

Article

Efficient Two-Step Synthesis of Novel Pyrimido[4,5-*d*] Pyrimidines with Potent Neuroprotective, Antioxidant, and A β Anti-Aggregation Properties

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Abstract: Eleven new differently substituted *N*,7-diphenylpyrimido [4,5-*d*]pyrimidin-4-amines **4a–k** were synthesized from readily available reagents in a simple and inexpensive two-step procedure with yields up to 57%. Neuroprotective analysis against H₂O₂ and analysis using ORAC assays identified compounds **4g**, **4i** and **4j** as promising antioxidant compounds. These compounds also showed potent inhibition of A β _{1–42} self-aggregation, and suitable physicochemical properties predicted by Datawarrior software V6.1.0, this biological activity and physicochemical property being of great interest for pathologies linked to oxidative stress, such as Alzheimer's disease.

Keywords: A β _{1–42} self-aggregation inhibition; antioxidant; neuroprotective agents; pyrimido [4,5-*d*] pyrimidines



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1. Introduction

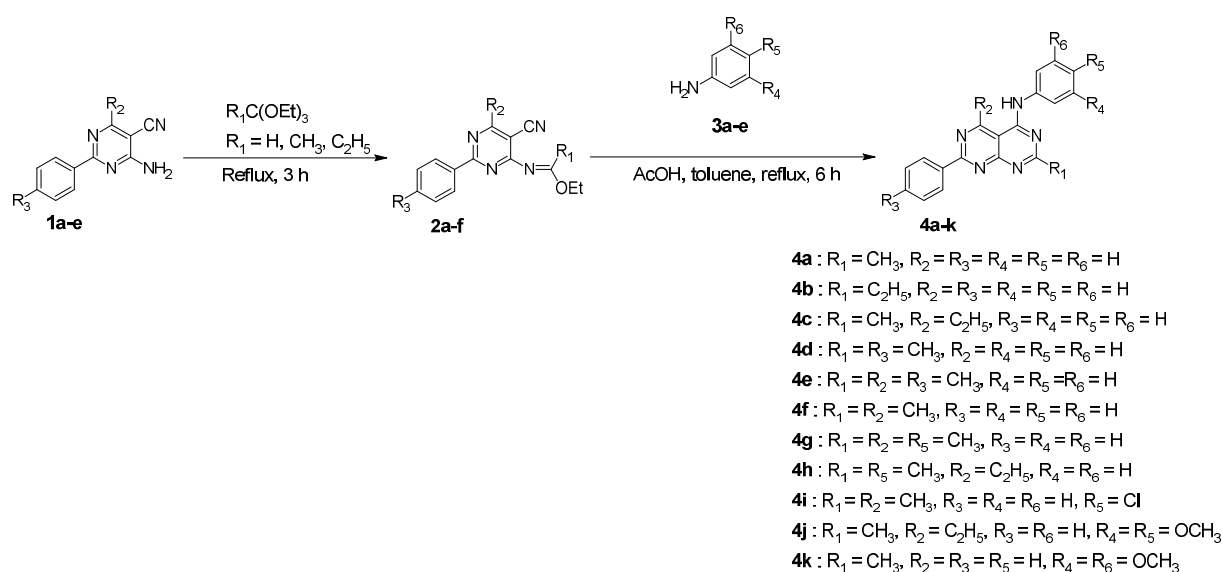
Over the past decade, we have been actively involved in the design, synthesis and pharmacological evaluation of diversely functionalized heterocyclic derivatives as multi-target small molecules for the potential treatment of Alzheimer's disease (AD) [1–3]. Our approach has always been motivated by preparing these molecules by using sustainable synthetic processes, either by reducing the number of steps (2 or 3) [4,5] or by favoring multicomponent reactions [2,3].

Among the many neuropathological features of AD (cholinergic deficit, intracellular neurofibrillary tangles and beta-amyloid (A β) deposition), oxidative stress (OS) plays a fundamental role in the biological events leading to this neurodegenerative disease [6,7]. In fact, histopathological data obtained from extracellular A β deposits have shown that the release of reactive oxygen species (ROS) can severely damage mitochondria and other cellular contents of neurons [8–10]. In light of these observations, the search for new and more effective neuroprotective and antioxidant agents for the treatment of AD has been intense in recent years [11–14].

In this context, we have developed several new compounds based on azaheterocyclic scaffolds [15,16]. In fact, nitrogen-containing heterocycles have been widely used in medicinal chemistry to find new bioactive molecules for AD [17–20]. In particular, our

work reported in 2020 [21] describes the discovery of a new pyrimidopyrimidine scaffold as dual-target small molecules for AD therapy. Continuing our work in this field, we were interested in exploring the nitrogen bioisosteres of pyrimidopyrimidines, in particular pyrimidopyrimidines. Indeed, compounds bearing the pyrimidopyrimidine scaffold have been reported to exhibit a wide range of biological activities, such as antibacterial, antiviral, antitumor, antiallergic, antihypertensive and hepatoprotective effects, making this scaffold of particular interest [22,23].

In this report, we describe the synthesis of eleven new pyrimidopyrimidines **4a–k** (Scheme 1) and the evaluation of their neuroprotective and antioxidant properties using ORAC assays. Our results have identified compounds **4g**, **4i** and **4j** as promising neuroprotective and antioxidant agents, which also exhibit potent inhibition of A β self-aggregation.



Scheme 1. Synthesis of pyrimidopyrimidines **4a–k**.

2. Results

2.1. Synthesis

The synthesis of the new diphenylpyrimido [4,5-*d*]pyrimidin-4-amines **4a–k** was carried out in two steps, as shown in Scheme 1, from moderate to good overall results.

The first step involves the reaction of readily available 4-amino-2,6-alkylpyrimidine-5-carbonitrile **1a–e** with triethyl orthoester derivatives at reflux. Next, in the second step, the resulting intermediate imidates **2a–f** were then reacted with various commercially available substituted anilines in refluxing toluene in the presence of a catalytic amount of acetic acid. It is interesting to note that precursors bearing no substituent at position 6 of the pyrimidinopyrimidine nucleus and on the aromatic ring attached at position 8 always gave to the best yields observed in this reaction, ranging between 47% and 57% (Table 1).

A *p*-tolyl substituent in position 7 (R₃ = Me) consistently leads to low yields, as shown by compounds **4d** (16%) and **4e** (20%). Similarly, the presence of a chlorine or methoxy group in *para* position of the aromatic ring attached to the amine also results in modest yields, as demonstrated by compounds **4i** (22%) and **4j** (20%).

All new compounds showed excellent analytical and spectroscopic data, in good agreement with the expected values (see Section 3, and Supplementary Materials).

Table 1. Synthesis of diphenylpyrimido [4,5-*d*] pyrimidin-4-amines derivatives **4a–k** using 2 steps.

Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	Yield (%)
4a	CH ₃	H	H	H	H	H	57
4b	C ₂ H ₅	H	H	H	H	H	50
4c	CH ₃	C ₂ H ₅	H	H	H	H	35
4d	CH ₃	H	CH ₃	H	H	H	16
4e	CH ₃	CH ₃	CH ₃	H	H	H	20
4f	CH ₃	CH ₃	H	H	H	H	50
4g	CH ₃	CH ₃	H	H	CH ₃	H	28
4h	CH ₃	C ₂ H ₅	H	H	CH ₃	H	30
4i	CH ₃	CH ₃	H	H	Cl	H	22
4j	CH ₃	C ₂ H ₅	H	OCH ₃	OCH ₃	H	20
4k	CH ₃	H	H	OCH ₃	H	OCH ₃	47

2.2. Biological Evaluation

2.2.1. In Cellulo Evaluation of the Neuroprotective Activity of Compounds **4a–k**

The neuroprotective potential of compounds **4a–k** was evaluated by examining their ability to inhibit cell death in the human neuroblastoma cell line SH-SY5Y induced by hydrogen peroxide (H₂O₂). H₂O₂ is known to generate exogenous free radicals, leading to a cytotoxic effect that reduces cell viability to 65% at a concentration of 200 mM compared to untreated cells. Therefore, compounds that can prevent or mitigate these damaging effects are considered to have neuroprotective properties [24].

Before performing the neuroprotection assay, we evaluated the direct toxicity of compounds **4a–k** over a concentration range from 0.1 to 10 µM. Cell viability was measured using the MTT assay [25], which indicated that there were no cytotoxic effects on SH-SY5Y cells at concentrations up to 5 µM.

Subsequently, compounds to be tested for neuroprotection were evaluated at 0.1, 1 and 5 µM. The results are summarized in Table 2.

These results highlight the significant efficacy of several compounds, particularly molecules **4g**, **4h–j**. Compound **4g** demonstrated a significant and strong neuroprotective effect in response to H₂O₂ with percentages of 47%, 68%, and 77% at concentrations of 0.1 µM, 1 µM, and 5 µM, respectively. Compound **4h** exerts its neuroprotective power mainly at 0.1 µM with 66%, but remains significantly neuroprotective, though at lower levels: 39% at 1 µM and 26% at 5 µM. Compound **4i**, undoubtedly the best neuroprotectant, completely or almost completely reversed the toxicity of H₂O₂ (118% at 1 µM and 95% at 5 µM). However, it remains an excellent neuroprotectant even at 0.1 µM with a percentage of 68%. Finally, compound **4j** shows a significant neuroprotective effect only at concentrations of 0.1 µM and 1 µM with 67% and 46%, respectively.

From a structure–activity relationship perspective, it is clear that these four compounds share similarities. Indeed, a double substitution at positions 2 and 6 with an alkyl group seems to be essential for the neuroprotective activity, combined with a substituent of whatever nature (alkyl, methoxy or chlorine) in the *para* position of the aromatic ring attached to the aromatic amine.

Table 2. Neuroprotective activity on H₂O₂ -induced cell death in SH-SY5Y cells and ORAC values of compounds **4a–k**.

Compound	Neuroprotection (%) against H ₂ O ₂ ^a at 0.1, 1 and 5 μ M			ORAC ^c (TE)
	0.1 μ M	1 μ M	5 μ M	
4a	– ^b	– ^b	– ^b	0.07 $\pm$ 0.00
4b	6.17 $\pm$ 0.01	4.95 $\pm$ 0.01	14.66 $\pm$ 0.04	– ^b
4c	6.96 $\pm$ 0.02	3.09 $\pm$ 0.2	8.64 $\pm$ 0.05	0.24 $\pm$ 0.02
4d	9.40 $\pm$ 0.01	– ^b	– ^b	0.14 $\pm$ 0.01
4e	– ^b	34.00 $\pm$ 0.09 *	8.45 $\pm$ 0.2	0.17 $\pm$ 0.01
4f	4.75 $\pm$ 0.01	6.98 $\pm$ 0.02	24.30 $\pm$ 0.02 *	0.19 $\pm$ 0.02
4g	47.03 $\pm$ 0.12 **	68.06 $\pm$ 0.22 **	77.55 $\pm$ 0.21 **	0.24 $\pm$ 0.02
4h	66.13 $\pm$ 0.17 **	38.77 $\pm$ 0.07 **	26.12 $\pm$ 0.16 **	– ^b
4i	68.35 $\pm$ 0.23 **	117.89 $\pm$ 0.04 ***	94.54 $\pm$ 0.33 ***	0.34 $\pm$ 0.01
4j	66.94 $\pm$ 0.23 **	45.89 $\pm$ 0.187 **	11.14 $\pm$ 0.15	1.64 $\pm$ 0.02
4k	0.13 $\pm$ 0.01	– ^b	– ^b	– ^b
Trolox	– ^d	– ^d	– ^d	0.99 $\pm$ 0.01
Melatonin	– ^d	– ^d	– ^d	2.45 $\pm$ 0.09

^a Data expressed as % neuroprotection $\pm$ SEM of quadruplicates from at least three different cultures; * p < 0.05, ** p < 0.01 and *** p < 0.001, as compared to the control cultures (one-way ANOVA). ^b not active, ^c Data are expressed as Trolox equivalents and are the mean (n = 3) $\pm$ SEM. ^d Not determined.

2.2.2. Antioxidant Analysis

The antioxidant capacity of compounds **4a–k** to scavenge peroxyl radicals was evaluated using the ORAC-FL (Oxygen Radical Absorbance Capacity by Fluorescence) assay [26,27]. Fluorescein (FL) was used as the fluorescent probe and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as the standard compound. Results were expressed in micromoles of Trolox equivalents (TE). Melatonin and Trolox were used as positive controls, with ORAC values of 2.4 TE and 0.99 TE, respectively, in agreement with previously reported values [1,28].

As shown in Table 2, three compounds showed no activity, while the remaining compounds showed a modest ability to trap peroxyl radicals. The ORAC values ranged from 0.07 TE (compound **4a**) to 1.64 TE (compound **4j**). Among the best neuroprotective compounds, **4g**, **4i** and **4j** showed antioxidant activities with ORAC values of 0.24, 0.34 and 1.64 TE, respectively. In particular, compound **4j** is 1.6-fold more active than Trolox and has almost 70% of the antioxidant activity of melatonin, one of the best-known antioxidants.

2.2.3. Inhibition of Self-Induced A β _{1–42} Aggregation by Compounds **4g**, **4i** and **4j**

The accumulation and aggregation of A β _{1–42} in the brain may lead to neurotoxicity and contribute to the development of AD. According to the amyloid cascade hypothesis, A β aggregation and deposition are responsible for the formation of senile plaques, cell damage and dementia in AD [29]. Therefore, compounds that can prevent the self-aggregation of A β _{1–42} may slow the progression of AD. Three compounds, selected for their neuroprotective and antioxidant activities, were tested for their ability to inhibit self-induced A β _{1–42} aggregation. The thioflavin T-based fluorometric assay [30] was used to evaluate this inhibitory effect and the results are summarized in Figure 1.

Based on the results of this experiment, compounds **4g**, **4i** and **4j** showed significant inhibition of A β _{1–42} aggregation, with inhibition rates of 16.3%, 25.7% and 56.6%, respectively, compared to 73.3% for curcumin used as a reference standard at a concentration of 2.5 μ M. These results suggest that our compounds have multifunctional properties.

The Thioflavin-T fluorescence method was used. The values are expressed as the mean $\pm$ SD of at least three independent measurements. All values were obtained at a compound concentration of 2.5 μ M.

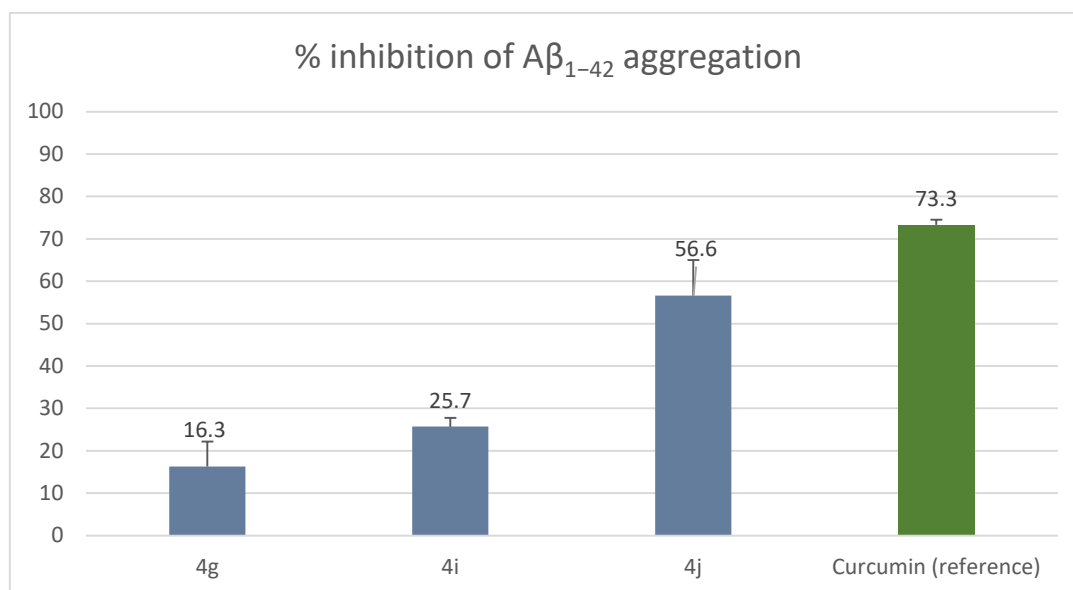


Figure 1. Inhibition of A β ₁₋₄₂ self-aggregation (%) by selected compounds.

2.2.4. ADME Studies

In order to predict the physicochemical properties of the compounds, we used 'Data Warrior V6.1.0', physical and chemical property visualization and analysis software developed by Idorsia Pharmaceuticals Ltd., 4123 Allschwil, Switzerland. This tool allows the prediction of drug-like properties using various parameters from Lipinski's rule of five, including molecular weight, LogP, LogS, hydrogen bond acceptors, hydrogen bond donors and topological polar surface area (TPSA).

As shown in Table 3, all compounds had molecular weights below 500 g/mol, a value typical of most oral drugs and used as the basis for Lipinski's rule of five. Lipophilicity, a critical physicochemical property, determines whether a molecule can cross biological membranes with a predicted CLogP less than 5. Interestingly, almost all the compounds showed suitable lipophilicity, with CLogP values ranging from 3.7393 to 4.8832, except for compound 4h which had a slightly higher CLogP of 5.0367. This value remains well below the upper limit of 6.5 for druggable compounds.

Most compounds had a number of hydrogen bond donors and acceptors in accordance with Lipinski's rule of five. The number of hydrogen bond donors was below 5 and the number of acceptors was also below 10. Notably, compounds 4j and 4k had slightly more hydrogen bond acceptors than the values suggested by Lipinski's rule.

TPSA represents the van der Waals surface area of the polar atoms of the molecule. In other words, PSA is the surface area associated with heteroatoms (such as oxygen, nitrogen and phosphorus atoms) and polar hydrogen atoms. According to Veber's rule, the polar surface area should not exceed 140 Å². It is noteworthy that all compounds had a TPSA of less than 75 Å².

Toxicity, particularly cardiac toxicity due to inhibition of the hERG (human Ether-à-go-go-Related Gene) channel, is a significant concern in drug development. The hERG channel plays a crucial role in the repolarization of the cardiac action potential, and its inhibition can lead to potentially fatal arrhythmias, which can result in sudden death [31,32]. In this context, hERG inhibition was predicted by PreADMET, one of several web-based applications developed to meet the need for rapid prediction of drug-likeness and ADME/Tox data, available as open source.

As shown in Table 3, no compounds showed a high risk of hERG inhibition. Three compounds showed a low risk, while eight compounds displayed a medium risk. It is worth noting that compounds with substituents on the aromatic rings attached to the pyrimidopyrimidine core always lead to a medium risk, whereas the absence of substituents leads to a low risk.

Table 3. Physicochemical properties of the synthesized compounds calculated by Data Warrior and hERG inhibition predicted by PreAdmet.

Compound	Molweight (g/mol)	CLogP	H-Acceptors	H-Donors	Topological Polar Surface Area (Å ²)	hERG Inhibition
4a	312.375	3.8793	4	0	51.56	low_risk
4b	326.402	4.2949	4	0	51.56	low_risk
4c	340.429	4.6928	4	0	51.56	medium_risk
4d	326.402	4.2232	4	0	51.56	medium_risk
4e	340.429	4.6211	4	0	51.56	medium_risk
4f	326.402	4.2772	4	0	51.56	low_risk
4g	340.429	4.6211	4	0	51.56	medium_risk
4h	354.456	5.0367	4	0	51.56	medium_risk
4i	360.847	4.8832	4	0	51.56	medium_risk
4j	400.481	4.5528	6	0	70.02	medium_risk
4k	372.427	3.7393	6	0	70.02	medium_risk

3. Materials and Methods

Melting points (°C) were determined using a Kofler hot bench and are uncorrected. Analytical thin-layer chromatography (TLC) was performed on silica gel precoated aluminium sheets (type 60 F254, 0.25 mm thickness, from Merck, Darmstadt, Germany) to monitor the progress of the reactions and to assess the purity and homogeneity of the synthesized products. High-resolution mass spectra (HRMS) were obtained using a Bruker micrOTOF-Q II spectrometer (Bruker Daltonics, Billerica, MA, USA) with positive electrospray ionization time-of-flight at the UCA Clermont Ferrand, France. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker DRX-400 Avance spectrometer (operating at 400 MHz for ¹H and 100 MHz for ¹³C) using dimethylsulfoxide (DMSO-d₆) as solvent and tetramethylsilane (TMS) as internal standard. Chemical shifts are given in parts per million (ppm) and the multiplicities of the ¹H NMR signals are given as follows: s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Coupling constants are given in hertz (Hz).

3.1. Synthesis of Compounds 2a–k

General experimental procedure for synthesis of imidate (2a–f)

A mixture of 4-amino-2,6-arylpyrimidine-5-carbonitrile **1a–e** (1 mmol) and triethylorthoester (4 mmol) was refluxed for 3 h without special atmospheric precautions. After cooling, the solvent was evaporated. The resulting solid was collected by filtration and recrystallized from ether.

Ethyl N-(5-cyano-6-methyl-2-phenylpyrimidin-4-yl)acetimidate 2a

Beige solid; Yield: 74%; mp: 66–68 °C; IR: ν (cm^{−1}) 2210, 1660; ¹H NMR (CDCl₃, 400 MHz) [δ ppm]: 8.38 (d, ³J = 8 Hz, 2 H), 7.45–7.39 (m, 3 H), 4.32 (q, ³J = 7.1 Hz, 2 H), 2.68 (s, 3 H), 2.07 (s, 3 H), 1.33 (t, ³J = 7.1 Hz, 3 H); ¹³C NMR (CDCl₃, 100 MHz) [δ ppm]: 170.39, 167.42, 165.02, 164.17, 135.39, 130.69, 127.91, 127.46, 114.25, 97.18, 62.68, 22.52, 16.96, 12.83. HRMS (ESI, M+H⁺) Calcd for C₁₆H₁₇N₄O: 281.13969. Found: 281.1396.

Ethyl N-(5-cyano-2-phenylpyrimidin-4-yl)acetimidate 2b

Beige solid; Yield: 94%; mp: 90–92 °C; IR: ν (cm^{−1}): 2210, 1660; ¹H NMR (CDCl₃, 400 MHz) [δ ppm]: 8.88 (s, 1 H), 8.47 (d, ³J = 8.3 Hz, 2 H), 7.56–7.50 (m, 3 H), 4.43 (q, ³J = 7.1

Hz, 2 H), 2.20 (s, 3 H), 1.44 (t, $^3J = 7.1$ Hz, 3 H); ^{13}C NMR (CDCl_3 , 100 MHz) [δ ppm]: 167.88, 166.59, 166.51, 161.11, 136.36, 132.08, 129.06, 128.68, 128.55, 115.07, 99.48, 64, 18.14, 13.92. HRMS (ESI, $\text{M}+\text{H}^+$) Calcd for $\text{C}_{15}\text{H}_{15}\text{N}_4\text{O}$: 267.12404. Found: 267.1238.

Ethyl N-(5-cyano-2-phenylpyrimidin-4-yl)Propionimide 2c

Viscous oil; Yield: 71%; IR: ν (cm^{-1}): 2227, 1650; ^1H NMR (CDCl_3 , 400 MHz) [δ ppm]: 8.87 (s, 1 H), 8.47 (d, $^3J = 8.2$, 2 H), 7.53 (m, 3 H), 4.42 (q, $^3J = 7.1$ Hz, 2 H), 2.48 (q, $^3J = 7.6$ Hz, 2 H), 1.44 (t, $^3J = 7.1$ Hz, 3 H), 1.26 (t, $^3J = 7.6$ Hz, 3 H); ^{13}C NMR (CDCl_3 , 100 MHz) [δ ppm]: 169.65, 167.85, 166.52, 161.04, 136.38, 132.06, 129.05, 128.90, 128.69, 128.58, 115.15, 99.29, 63.89, 25.47, 13.90, 10.67. HRMS (ESI, $\text{M}+\text{H}^+$) Calcd for $\text{C}_{16}\text{H}_{17}\text{N}_4\text{O}$: 281.13969. Found: 281.1397.

Ethyl N-(5-cyano-6-ethyl-2-phenylpyrimidin-4-yl)acetimidate 2d

Beige solid; Yield: 90%; mp: 90–92 °C; IR ν (cm^{-1}): 2220, 1625; ^1H NMR (CDCl_3 , 400 MHz) [δ ppm]: 8.50 (d, $^3J = 8.1$ Hz, 2 H), 7.56–7.48 (m, 3 H), 4.42 (q, $^3J = 7.1$ Hz, 2 H), 3.07 (q, $^3J = 7.5$ Hz, 2 H), 2.17 (s, 1 H), 1.46–1.41 (m, 6 H); ^{13}C NMR (CDCl_3 , 100 MHz) [δ ppm]: 176.00, 168.06, 166.09, 165.51, 136.73, 131.78, 129.10, 128.56, 115.25, 97.58, 63.77, 30.18, 18.06, 13.97, 12.24. HRMS (ESI, $\text{M}+\text{H}^+$) Calcd for $\text{C}_{17}\text{H}_{19}\text{N}_4\text{O}$: 295.15534. Found: 295.1553.

Ethyl N-(5-cyano-2-(p-tolyl)pyrimidin-4-yl)acetimidate 2e

Beige solid; Yield: 53%; mp: 254–256 °C; IR ν (cm^{-1}): 2225, 1641; ^1H NMR (CDCl_3 , 400 MHz) [δ ppm]: 8.85 (s, 1 H), 8.36 (d, $^3J = 8.2$ Hz, 2 H), 7.32 (d, $^3J = 8.2$ Hz, 2 H), 4.42 (q, $^3J = 7.1$ Hz, 2 H), 2.45 (s, 3 H), 2.18 (s, 3 H), 1.43 (t, $^3J = 7.1$ Hz, 3 H); ^{13}C NMR (CDCl_3 , 100 MHz) [δ ppm]: 167.84, 166.55, 166.51, 161.08, 142.79, 133.66, 129.49, 129.08, 115.23, 99.05, 63.96, 21.62, 18.1, 13.95. HRMS (ESI, $\text{M}+\text{H}^+$) Calcd for $\text{C}_{16}\text{H}_{17}\text{N}_4\text{O}$: 281.13969. Found: 281.1394.

Ethyl N-(5-cyano-6-methyl-2-(p-tolyl)pyrimidin-4-yl)acetimidate 2f

White solid; Yield: 45%; mp: 220–222 °C; IR ν (cm^{-1}): 2212, 1662; ^1H NMR (CDCl_3 , 400 MHz) [δ ppm]: 8.37 (d, $^3J = 7.4$ Hz, 2 H), 7.30 (d, $^3J = 7.4$ Hz, 2 H), 4.41 (q, $^3J = 7.1$ Hz, 2 H), 2.76 (s, 3 H), 2.45 (s, 3 H), 2.15 (s, 3 H), 1.43 (t, $^3J = 7.1$ Hz, 3 H); ^{13}C NMR (CDCl_3 , 100 MHz) [δ ppm]: 171.40, 168.53, 166.03, 165.38, 142.42, 133.87, 129.37, 129.08, 115.49, 97.92, 63.75, 23.64, 21.59, 18.05, 13.96. HRMS (ESI, $\text{M}+\text{H}^+$) Calcd for $\text{C}_{17}\text{H}_{19}\text{N}_4\text{O}$: 295.15534. Found: 295.1553.

3.2. Synthesis of Compounds 4a–k

General experimental procedure for synthesis of pyrimido [4,5-d]pyrimidin-4-amines 4a–k

A mixture of imidate 2 (1 mmol) and aniline derivatives 3 (1 mmol) was refluxed for 6 h with acetic acid (1 mmol) as catalyst in anhydrous toluene (3 mL) without special atmospheric precautions. After evaporation of the solvent, the resulting product was filtered and recrystallized from ether to give the desired compounds 4a–k.

2-Methyl-N,7-diphenylpyrimido [4,5-d]pyrimidin-4-amine (4a):

Beige solid; Yield: 57%; mp > 260 °C; IR ν (cm^{-1}): 3326, 1610; ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) [δ ppm]: 10.38 (s, 1 H), 10.01 (s, 1 H), 8.53 (d, $^3J = 7.2$ Hz, 2 H), 7.89 (d, $^3J = 7.2$ Hz, 2 H), 7.59 (m, 3 H), 7.44 (t, $^3J = 7.5$ Hz, 2 H), 7.19 (t, $^3J = 7.5$ Hz, 1 H), 2.55 (s, 3 H); ^{13}C NMR ($\text{DMSO}-d_6$, 100 MHz) [δ ppm]: 172.53, 166.05, 163.27, 158.80, 158.19, 138.89, 137.24, 132.16, 129.27, 129.10, 129.01, 124.93, 122.85, 105.07, 27.47. HRMS (ESI, $\text{M}+\text{H}^+$) Calcd for $\text{C}_{19}\text{H}_{16}\text{N}_5$: 314.14002. Found: 314.1400.

2-Ethyl-N,7-diphenylpyrimido [4,5-d]pyrimidin-4-amine (4b):

Beige solid; Yield: 50%; mp: 264–266 °C; IR ν (cm^{-1}): 3414, 1626; ^1H NMR ($\text{DMSO}-d_6$, 400 MHz) [δ ppm]: 10.40 (s, 1 H), 10.04 (s, 1 H), 8.55 (d, $^3J = 7.8$ Hz, 2 H), 7.92 (d, $^3J = 7.8$ Hz, 2 H), 7.61–7.59 (m, 3 H), 7.45 (t, $^3J = 7.7$ Hz, 2 H), 7.19 (t, $^3J = 7.7$ Hz, 1 H), 2.84 (q, $^3J = 7.5$ Hz, 2 H), 1.32 (t, $^3J = 7.5$ Hz, 3 H); ^{13}C NMR ($\text{DMSO}-d_6$, 100 MHz) [δ ppm]: 166.12, 158.91, 158.26, 137.24, 132.21, 129.31, 129.09, 129.04, 124.89, 122.72, 105.33, 33.43, 12.40. HRMS (ESI, $\text{M}+\text{H}^+$) Calcd for $\text{C}_{20}\text{H}_{18}\text{N}_5$: 328.15567. Found: 328.1555.

5-Ethyl-2-methyl-N,7-diphenylpyrimido [4,5-d]pyrimidin-4-amine (4c):

Beige solid; Yield: 35%; mp: 224–226 °C; IR ν (cm⁻¹): 3484, 1605; ¹H NMR (CDCl₃, 400 MHz) [δ ppm]: 8.74 (d, ³J = 7.6 Hz, 2 H), 7.77 (d, ³J = 7.6 Hz, 2 H), 7.73 (s, 1 H), 7.54–7.52 (m, 3 H), 7.46 (t, ³J = 7.5 Hz, 2 H), 7.25 (t, ³J = 7.5 Hz, 1 H), 3.45 (q, ³J = 7.0 Hz, 2 H), 2.73 (s, 3 H), 1.67 (t, ³J = 7.0 Hz, 3 H); ¹³C NMR (CDCl₃, 100 MHz) [δ ppm]: 171.42, 169.48, 165.14, 164.91, 158.20, 137.82, 136.90, 131.67, 129.34, 129.17, 128.49, 125.23, 121.91, 104.17, 32.38, 26.85, 11. HRMS (ESI, M+H⁺) Calcd for C₂₁H₂₀N₅: 342.17132. Found: 342.1714.

2-Methyl-N-phenyl-7-(p-tolyl)pyrimido [4,5-d]pyrimidin-4-amine (4d):

White solid; Yield: 16%; mp: 234 °C; IR ν (cm⁻¹): 3462, 1611; ¹H NMR (DMSO-d₆, 400 MHz) [δ ppm]: 10.37 (s, 1 H), 10.03 (s, 1 H), 8.43 (d, ³J = 7.9 Hz, 2 H), 7.90 (d, ³J = 7.9 Hz, 2 H), 7.44 (t, ³J = 7.7 Hz, 2 H), 7.38 (d, ³J = 7.8 Hz, 2 H), 7.19 (t, ³J = 7.7 Hz, 1 H), 2.56 (s, 3 H), 2.41 (s, 3 H); ¹³C NMR (DMSO-d₆, 100 MHz) [δ ppm]: 172.40, 166.21, 163.20, 158.83, 158.15, 142.23, 138.98, 134.63, 129.89, 129.08, 129.05, 124.93, 122.88, 104.91, 27.38, 21.57. HRMS (ESI, M+H⁺) Calcd for C₂₀H₁₈N₅: 328.15567. Found: 328.1555.

2,5-Dimethyl-N-phenyl-7-(p-tolyl)pyrimido [4,5-d]pyrimidin-4-amine (4e):

Beige solid; Yield: 20%; mp: 226 °C; IR ν (cm⁻¹): 3478, 1607; ¹H NMR (CDCl₃, 400 MHz) [δ ppm]: 8.58 (m, 2 H); 7.76 (m, 2 H), 7.45 (m, 2 H), 7.32–7.25 (m, 3 H), 3.20 (s, 3 H), 2.71 (s, 3 H), 2.44 (s, 3 H); ¹³C NMR (CDCl₃, 100 MHz) [δ ppm]: 21.58, 26.84, 27.33, 104.46, 121.90, 125.19, 128.90, 129.12, 129.28, 133.94, 137.80, 142.19, 158.47, 164.76, 165.13, 165.44, 171.56. HRMS (ESI, M+H⁺) Calcd for C₂₁H₂₀N₅: 342.17132. Found: 342.1714.

2,5-Dimethyl-N,7-diphenylpyrimido [4,5-d]pyrimidin-4-amine (4f):

Beige solid; Yield: 50%; mp: 230 °C; IR ν (cm⁻¹): 3291, 1606; ¹H NMR (CDCl₃, 400 MHz) [δ ppm]: 8.70 (d, ³J = 7.2 Hz, 2 H), 7.78–7.74 (m, 3 H), 7.52 (d, ³J = 7.7 Hz, 2 H), 7.46 (t, ³J = 7.7 Hz, 2 H), 7.25 (t, ³J = 0.77, 1 H), 3.21 (s, 3 H), 2.73 (s, 3 H); ¹³C NMR (CDCl₃, 100 MHz) [δ ppm]: 171.77, 165.41, 165.26, 164.76, 158.52, 137.70, 136.63, 131.68, 129.29, 129.15, 128.50, 125.30, 121.95, 104.62, 27.35, 26.90. HRMS (ESI, M+H⁺) Calcd for C₂₀H₁₈N₅: 328.15567. Found: 328.1554.

2,5-Dimethyl-7-phenyl-N-(p-tolyl)pyrimido [4,5-d]pyrimidin-4-amine (4g):

Beige solid; Yield: 28%; mp: 176–178 °C; IR ν (cm⁻¹): 3467, 1606; ¹H NMR (CDCl₃, 400 MHz) [δ ppm]: 8.65 (d, ³J = 6.6 Hz, 2 H), 7.64 (d, ³J = 8.1 Hz, 2 H), 7.54–7.47 (m, 3 H), 7.24 (d, ³J = 8.1 Hz, 2 H), 3.20 (s, 3 H), 2.66 (s, 3 H), 2.41 (s, 3 H). ¹³C NMR (CDCl₃, 100 MHz) [δ ppm]: 171.65, 165.32, 158.51, 136.59, 135.22, 135.06, 131.66, 129.66, 129.26, 128.49, 122.09, 104.60, 27.35, 26.82, 21.02. HRMS (ESI, M+H⁺) Calcd for C₂₁H₂₀N₅: 342.17132. Found: 342.1713.

5-Ethyl-2-methyl-7-phenyl-N-(p-tolyl)pyrimido [4,5-d]pyrimidin-4-amine (4h):

Beige solid; Yield: 30%; mp: 148–150 °C; IR ν (cm⁻¹): 3460, 1604; ¹H NMR (CDCl₃, 400 MHz) [δ ppm]: 8.71 (d, ³J = 6.8 Hz, 2 H), 7.63 (d, ³J = 8.1 Hz, 2 H), 7.54–7.50 (m, 3 H), 7.25 (d, ³J = 8.1 Hz, 2 H), 3.44 (q, ³J = 7.2 Hz, 2 H), 2.69 (s, 3 H), 2.40 (s, 3 H), 1.65 (t, ³J = 7.2 Hz, 3 H); ¹³C NMR (CDCl₃, 100 MHz) [δ ppm]: 171.12, 169.71, 165.05, 158.18, 136.80, 135.14, 131.64, 129.65, 129.32, 128.46, 122.06, 104.15, 32.29, 26.67, 21.02, 11.57. HRMS (ESI, M+H⁺) Calcd for C₂₂H₂₂N₅: 356.18697. Found: 356.1869.

N-(4-Chlorophenyl)-2,5-dimethyl-7-phenylpyrimido [4,5-d]pyrimidin-4-amine (4i):

Beige solid; Yield: 22%; mp: 162–164 °C; IR ν (cm⁻¹): 3472, 1606; ¹H NMR (CDCl₃, 400 MHz) [δ ppm]: 8.63 (d, ³J = 6.7 Hz, 2 H), 7.71 (d, ³J = 8.4 Hz, 2 H), 7.55–7.46 (m, 4 H), 7.40 (d, ³J = 8.4 Hz, 2 H), 3.20 (s, 3 H), 2.66 (s, 3 H); ¹³C NMR (CDCl₃, 100 MHz) [δ ppm]: 171.54, 165.38, 158.24, 136.42, 131.80, 130.30, 129.25, 129.12, 128.52, 123.27, 104.61, 27.31, 26.59. HRMS (ESI, M+H⁺) Calcd for C₂₀H₁₇N₅Cl: 362.11670. Found: 362.1166.

N-(3,4-Dimethoxyphenyl)-5-ethyl-2-methyl-7-phenylpyrimido [4,5-d]pyrimidin-4-amine (4j):

Beige solid; Yield: 20%; mp: 178–180 °C; IR ν (cm⁻¹): 3420, 1613; ¹H NMR (CDCl₃, 400 MHz) [δ ppm]: 8.69 (d, ³J = 2.6 Hz, 2 H), 7.64 (s, 1 H), 7.52–7.49 (m, 3 H), 7.10 (d, ³J = 8.5 Hz, 1 H), 6.91 (d, ³J = 8.5 Hz, 1 H), 3.95 (m, 6 H), 3.47 (q, ³J = 6.8 Hz, 2 H), 2.65 (s, 3 H), 1.65 (t, ³J = 6.8 Hz, 3 H); ¹³C NMR (CDCl₃, 100 MHz) [δ ppm]: 165.03, 158.18, 8.95, 146.66, 136.78, 131.80, 131.59, 129.31, 128.59, 128.38, 114.15, 113.97, 111.20, 111, 107.08, 106.84, 104.11, 55.68,

56.33, 32.33, 11.77, 11.27. HRMS (ESI, $M+H^+$) Calcd for $C_{23}H_{24}N_5O_2$: 402.19245. Found: 402.1926.

***N*-(3,5-Dimethoxyphenyl)-2-methyl-7-phenylpyrimido [4,5-*d*]pyrimidin-4-amine (4k):**

Beige solid; Yield: 47%; mp > 266 °C; IR ν (cm^{-1}): 3326, 1620; ^1H NMR (DMSO- d_6 , 400 MHz) [δ ppm]: 10.27 (s, 1 H), 10.06 (s, 1 H), 8.56 (d, 2 H), 7.61 (m, 3 H), 7.27 (d, 2 H), 6.35 (s, 1 H), 3.80 (s, 6 H), 2.61 (s, 3 H); ^{13}C NMR (DMSO- d_6 , 100 MHz) [δ ppm]: 172.38, 166.11, 163.25, 160.78, 158.77, 158.17, 140.65, 137.27, 132.17, 129.27, 129.03, 105.11, 100.78, 96.84, 55.66, 27.46. HRMS (ESI, $M+H^+$) Calcd for $C_{21}H_{20}N_5O_2$: 374.16115. Found: 374.1609.

3.3. Biological Evaluation

Effect of compounds 4a–k on H_2O_2 (200 μM)-induced cell death in SH-SY5Y cells. SH-SY5Y cells were seeded in 96-well culture plates at a density of 8×10^4 cells per well in DMEM/F12 (1:1) medium supplemented with 10% fetal bovine serum, 1X non-essential amino acids, 100 units/mL penicillin and 10 mg/mL streptomycin (Dutscher, France). After 48 h of incubation, the cultures were treated with 100 μL of the test compounds or DMSO (0.1%) in the same medium. Following 24 h, the cells were co-incubated with H_2O_2 (200 μM) with or without the tested compounds at noncytotoxic concentrations for an additional period of 24 h. The percent of cell viability was measured using CellTiter 96 Aqueous Non-Radioactive Cell Proliferation (MTS) Assay (Promega, France).

Oxygen Radical Absorbance Capacity Assay. The antioxidant capacity of compounds 4a–k was evaluated by the ORAC-FL assay using fluorescein as a probe [26]. Samples were incubated in a black 96-well plate at 37 °C for 15 min followed by the addition of AAPH and fluorescence was measured every minute for 60 min. Each reaction was run in triplicate. The area under the fluorescence decay curve (AUC) was calculated and normalised to a blank. The ORAC value was derived by comparing the net AUC of the sample with that of Trolox, with results expressed as Trolox equivalents (μmol Trolox equivalent/ μmol adduct). Data are expressed as mean $\pm$ SD.

Inhibition of $A\beta_{1-42}$ aggregation. $A\beta_{1-42}$ was stocked in ultra pure water ($[A\beta_{1-42}] = 50 \mu M$). Experiments were performed by incubating the peptide in 50 mM phosphate buffer (pH 7.4) at 37 °C for 24 h (final $A\beta$ concentration = 25 μM) with and without inhibitor (25 μM). The inhibitor was dissolved in DMSO (50 μM) and diluted in the assay buffer (final concentration = 25 μM). Blanks containing inhibitor and ThT were also prepared and evaluated to account for quenching and fluorescence properties. To quantify amyloid fibril formation, the ThT fluorescence method was used.

After incubation, samples were diluted to a final volume of 400 μL ($[A\beta_{1-42}] = [inhibitor] = 2.5 \mu M$) with 50 mM glycine-NaOH buffer (pH 8) containing 5 μM ThT. A 300 scan of fluorescence intensity was carried out ($\lambda_{exc} = 450$ nm; $\lambda_{em} = 485$ nm) and values at plateau were averaged after subtracting the background fluorescence of 5 μM ThT solution. The fluorescence intensities were compared, and the % inhibition was calculated.

4. Conclusions

In this study we have successfully designed and synthesized eleven new *N*,7-diphenylpyrimido [4,5-*d*]pyrimidin-4-amines 4a–k by a simple two-step process from moderate to good overall chemical yields. Based on the biological and physicochemical results, compounds 4g, 4i and 4j were identified as multifunctional agents with neuro-protective and antioxidant activities and the ability to inhibit $A\beta$ self-aggregation. In addition, these compounds exhibited suitable physicochemical properties as predicted by the DataWarrior software V6.1.0. Our results suggest that compounds 4g, 4i and 4j hold promise for further research in the treatment of AD. Ongoing work in our laboratories is aimed at developing analogues with improved pharmacological profiles and the results will be reported in due course.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/chemistry6040041/s1>, The NMR, IR and HRMS spectras.

Author Contributions: G.B.A. carried out the synthesis of the molecules. A.-S.J., performed the neuroprotective study and ORAC assay. P.J.B. provided the Physicochemical properties. N.B. carried out the Self-Induced A β _{1–42} Aggregation assay. H.M. supervised the biological assays. E.M. supervised the synthesis of compounds. F.C. and J.M.-C., supervised the project and edited the manuscript. L.I. supervised the project and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Samples of the compounds are available from the authors.

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