESIMS, consistent with the protonated molecular formula $C_9H_{12}N_5O^+$ (calcd. 206.1036). Analysis of the ^{1}H NMR (Table 1) indicated **1** contained proton resonances associated with three olefinic methine protons (δ_H 8.06, 7.71, 6.91) and two deshielded methyl groups (δ_H 3.74, 3.14). The ^{13}C NMR and HSQC data (Table 1) indicated **1** contained carbon resonances associated with three olefinic methines (δ_C 140.1, 126.0, 107.9), an amide carbonyl carbon (δ_C 162.6), a guanidine carbon (δ_C 155.2), two tertiary olefinic carbons (δ_C 134.8, 126.0) and two *N*-methyl groups (δ_C 33.6, 26.0). The characteristic *N*-methyl proton resonance at δ_H 3.74 (H_3-12) and methine proton resonances at δ_H 7.71 (H-4) and 8.06 (H-2) suggested the presence of an *N*-methylimidazole moiety. HMBC correlations (Fig. 2) to C-2 and C-4 from H-12 and from H-2 and H-4 to C-5 confirmed the assignment of the *N*-methylimidazole moiety. The amide and guanidino groups C-8 and C-10 were assigned based upon $^{3}J_{CH}$ HMBC correlations from the remaining *N*-methyl proton resonance H_3-13 to C-8 and C-10. The amide (C-8), imine (C-10) and amine functionalities (N-11) were assigned based upon the molecular formula provided by HRESIMS and the deshielded chemical shifts of H-14, H-11, C-8, C-10 and C-7, confirming the presence of the imidazolone moiety. The olefinic methine carbon (C-6) linking the imidazole and imidazolone moieties was assigned based upon HMBC correlations from H-6 to C-4, C-7, and C-8 paired with the deshielded chemical shifts of C-6 (δ_C 107.9) and C-7 (δ_C 126.0), confirming the assignment of hesperine (**1**).

To determine the geometry of the C-6/C-7 double bond in **1,** its NMR data was compared to that of an analogue, corallistine (**8**) (Fig. 3), isolated from the marine sponge *Corallistes fulvodesmus* [26]. Corallistine (**8**) is the 2-*S*-methyl analogue of hesperine (**1**). NMR data reported for **8**, contained an olefinic resonance at δ_C 99.0 that can be assigned to C-6 and this suggests **1** had a different geometry about the C-6/C-7 double bond. Data reported for *Z/E* arylmethylenehydantoins shows that C-6 in *E* regioisomers is consistently 5–8 ppm more deshielded compared to C-6 in *Z* isomers [27]. Likewise in aplysinopsin alkaloids (such as **4**), C-6 is more deshielded by 3–7 ppm in *E* regioisomers compared to *Z* regioisomers [28]. Since C-6 in **1** is 8 ppm more deshielded compared in C-6 in **8** this suggests that **1** has 6*E* geometry.

All compounds (**1**–**7**) were evaluated for both α-syn binding and anti-aggregation activity using *in vitro* MS binding and thioflavin T (ThT) amyloid dye assays respectively. The compounds were incubated with α-syn for three h at a 10:1 (compound 100 μM:protein 10 μM) molar ratio before the acquisition of a mass spectrum. The addition of peaks in the mass spectrum for **2** (See SI) mirroring the unique distribution pattern of α-syn's charged states indicated the presence of a complex that had formed between **2** and α-syn, confirming that 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) binds to the protein. All other compounds (**1**,**3**–**6**) displayed no binding activity.

The ThT amyloid dye assay was then used to evaluate how direct binding of 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) affects the aggregation of α-syn, which is considered a crucial step of the amyloid aggregation cascade. Compound **2** was incubated with α-syn at a 5:1 (compound 400 μM:protein 80 μM) molar ratio for 36 h under conditions that promote aggregation; ThT was used to quantify the

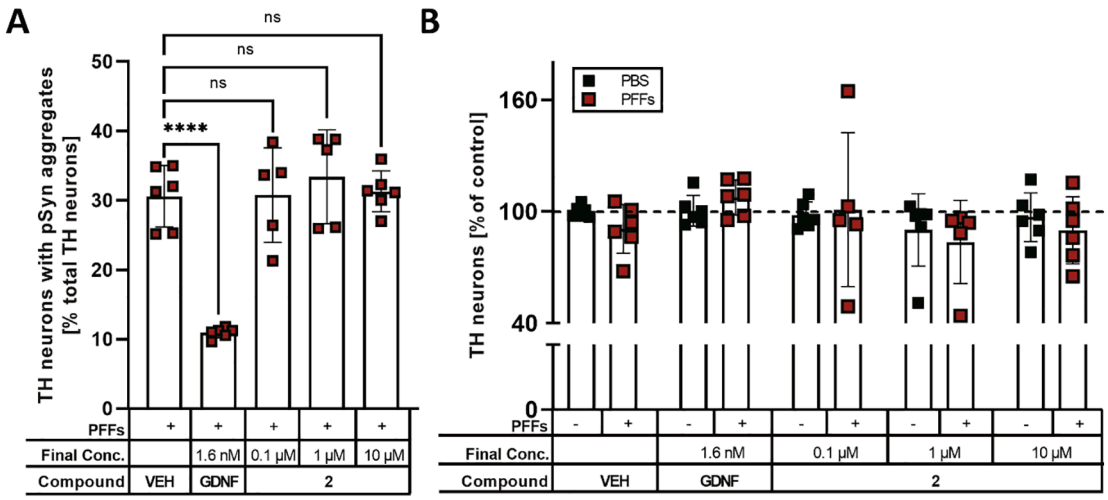

Fig. 4. (a) The effect of 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) on intracellular α-syn aggregation. The amount of primary dopaminergic mouse neurons (TH + neurons) containing pSyn aggregates (normalised to total numbers of TH neurons/well) was unchanged after treatment with (**2**) (not significant, ns). Treatment with 1.6 nM GDNF was used as a positive control (****$p < 0.0001$) (mixed-effects analysis, followed by a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli) (b) Toxicity of 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) against TH neurons. None of the compounds tested, including tested doses of (**2**), were toxic to TH neurons with or without pSyn aggregates (mixed-effects analysis, followed by a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli). n = 5–6 technical repeats, all data are mean ± SD.

presence of amyloid species. The addition of 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) did not affect the fluorescence of the amyloid dye ThT compared to the negative control (untreated α-syn), indicating that the binding of **2** has no direct effect on α-syn aggregation. All other compounds (**1**,**3**–**6**) displayed no activity in the aggregation assay (results not shown). Since the aggregation of α-syn in a functioning biological system is potentially different to induced extracellular aggregation (such as the ThT aggregation assay), **2** was also screened in a cell-based model for α-syn aggregation [17].

Midbrain dopaminergic neurons, one of the most affected neuronal populations in Parkinson's disease [29], were used to assess toxicity and α-syn aggregation. After eight days of *in vitro* culture (DIV-8), preformed α-syn fibrils (2.5 μg/mL) were introduced to cultured primary dopaminergic mouse neurons (tyrosine hydroxylase, TH, positive neurons), 15 min later 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) (0.1 μM, 1 μM and 10 μM) was introduced and the cells further incubated for seven days until DIV-15. At the end of the incubation period, neuronal cultures were fixed, and the presence of intracellular phosphorylated at Serine129 α-syn aggregates (pSyn aggregates) in TH neurons was analysed by immunostaining and compared to the negative (vehicle, VEH) and positive (glial cell-line derived neurotrophic factor, GDNF) controls. 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) did not affect the intracellular aggregation of α-syn in dopaminergic cells, or the survival of the dopaminergic neurons (Fig. 4).

Hesperine (**1**) was also evaluated for anti-bacterial activity against a methicillin-susceptible *Staphylococcus aureus* strain (ATCC25923), a methicillin-resistant *Staphylococcus aureus* strain (ATCC43300) and a Gram-negative *Pseudomonas aeruginosa* strain (ATCC27853) with a concentration range of 32 to 0.25 ug/mL, though no activity was detected. Since the close analogue corallistine (**8**), has previously been shown to be inactive against KB and P388 cells.[26] and the results obtained in our antibacterial assays suggest that compounds of this class are not likely to exhibit toxicity to cells.

1-Methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) was previously isolated from the Irish marine sponge *Clathria (Microciona) strepsitoxa* and screened for anti-microbial activity (Gram-positive - *Staphylococcus aureus* (MSSA, MRSA), Gram-negative - *Klebsiella pneumoniae*, *Escherichia coli* and *Acinetobacter baumannii*), anti-fungal activity (*Aspergillus fumigatus*) and cytotoxic activity (hepatic tumoral cell line hep_G2), though no significant activity was detected.[20] The isolation of 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) from *Clathria (Thalysias) cf. hesperia* demonstrates a potential chemotaxonomic relationship between these sponges, both of which belong to the *Clathria* genus. Doridosine, a structurally similar guanosine derivative, was isolated from the Mediterranean/Atlantic marine sponge *Tedania digitata*, currently known as *Tedania (Tedania) anhelans* [30]. Interestingly, *Tedania* belongs to the same order (Poecilosclerida) as *Clathria* sponges, which also suggests a chemotaxonomic link between these sponges [20,30].

Fluorescent probes are commonly used to monitor the interactions and behaviours of specific proteins in cellular environments [31]. The α-syn binding activity and low cytotoxicity of 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) suggest that it may be modified to fit this purpose. The addition of a fluorophore to **2** that does not affect its α-syn binding properties could yield a probe that is useful for studying the events that precede α-syn aggregation, such as the misfolding and oligomerisation. Such probes could be combined with ThT or antibodies against α-syn to provide a more detailed picture of the α-syn aggregation cascade. Similarly, modification of 1-methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) to include a bulkier component could produce a molecule that could inhibit the α-syn aggregation pathway.

In summary, a new imidazole derivative, hesperine (**1**), has been isolated from the marine sponge *Clathria (Thalysias) cf. hesperia* and its structure has been elucidated by the analysis of HRMS and 1D and 2D NMR experiments. Biological screening of hesperine (**1**) indicates that it does not have anti-bacterial or α-syn inhibitory properties. 1-Methyl-1,2,7,8-tetrahydro-2,8-dioxoadenosine (**2**) was shown to bind to the amyloid protein α-syn. This, paired with its low toxicity, suggests that it may be a promising candidate for modification to produce a fluorescent probe. Such probes could be used in combination with ThT or α-syn antibodies to monitor the aggregation of α-syn and the events that precede it.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rechem.2022.100302.

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