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α-synuclein aggregation inhibitory activity of the bromotyrosine derivatives aerothionin and aerophobin-2 from the subtropical marine sponge *Aplysinella sp*

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A B S T R A C T

The neuronal protein α-synuclein (α-syn) is one of the main constituents of intracellular amyloid aggregations found in the post-mortem brains of Parkinson's disease (PD) patients. Recently, we screened the MeOH extracts obtained from 300 sub-tropical marine invertebrates for α-syn binding activity using affinity MS and this resulted in the extract of the Verongida marine sponge *Aplysinella* sp. 1194, (QM G339263) displaying molecules that bind to the protein. The subsequent bioassay-guided separation of the *Aplysinella* sp. extract led to the isolation of the known bromotyrosine derivatives (+)-aerothionin (**1**) and (+)-aerophobin-2 (**2**). Both compounds bind to α-syn as detected by a MS affinity assay and inhibit α-syn aggregation in an assay that uses the fluorescence probe, thioflavin T, to detect aggregation. (+)-Aerothionin (**1**) was toxic to primary dopaminergic neurons at its expected α-syn aggregation inhibitory concentration and so could not be tested for inhibition of pSyn aggregates in this functional assay. (+)-Aerophobin-2 (**2**) was not toxic and shown to weakly inhibit pSyn aggregation in primary dopaminergic neurons at 10 μM.

Introduction

The neuronal protein α-synuclein (α-syn) is one of the main constituents of intracellular amyloid aggregates found in the post-mortem analyses of numerous brain regions, including the substantia nigra, of patients with Parkinson's disease (PD). These intracellular, perinuclear aggregates, commonly referred to as Lewy bodies, and similar aggregates in neurites, known as Lewy neurites, are considered to be neuropathological hallmarks of PD.[1] Although the role of α-syn aggregation in the development and progression of PD is not well understood, several studies have shown that the initiation of the α-syn aggregation cascade leads to cellular toxicity and, eventually, neurodegeneration.[2–4] The α-syn aggregation cascade describes the process by which intrinsically disordered, monomeric α-syn misfolds into several β-sheet-containing oligomeric species which further aggregate into mature amyloid fibrils.[5,6] It is now widely accepted that the oligomeric species of α-syn is the true source of toxicity associated with PD with suggestions that late-stage aggregates may form as part of a protective mechanism aimed at detoxifying the oligomeric species.[2,3,7,8] The complex events that lead to the initiation of the α-syn aggregation cascade are also not well understood, although genetic and environmental factors are thought to be involved. Familial cases of PD account for only ~5% of cases; among them rare point mutations in, and gene multiplications of the α-syn gene (*SNCA*) account for a very small proportion of cases.[9–11] Interestingly, several environmental factors such as pesticides (i.e., rotenone) have also been identified as risk factors linked to the development of PD.[12] Unlike some other drug-induced models of PD, abnormal protein aggregation is one of the phenotypes observed in rotenone-induced animal PD models.[13] Although it accounts for ~1% of cytosolic protein, α-syn's biological functions remain elusive. However, mounting evidence suggests that it is intrinsically involved with the maintenance of synaptic regulation through processes such as molecular chaperoning,[14] and vesicle trafficking.[15,16] As the incidence of PD continues to increase, so does the demand for new and improved treatments. As a result, over the last decade, inhibition of the α-syn aggregation cascade by small molecules has received more attention as a therapeutic target

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for PD.[17–19] Recent reviews have shown that the natural environment is a viable source of diverse small molecule amyloid aggregation inhibitors.[20,21].

Several assays have now been developed to identify small molecules that bind directly to α-syn and inhibit its aggregation. This includes mass spectrometry-based binding assays [22] and thioflavin T (ThT) amyloid dye aggregation assays.[23] In related studies we have used the MS based binding assay to identify molecules from complex mixtures that bind to α-syn.[24] We have also utilized the ThT aggregation assay to demonstrate the inhibition of the α-syn aggregation cascade by several of these binding molecules.[25–27] Recently, we screened the MeOH extracts obtained from 300 sub-tropical marine invertebrates for α-syn binding activity, resulting in the extract of the Verongida marine sponge *Aplysinella* sp. 1194, (QM G339263) displaying activity. The subsequent bioassay-guided separation of the *Aplysinella* sp. extract led to the isolation of the known bromotyrosine derivatives (+)-aerothionin (**1**) and (+)-aerophobin-2 (**2**) as the constituents that bind to α-syn.[28–33] (Fig. 1). Aerothionin (**1**) has previously been isolated from several Verongida sponges and shown to possess antibacterial, cytotoxic and anticancer activities.[32–34] Aerophobin-2 (**2**) has also been isolated from several Verongida sponges.[30,31] Interestingly, aerothionin (**1**) has been detected in the extract of the bacterium *Pseudovibrio denitrificans* Ab134 suggesting a microbial origin.[35] Herein, we report the α-syn binding and aggregation inhibitory activity of (+)-aerothionin (**1**) and (+)-aerophobin-2 (**2**).

The freeze-dried *Aplysinella* sp. (#1194) material was exhaustively extracted with MeOH. Mass-spectrometry based screening of the *Aplysinella* sp. extract with α-syn indicated the presence of a compound with a mass of 818 Da that binds to α-syn. The *Aplysinella* sp. extract was then separated using a preparative C_{18} silica gel HPLC column, eluted with a gradient from H_2O to MeOH. The resulting fractions were screened for α-syn binding activity yielding two pure compounds that displayed binding activity towards α-syn, aerothionin (**1**) and aerophobin-2 (**2**). Both compounds were incubated with α-syn at a 5:1 (compound 50 μM: protein 10 μM) molar ratio for 3 h before the acquisition of a mass spectrum. The addition of peaks in the mass spectrum that mirrored the unique distribution pattern of α-syn's charged states indicated the presence of complexes that had formed between α-syn and aerothionin (**1**) (Fig. 2) and α-syn and aerophobin-2 (**2**) (see supplementary file).

To assess the effect that the binding of aerothionin (**1**) and aerophobin-2 (**2**) has on the α-syn aggregation cascade, aerothionin (**1**) was incubated with α-syn at 1:1 (compound 80 μM: protein 80 μM) and 5:1 (compound 400 μM: protein 80 μM) molar ratios under conditions that promote aggregation using a ThT amyloid dye assay.[36,37] Aerophobin-2 was incubated with α-syn at 5:1 (compound 400 μM: protein 80 μM), 10:1 (compound 800 μM: protein 80 μM) and 20:1 (compound 1.60 mM: protein 80 μM) molar ratios under conditions that promote aggregation. After 30 h, aerothionin (**1**) inhibited aggregation by 50.7% (±9.7%) ($P < 0.0001$) when screened at a 1:1 M ratio and 71.6 % (±5.4 %) ($P < 0.0001$) when screened at a 5:1 M ratio (Fig. 3).

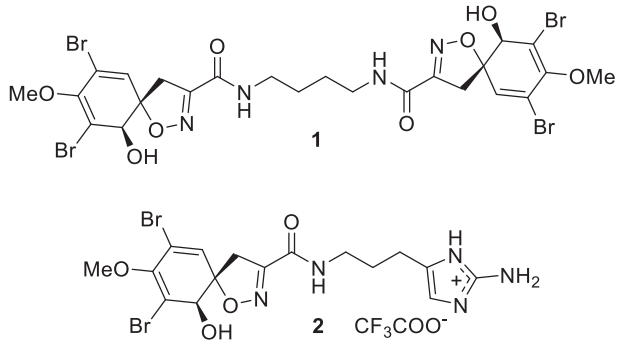

Fig. 1. Aerothionin (**1**) and aerophobin-2 (**2**) isolated from the marine sponge *Aplysinella* sp. (#1194).

Aerophobin-2 (**2**) inhibited aggregation by 42.8% (±10.6%) ($P < 0.0001$) when screened at a 10:1 M ratio and 46.0 % (±0.6 %) ($P < 0.0001$) when screened at a 20:1 M ratio (Fig. 3), aerophobin-2 (**2**) did not inhibit α-syn aggregation when screened at a 5:1 M ratio (see supplementary file).

The effect of aerothionin (**1**) and aerophobin-2 (**2**) on α-syn aggregation was then assessed using a modified version of a cell-based model for α-syn aggregation.[38] Prior to undertaking this assay the toxicity of **1** and **2** towards naïve TH neurons without fibrils after 72 h treatment was assessed so that suitable dosages below cell toxicity levels could be used in the aggregation assay. Aerothionin (**1**) was toxic at doses of 1 μM and above (see supplementary file) while aerophobin-2 (**2**) was not toxic up to the highest dose tested (10 μM). The cell-based model for α-syn aggregation utilizes pre-formed α-syn fibrils (PFFs), relatively short fibrils of recombinant α-syn, which can be internalized by cells, e.g. midbrain dopamine neurons, and then induce the formation of Lewy body-like, phosphorylated at Serine 129 α-syn (pSyn) aggregates in neurons.[39,40] Phosphorylation at Serine129 of α-syn is routinely used to detect Lewy bodies in post-mortem PD patient brains,[41] and it has also been suggested as a marker of severity of PD.[42] In this experiment, cultured primary dopaminergic neurons (Tyrosine Hydroxylase, TH positive neurons) are kept without an external supply of survival increasing neurotrophic factors (such as glial cell-line derived neurotrophic factor, GDNF) [43] for eight days of *in vitro* growth (DIV8). On DIV-8, α-syn PFFs (2.5 μg/mL) were introduced to cultured primary dopaminergic mouse neurons, and then 15 min later, positive control (GDNF), aerothionin (**1**) (0.1 μM and 0.01 μM) or aerophobin-2 (**2**) (1.0 μM and 10 μM) were introduced. Cultures were further incubated for seven days with both PFFs and compounds. At DIV15, the presence of intracellular pSyn aggregates in TH-positive neurons was analysed by immunostaining. The numbers of TH neurons with or without pSyn aggregates in compound-treated cultures were quantified by unbiased image analysis and compared to the vehicle control (VEH). Aerothionin (**1**) was inactive in preventing pSyn accumulation at its highest non-toxic dose (0.1 μM) (Fig. S9). In contrast, aerophobin-2 (**2**) was found to have a statistically significant ($p < 0.01$) effect on pSyn aggregation at 10 μM, but not at lower concentrations (Fig. 4).

Aerophobin-2 (**2**) is active in the ThT aggregation assay and we have further shown that it can effectively inhibit the intracellular aggregation of α-syn at 10 μM in dopaminergic mouse neurons. We believe these complementary assays support the hypothesis that aerophobin-2 is a α-syn aggregation inhibitor and that its cell activities suggests that it may block the internalization of short fibrils of α-syn. The toxicity of **1** in the cell assay at concentrations of 1 μM and above precluded our ability to test its intracellular aggregation inhibitory activity but there is potential that it could display significant inhibition of α-syn internalization if not for its toxicity. The observation that the weaker ThT assay aggregation inhibitor aerophobin-2 (**2**) does inhibit internalization and aggregation of α-syn in neurons provides promise for other bromotyrosine derivatives to prevent α-syn's prion-like spread and aggregation, but there is also a possibility that **2** has a different mechanism of inhibitory action in the intracellular aggregation assay compared to **1.** We have previously shown that the related compounds, aplysamine-2 (**3**) and purealidine-Q (**4**) (Fig. 5) cure prion infections in yeast, pointing to a common amyloid protein anti-aggregation mechanism,[44] while ThT α-syn aggregation inhibition screening of **3** and **4** showed that **4** inhibited aggregation.[45] This combined evidence suggest that bromotyrosine derivatives provide a viable starting point to design analogues for PD drug development with improved activity and low toxicity. Chemical modification of **2** to incorporate features present in **1** and **4** leading to an increase in *in vitro* aggregation inhibition to levels similar to that observed for aerothionin's (**1**) in the ThT aggregation assay while maintaining low toxicity would be ideal and this would also help to answer questions relating to shared or different modes of action.

A MarinLit database search has revealed 109 sponge derived compounds that contain the spirodibromocyclohexyl system that is shared

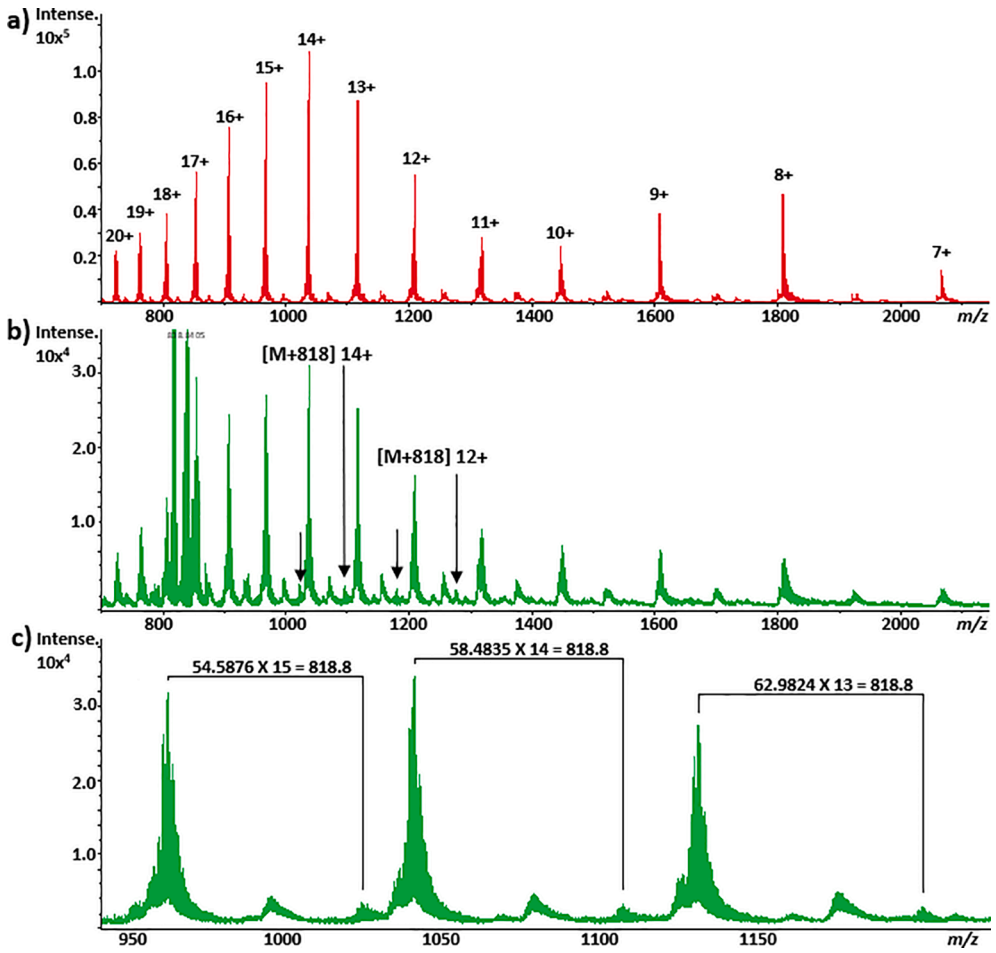

Fig. 2. α-synuclein binding assay. (a) Mass spectrum for untreated α-syn, with the peaks marked by their charged state. (b) Mass spectrum for α-syn treated with aerothionin (**1**), arrows indicating the additional peaks representing a 1:1 α-synuclein-aerothionin (**1**) complex. (c) The deconvolution process generating the mass of the bound compound.

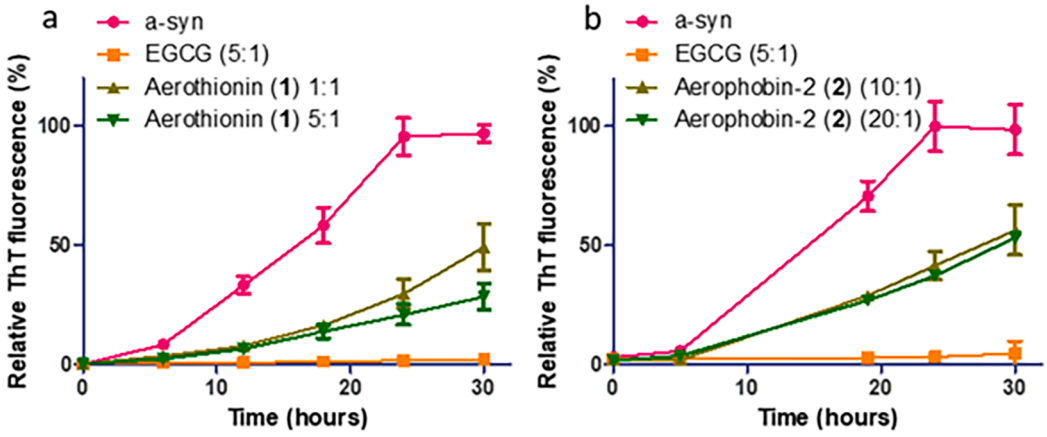

Fig. 3. a) Relative ThT fluorescence of α-syn alone and after being incubated for 30 h with the positive control EGCG at a 5:1 (EGCG 400 μM: protein 80 μM) molar ratio and aerothionin (**1**) at 1:1 (compound 80 μM: protein, 80 μM) and 5:1 (compound 400 μM: protein 80 μM) molar ratios, (Error bars = ± SD). b) Relative ThT fluorescence of α-syn alone and after being incubated for 30 h with the positive control EGCG at a 5:1 (EGCG 400 μM: protein 80 μM) molar ratio and aerophobin-2 (**2**) at 10:1 (compound 800 μM: protein, 80 μM) and 20:1 (compound 1.60 mM: protein 80 μM) molar ratios, (Error bars = ± SD).

with **1, 2** and **4**.[49] and we suggest that compounds containing the spirodibromocyclohexyl system such as fistularin-3 (**5**) and hexadellin A (**6**) be investigated for their aggregation inhibitory activity towards α-syn (Fig. 5).

Access to derivatives via synthesis would be ideal and importantly the complete asymmetric synthesis of compounds related to aerophobin-2 (**2**), such as (+) aerothionin (**1**), has now been reported.[46] Furthermore, aerothionin (**1**) is patented for the treatment of stroke, brain ischemia, cognitive disorders such as senile dementia, attention deficit disorder, cerebrovascular dementia, mild recognition

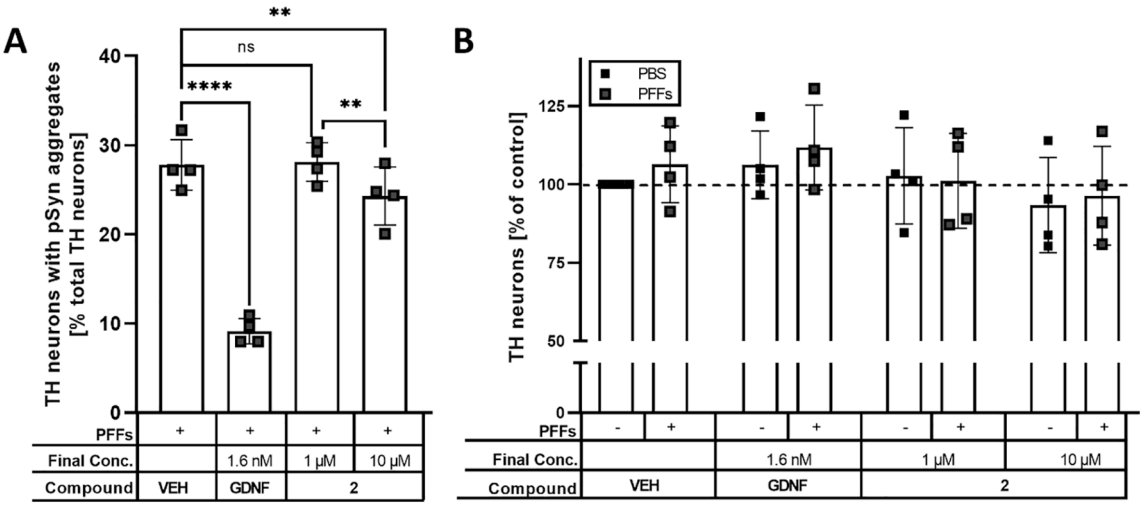

Fig. 4. (a) The effect of aerophobin-2 (**2**) on intracellular α-syn aggregation. Compared to a vehicle (VEH) control, numbers of tyrosine hydroxylase (TH) neurons containing pSyn aggregates were significantly reduced by treatment with 10 μM aerophobin-2 (**2**) (**$p < 0.01$); this effect was dose-dependent (**$p < 0.01$) and not present at the lower dose (1 μM) of (**2**) (not significant, ns). Treatment with 1.6 nM GDNF was used as a positive control (****$p < 0.0001$) (one-way ANOVA, followed by a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli). (b) Toxicity of aerophobin-2 (**2**) against primary dopaminergic mouse neurons. Neither controls (VEH and GDNF), nor the compound (**2**), regardless of inoculation with PFFs, affected the number of TH neurons in a statistically significant manner. (two-way ANOVA, followed by a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli). N = 4 independent experiments, all data are mean ± SD.

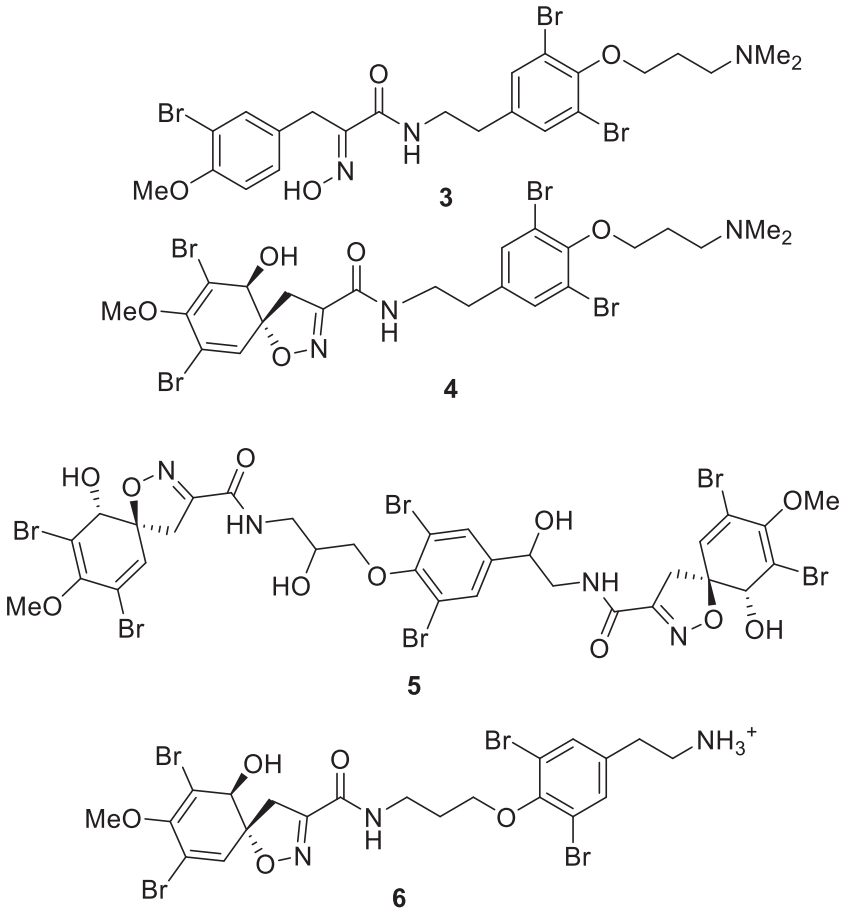

Fig. 5. Marine-derived bromotyrosine compounds aplysamine-2 (**3**) and purealidine-Q (**4**), fistularin-3 (**5**) [35] and hexadellin A (**6**) [30].

impairment, and/or neurodegenerative dementing disease with aberrant protein aggregations, especially Alzheimer's disease or condition, or prion diseases such as Creutzfeld-Jacob disease and Gerstmann-Straussler-Scheinher disease.[47] This is claimed to be due to **1**'s voltage-dependent calcium channel (VDCC) blocking activity in SH-SY5Y neuroblastoma cells (6 μM), *in vitro* inhibition of acetylcholinesterase (10 μM) and *in vitro* inhibition of butyrylcholinesterase (10 μM). [47] However, since α-syn is expressed at low basal levels in SH-SY5Y

cells and its overexpression in these cells has been shown to lead to the formation of α-syn aggregates (demonstrating the presence of the α-syn aggregation cascade),[48] the neuroprotective effect observed by Gil et al.[47] could also be attributed to interactions with α-syn. We propose that future work in this area should also consider this possibility. Aerothionin's lower reported toxicity in SH-SY5Y neuroblastoma cells also provides an opportunity to test its α-syn intracellular aggregation inhibition in a different cell line.

In summary, we have demonstrated that the bromotyrosine derivatives aerothionin (**1**) and aerophobin-2 (**2**) bind to α-syn and inhibit its aggregation in a ThT aggregation assay. We have also demonstrated that aerophobin-2 (**2**) inhibits the aggregation of α-syn in primary dopaminergic mouse neurons. We believe the activity displayed by aerothionin (**1**) and aerophobin-2 (**2**) suggests that other bromotyrosine derivatives may show activity towards inhibition of spreading prion-like forms of α-syn. For this reason, we suggest that related bromotyrosine derivatives be screened for their activity against α-syn.

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.

Data availability

Data will be made available on request.

Acknowledgments

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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.2022.100472.

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