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Enhancing Inhibition of Both α -Synuclein Aggregation and Neuroinflammation: New Insights into the C-9 Modification of Doxycycline

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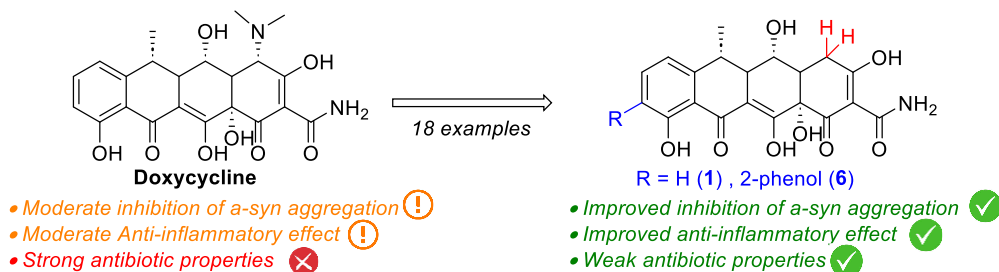
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Equal contribution

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ABSTRACT:

Doxycycline, a semi-synthetic tetracycline, is a widely used antibiotic for mild-to-moderate infections. However, its pleiotropic effects, such as anti-inflammatory and antioxidant properties, combined with its ability to interfere with α -synuclein inhibiting its aggregation, make it an attractive candidate for repositioning in Parkinson's disease treatment. Nevertheless, the antibiotic activity of doxycycline restricts its potential for long-term treatment of Parkinsonian patients. Eighteen novel doxycycline derivatives were designed with substitution at C₉. Specifically, the dimethylamino group at C₄ was reduced to significantly diminish the antibiotic activity, and several coupling reactions were performed at position C₉ of the D ring. Using biophysical models, we found that seven compounds were more effective than the parent compound in inhibiting α -synuclein aggregation. Furthermore, two of these derivatives exhibited better anti-inflammatory effects at non-cytotoxic concentrations on microglial cell culture. Thus, we identified two design-based tetracyclines as the most promising candidates for further preclinical investigations. In addition, our study provides new insights into the structure-activity relationship of tetracyclines as neuroprotective molecules.

1. INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons, resulting in motor and non-motor symptoms.^{1,2} The prevalence of PD has been increasing rapidly, with projections indicating a significant rise in cases in the coming decades.³ The aggregation of the presynaptic protein α -synuclein (α -Syn) has been identified as a key pathological event in PD, making it a therapeutic target for neuroprotective drug development.^{4,5} In addition, neuroinflammatory processes have been implicated as etiological factors in PD, and α -Syn aggregates can induce and amplify these processes, leading to neuronal damage.⁶

Tetracyclines have a long history of clinical use due to their antibiotic, antifungal, and antineoplastic properties, but they have also shown potential for matrix metalloproteinase inhibition, as well as antioxidant and anti-inflammatory activities.^{7,8} For instance, doxycycline (**DOX**) has demonstrated excellent neuroprotective effects in experimental models without significant toxicity signs.⁹ Among these neuroprotective effects, **DOX** was able to prevent the aggregation of α -Syn;¹⁰ reduce oxidative stress, act as an anti-inflammatory agent,¹¹ induce cellular redistribution of aggregates in an animal model of Parkinson's disease¹² among other activities in *ex vitro* and *in cellulo* assays.^{10,13–15} These pieces of evidence, along with **DOX**'s ability to cross the blood-brain barrier,¹⁶ make **DOX** a good candidate as a novel therapeutic agent not only for PD but also for Alzheimer's disease.¹⁷ However, using **DOX** for long-term treatment in PD patients may lead to potential antibiotic resistance and disruption of microbiota. Indeed, tetracyclines are well-known antimicrobial therapeutic agents with excellent and safe toxicological profiles and well-understood structure-activity relationships that pave the way for new pharmacomodulation studies.¹⁸ Recently, we showed that a new tetracycline derivative, a "reduced" demeclocycline (**DMC**) derivative called **DDMC**, showed promising neuroprotective activity by interfering with α -Syn aggregation without exhibiting significant antibiotic properties.¹⁴ Moreover, **DMC**, **DOX**, and their corresponding reduced analogs **DDMC** and **DDOX** have also shown neuroprotective properties through their ability to chelate iron, preventing oxidative stress (Figure 1).¹⁹

In the present study, eighteen novel non-antibiotic **DOX** derivatives were synthesized, with pharmacomodulation at position C₉. Our goal was to identify promising compounds that exhibit enhanced anti-aggregative properties against α -Syn and diminished neuroinflammation compared to parent **DOX** compound, but with no antibiotic properties. The ultimate objective is to halt or slow down the progression of the underlying pathology.

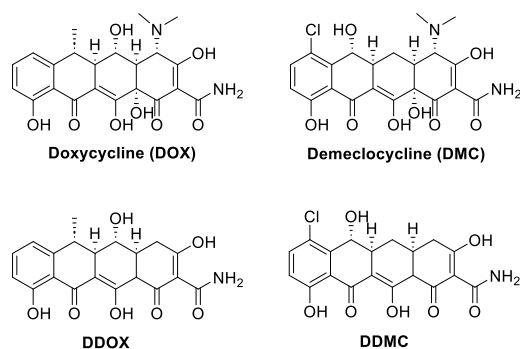


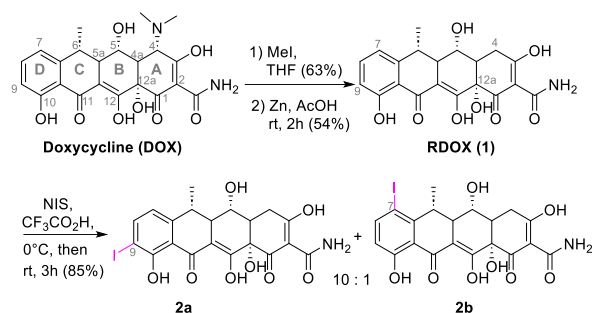
Figure 1. Structures of previously studied tetracyclines: **DOX**, **DMC**, **DDOX** and **DDMC**.

2. RESULTS AND DISCUSSION

2.1. Chemical Synthesis.

It is well accepted that the *N*-dimethylamino function at the C₄ position on the upper half of the tetracycline core structure in ring A is crucial for the antibacterial properties.^{20,21} Additionally, previous research has established a strong correlation between function and structure, emphasizing the importance of maintaining a specific structural motif in tetracyclines, which is crucial to interact with cross-beta structures characteristic of toxic aggregates.¹⁹ To prepare new analogs, the aromatic ring D appears to be the most accessible option due to the phenol moiety's ability to promote aromatic electrophilic substitutions at either position C₉ or C₇. Specifically, halogenation of these positions would lead to further cross-coupling reactions.

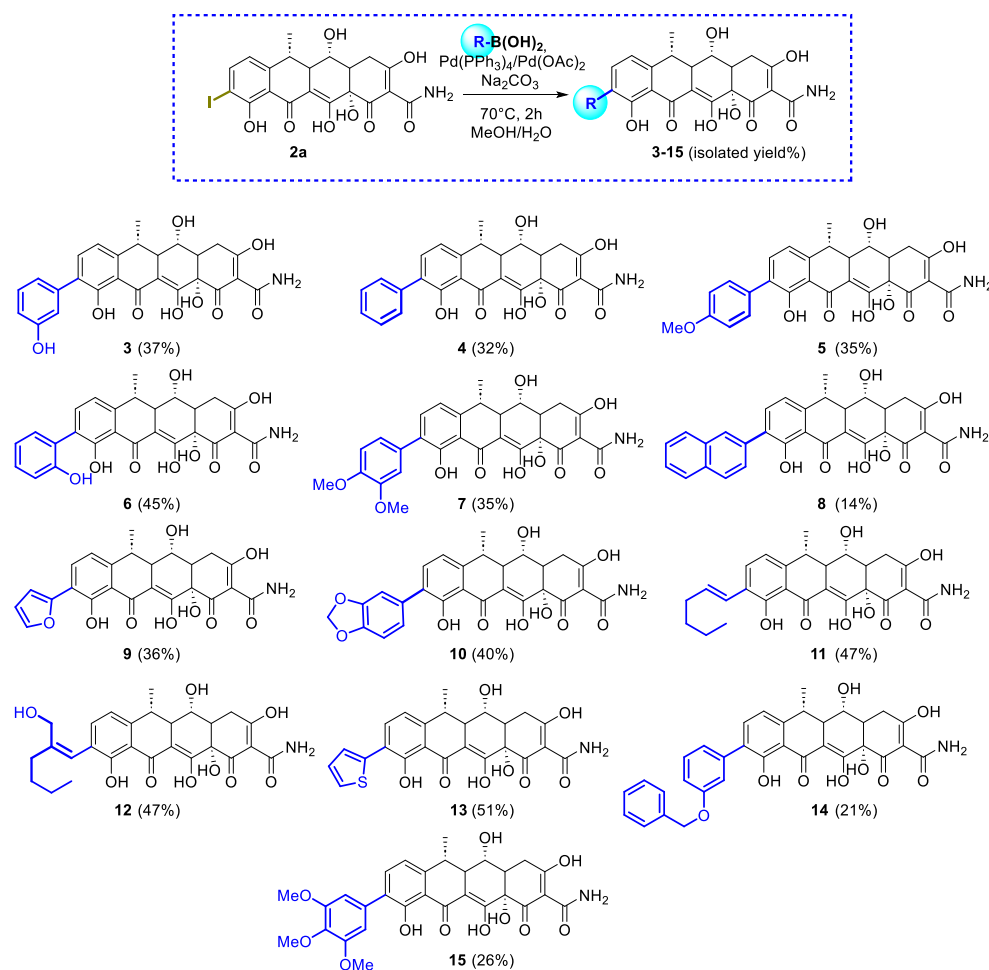
Thus, we first targeted the dimethylamino group's reduction using previously reported methods.^{20,23–25} The compound 4-des-*N*-dimethylaminodoxycycline **1** (**RDOX**) was then prepared in two steps from **DOX**, after quaternarization of the amino group with methyl iodide in THF, followed by its reduction with zinc dust in aqueous acetic acid over 2 h. **RDOX** was selectively obtained using this two-step procedure without the significant formation of **DDOX**.¹⁹ In a second step, we focused our attention on the functionalization of the aromatic ring. After several attempts, we found that CF₃COOH and NIS at 0 °C were the best conditions to selectively iodinate **RDOX**²⁶ on position C₉ (**2a**) over position C₇ (**2b**) with about a 10:1 ratio. Preparative HPLC then separated the two regioisomers **2a** and **2b**. Attempts towards bromination of **RDOX** were unsuccessful, leading to unselective reactions and/or an inseparable mixture of products. (Scheme 1).



Scheme 1. Synthesis of 4-*des-N*-dimethyl-aminodoxycycline RDOX (1) and 9-iodo derivative 2a.

The final step consisted of performing a cross-coupling reaction at the C9 position of the D ring to introduce

various new functional groups. Suzuki cross-coupling reaction was an attractive transformation due to the high functional tolerance of the reaction (*e.g.*, ketones, alcohols, carboxylic acid, amide) and the low toxicity of boronic acids. The use of MeOH as a solvent for this transformation^{27,28} was crucial for substrate 2a: THF, dioxane, or DMF did not give any conversion. We thus obtained 13 original tetracyclines 3-15 with moderated yields after isolation by preparative HPLC (14-47% yield). Different aromatic rings were installed (3-8, 10, 14-15), as well as heteroaromatic rings (9, 13). Surprisingly, the insertion of nitrogen-containing heterocycles such as pyridine, quinoline, or pyrrole derivatives was ineffective in our hands. We were also pleased to enable the insertion of alkene derivatives (11-12). (Scheme 2)

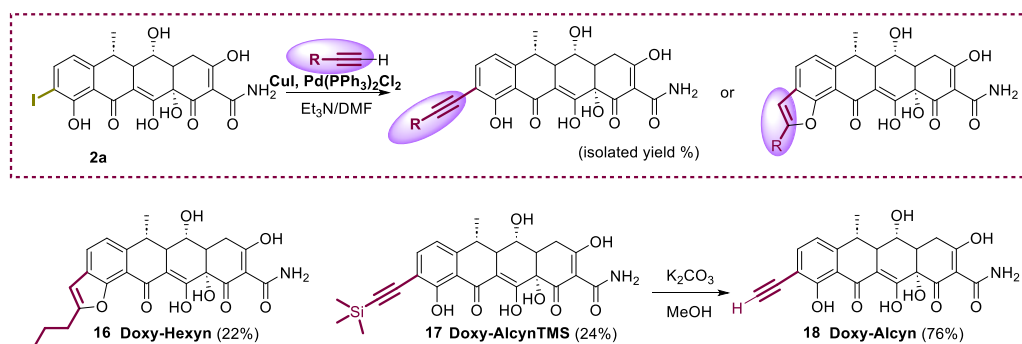


Scheme 2. Preparation of 9-substituted doxycyclines by Suzuki cross-coupling from 2a

The insertion of alkyne derivatives was also investigated through Sonogashira cross-coupling reaction. DMF was crucial to solubilize substrate 2a efficiently, leading to reproducible conversions. The

reaction outcome was also different depending steric and electronic properties of the alkyne. Hexyne afforded only the product of a subsequent cycloisomerization with phenol (16), whereas TMS-acetylene gave only the

cross-coupling product **17**. Further deprotection of the TMS group furnished the free acetylene function in compound **18**. (Scheme 3)



Scheme 3. Preparation of benzofuranyl derivative **16** and alkynes derivatives **17** and **18** from a Sonogashira cross-coupling reaction with **2a**.

2.2. Antibacterial activity.

Table 1. MIC of synthesized products **1-18** against Gram + and Gram – bacterial strains

Compounds	MIC (μM)		
	<i>P. aeruginosa</i> PAO1	<i>E. coli</i> ATCC 25922	<i>S. aureus</i> ATCC 25923
1	>2000	200	25
2a	>200	200	50
3	>200	>200	50
4	>200	>200	6.25
5	>200	>200	6.25
6	>200	>200	50
7	>200	>200	12.5
8	>200	>200	6.25
9	>200	>200	25
10	>200	3.125	3.125
11	>200	>200	6.25
12	>200	>200	3.125
13	>200	>200	6.25
14	>200	>200	100
15	>200	>200	12.5
16	>200	>200	25
17	>200	>200	3.125
18	>200	200	100
DOX	12.5	3.125	0.4

One of the study's main objectives was to suppress the antibiotic activity of tetracyclines as a requirement for neurodegenerative diseases' chronic administration; this was to prevent any potential interference with other medications and to avoid the development of antibiotic

resistance.^{21,29} Thus, all synthesized compounds were evaluated for their antibacterial activity against several Gram-negative (*i.e. Pseudomonas aeruginosa* PA01 *E. coli* ATCC25922) and a Gram-positive strains (*Staphylococcus aureus* ATCC25923), and their minimal inhibitory concentrations (MIC) were determined. Interestingly all newly synthesized products exhibit mainly no antibacterial activity below 200 μM against Gram-negative *P. aeruginosa* and *E. coli*. The antibiotic activity of our novel compounds against Gram-positive bacteria *S. aureus* was decreased by at least 8-fold and up to 250-fold compared to **DOX**, indicating moderate to low activity. On the one hand, removing the dimethylamino group dramatically reduces antibiotic activity, highlighting the role of this substituent. On the other hand, the nature of the substituent at C₉ does not seem to improve antibacterial activity. Indeed, 9-*t*Bu-DOX, which possesses both the *tert*-butyl group at C₉ and the dimethylamino group at C₄, showed an increase of the MIC compared to **DOX** (>200, 50, 6.25 μM against *P. aeruginosa*, *E. coli*, and *S. aureus*, respectively),³⁰ meaning that the substitution at C₉ also decreases the antibacterial activity (Table 1).

2.3. Inhibition of α -synuclein aggregation.

It has been previously reported that some tetracyclines, specifically **DOX**, can inhibit α -Syn aggregation.^{9,10,22} To analyze the capacity of all the novel synthesized compounds to interfere with the fibril assembly process of α -Syn, we incubated 70 μM of the protein in the absence or the presence of 20 μM of each tetracycline at 37 °C under orbital agitation for 120 hours. The cross- β structure, which is the hallmark of

amyloid aggregation,³¹ was monitored by Thioflavin T (ThT) fluorescence emission at 482 nm (λ_{exc} 450 nm).³²

The results indicated that seven of the eighteen new tetracyclines tested showed a decrease in ThT fluorescence intensity compared to the control. It is noteworthy that each molecule displayed a varying degree of antiaggregant potential. Compound **17** was the most effective, reducing the ThT signal by approximately 95.6% compared to the control. The ranking was followed by compounds **16**, **14**, **6**, **12**, **RDOX**, and **4**, with reductions of 93.7%, 90.7%, 89.3%, 81.3%, 70.25%, and 62% respectively (Figure 2). Interestingly, the lipophilic substituent at the C₉ position appears to have a favorable effect on inhibiting α -Syn aggregation. Compounds **16** and **17** exhibited the highest activities in this regard. Conversely, compound **18**, which lacks the trimethylsilyl group, showed limited effectiveness, reducing the ThT level to 14.3% compared to the control. Interestingly, **RDOX** displayed a significant ThT signal reduction of 70.2% in inhibiting of α -Syn aggregation, whereas the iodo-derivative **2a**, containing an iodine atom at position C-9 instead of hydrogen, exhibited no inhibition (2.63%).

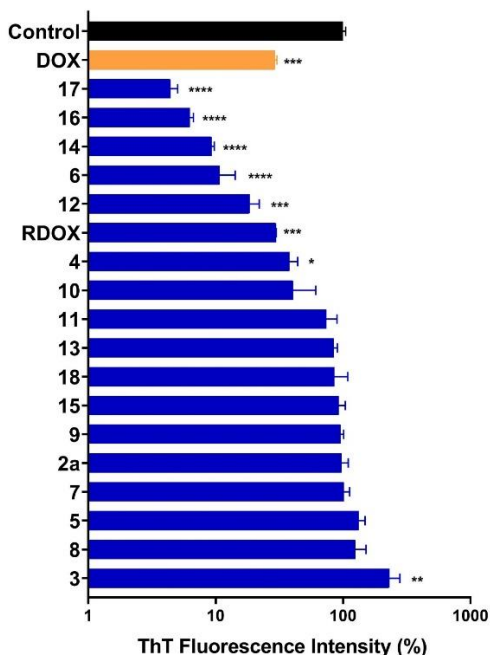


Figure 2. Effect of the new tetracyclines derivatives RDOX, 2a, 3-18 on the α -Syn aggregation. The fluorescence emission intensity of 25 μ M thioflavin T (ThT) was measured in a solution containing 70 μ M α -Syn alone (Control) and in the presence of 20 μ M of DOX or each compound (RDOX, 2a, 3-18) after 120 hours of incubation. The ThT fluorescence intensity of the Control was considered 100%, and the values obtained in the presence of the different molecules tested were referred to this Control. The data are presented as mean \pm SEM (n= 3), and statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test. The figure indicates significant

differences: ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05 vs. Control.

Conformation structure also played a role, with alkene **11** being less effective (26%), indicating that suppressing conformational rotation adjacent to the D ring was beneficial for compounds **16** and **17** (Figure 2). Another noteworthy case was *meta*-benzyloxy derivative **14**, which proved to be one of the most effective compounds, while free *meta*-phenol **3** resulted in a negative outcome, acting as a pro-aggregative molecule. According to previous observations, protection as benzyl ether provides lipophilic properties, and the aromatic ring restrained conformational rotation adjacent to the D ring of tetracycline.

Moreover, a supplementary hydroxyl group adjacent to the phenol of the D ring was beneficial for antiaggregant activity (compound **6**). This result is not unexpected, as it provides additional hydrogen bonding for antiaggregatory properties. As for the remaining compounds, no significant differences were observed in ThT signal reduction compared to the control. The percentage reductions were as follows: **10** (59.4%), **13** (15.4%), **15** (7.2%), **9** (4.7%), **7** (-1.6%), **5** (-32.7%), and **8** (-24.4%) (Figure 2).

2.4. Analysis of cytotoxic effects

The appropriate safety profile of DOX has allowed its administration for decades,³³ however, new chemical modifications could impact its cytotoxicity. Consequently, a lactate dehydrogenase (LDH) assay was performed, which is commonly used as a measure of cytotoxicity by membrane impairment. We used untreated cells as the 100% release of physiological LDH. In contrast, the positive control with 1% Triton X-100 displayed high cytotoxicity, with a +150% increase in the release of LDH into the culture medium compared to the control (Figure 3). Cells were also treated with 20 μ M of the new tetracycline derivatives that exhibit significant inhibition of α -Syn aggregation (Figure 2). We observed that **6**, **12**, and **RDOX** exhibited no cytotoxicity, as their LDH values were not significantly different from the control condition. Additionally, we tested cell viability using the Methyl Thiazolyl Tetrazolium (MTT) assay³⁴ after exposure to the tetracyclines derivatives **RDOX**, **4**, **6**, **12**, **14**, **16**, **17**. Results showed that two tetracycline derivatives, **6** and **RDOX** do not influence cell viability up to 20 μ M, reinforcing the non-toxicity of both compounds (Figure S-1, see supplementary information).

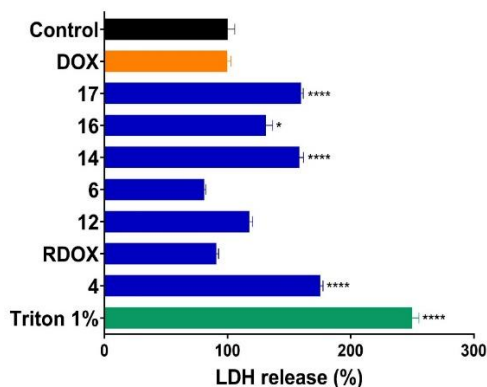


Figure 3. Cytotoxicity of α -Syn antiaggregant compounds. LDH release of primary microglial cells culture in the absence (Control) or after adding 20 μ M of each compound (DOX, 17, 16, 14, 6, 12, RDOX or 4). The bars represent the mean values expressed in percentage, normalized to the Control condition. Triton 1% was used as a cytotoxic control, inducing a total disruption of the cells. Data are presented as mean \pm S.E.M (n = 6), and statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test. Significant differences are indicated in the figure: ****p < 0.0001; *p < 0.05 vs. Control.

2.5. Anti-inflammatory effect.

Tumor necrosis factor alpha (TNF- α) is a cytokine that plays a crucial role in the response to various inflammatory insults.³⁵ While acute activation results in tissue repair and the induction of a protective immune response, chronic activation can be detrimental to the brain, leading to neurodegeneration. This study evaluated the potential anti-inflammatory effects of the non-cytotoxic compounds **6** and **RDOX** on primary microglial cells activated by lipopolysaccharide (LPS) compared to the precursor **DOX**. As previously reported,¹¹ **DOX** does not exhibit anti-inflammatory properties at a concentration of 20 μ M, only becoming effective at 50 μ M. In contrast, when cells were pre-treated with either **6** or **RDOX** at 20 μ M, the TNF- α release was significantly reduced by 77 and 47 %, respectively, compared to LPS (Figure 4). These results showed that **6** or **RDOX** are more effective than **DOX** in inhibiting TNF- α release induced by LPS stimulation in primary microglia cells.

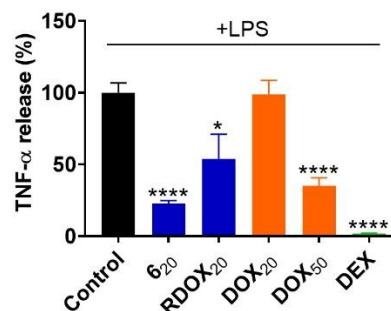


Figure 4. Anti-inflammatory properties of α -Syn antiaggregant and non-toxic compounds. TNF- α release of primary microglial cell cultures upon pre-treatment for 4 h with **DOX**, **6**, or **RDOX** at 20 μ M; **DOX** at 50 μ M; dexamethasone (DEX) at 2.5 μ M, followed by stimulation with 10 ng/ml of LPS for 24 h. The bars represent the mean \pm S.E.M (n = 3). Statistical analysis was performed using one-way ANOVA followed by Dunn's multiple comparison test. Significant differences are indicated in the figure: ****p < 0.0001; *p < 0.05 compared to the LPS control group.

3. CONCLUSIONS

Previous studies have demonstrated that **DOX** has beneficial effects in several *in vitro* and *in vivo* models of PD by targeting key pathomechanisms involved in the degenerative process of dopaminergic neurons, such as α -Syn aggregation and neuroinflammation. However, its antibiotic property could lead to antimicrobial resistance and interfere with chronic treatment. To address this issue, we designed a chemical library of eighteen novel tetracyclines through the Suzuki or Sonogashira cross-coupling reaction of 9-Iodo-**RDOX 2a** with the corresponding nucleophiles. Reducing the dimethylamino group significantly decreased the antibiotic activity of most compounds, making these novel tetracyclines suitable for chronic prescriptions in neurodegenerative diseases. We hypothesized that novel chemical entities with both α -Syn antiaggregant and anti-inflammatory activities could have a disease-modifying effect and be promising therapeutic agents. All synthesized compounds were therefore tested for α -Syn aggregation in a biophysical *in vitro* model, and eight of them (**17**, **16**, **14**, **6**, **12**, **10**, **4**, and **RDOX**) exhibited a decrease in the ThT fluorescence intensity. The lipophilic properties of the substituent at position C₉ generally induce α -Syn anti-aggregation, but a reduced degree of liberty adjacent to the aromatic ring of tetracycline is required. Positioning an adjacent hydroxy group, such as compound **6**, improves the anti-aggregation properties by adding a supplementary hydrogen bond to the south part of the tetracycline. Before determining the anti-inflammatory effect of those active compounds on microglial cells, an LDH assay was performed to ensure their non-cytotoxicity.

Therefore, only compounds **6** and **RDOX** showed no cytotoxicity at 20 μM . Moreover, compounds **6** and **RDOX** showed greater anti-aggregating and anti-inflammatory properties than **DOX** by acting at lower concentrations. These findings highlight the promising drug candidacy of **6** and **RDOX**, warranting further *in vivo* studies on PD models before embarking on preclinical trials.

4. EXPERIMENTAL PROCEDURES

4.1. Chemistry

General. All the reactions were performed under an inert atmosphere (Ar). THF was distilled over sodium/benzophenone mixture. DMF was purchased as an anhydrous grade from Acros Organics and used as received. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F₂₅₄ (0.25 mm) plates purchased from Merck. Doxycycline was purchased from Alfa Aesar. Compounds were visualized by exposure to a UV lamp ($\lambda = 254$ and 365 nm). All Preparative chromatographies were performed on an Xbridge (Waters) C18 5 μm , [\varnothing 19 mm x 150 mm or \varnothing 30mmx150 mm, 42 mL/min]. All reagents were commercial and used as received, except for *E*-hexenyl boronic acid and 4-butyl-1,2-oxaborol-2(5H)-ol, needed to synthesize tetracyclines **11** and **12**, the synthesis of which was reported by us.^{36,37} ¹H and ¹³C NMR spectra were recorded using a Bruker Advance 300 (300 MHz) or a Bruker Advance 400 (400 MHz) spectrometers in the indicated solvent. Chemical shifts (δ) are given in ppm, and the coupling constants (*J*) in Hz. The solvent signals were used as reference (CDCl₃: $\delta_{\text{C}} = 77.16$ ppm unless notified, residual CHCl₃ in CDCl₃: $\delta_{\text{H}} = 7.26$ ppm; C₆D₆: $\delta_{\text{C}} = 128.06$ ppm unless notified, residual C₆H₅ in C₆D₆: $\delta_{\text{H}} = 7.16$ ppm. Multiplicities are described by the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, p = pentuplet, h = hexuplet, m = multiplet, br = broad. An optimized sequence, called UDEFT,³⁸ was used for 1D ¹³C{¹H} spectra. HPLC chromatograms and mass spectra were obtained on a Waters LCT Premier (ESI-TOF) spectrometer, Agilent QTOF 6530, or Agilent QTOF 6546 in BioCIS, at Université Paris-Saclay.

RDOX (1). In a 100mL round-bottom flask, **doxycycline (DOX)** monohydrate (2.0 g, 4.3 mmol, 1.0 eq) was suspended in dry THF (20 mL), and CH₃I (2.7 mL, 43.2 mmol, 10 eq) was added. The reaction mixture was stirred at 40 °C for 24 h under an argon atmosphere. After cooling at room temperature, the solvent was evaporated under reduced pressure. The residue was dissolved in a minimum of MeOH, and Et₂O was added. Filtration of the obtained precipitate afforded doxycycline-trimethylammonium iodide salt (1.6 g,

63%). ¹H NMR (300 MHz, DMSO-*d*₆) δ 15.41 (brs, 1H), 11.46 (s, 1H), 9.25 (brs, 2H), 7.70 (s, 1H), 7.54 (t, *J* = 8.1 Hz, 1H), 6.94 (d, *J* = 7.7 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, 1H), 5.71 (d, *J* = 8.2 Hz, 1H), 4.58 (s, 1H), 3.58 – 3.16 (m, 3H), 3.37 (s, 9H), 3.10 (d, *J* = 11.0 Hz, 1H), 2.78 – 2.66 (m, 1H), 1.45 (d, *J* = 6.3 Hz, 3H). ¹³C NMR (75 MHz, DMSO) δ 192.25, 191.90, 185.97, 174.40, 172.07, 161.07, 147.82, 136.77, 115.96, 115.73, 115.46, 106.74, 97.86, 72.32, 72.22, 68.24, 54.69, 45.70, 43.48, 38.22, 16.02. HRMS (ESI): calculated for C₂₃H₂₇N₂O₈ [M]⁺: 459.1762, found 459.1765.

In a 50mL round-bottom flask, doxycycline-trimethylammonium iodide salt (600 mg, 1.02 mmol, 1.0 eq) was suspended in AcOH (50%) (9.6 mL), then zinc (powder) (669 mg, 10.2 mmol, 10 eq) was added, and the reaction mixture was stirred at room temperature for 1 h. The resulting solution was filtered through a small pad of Celite with AcOH. The organic phase was extracted with CH₂Cl₂, washed with HCl (1 M) and brine, dried over MgSO₄, filtered off, and concentrated *in vacuo*. Precipitation in EtOAc/*n*-pentane afforded compound **RDOX (1)** as a yellow solid in 54% yield (220 mg). ¹H NMR (300 MHz, DMSO-*d*₆): δ 15.36 (s, 1H, C₁₂-OH), 11.53 (s, 1H, C₁₀-OH), 8.85, 8.74 (2brs, each 1H, NH₂), 7.51 (t, *J* = 8.0 Hz, 1H, H₈), 6.91 (d, *J* = 8.0 Hz, 1H, H₇), 6.86 (d, *J* = 8.0 Hz, 1H, H₉), 6.75 (brs, 1H, C_{12a}-OH), 5.25 (brd, *J* = 5.4 Hz, 1H, C₅-OH), 3.47 (m, 1H, H₅), 2.98 – 2.75 (m, 2H, H₄), 2.60 (p, *J* = 6.7 Hz, 1H, H₆), 2.31 (dd, 1H, *J* = 12.2, 8.4 Hz, H_{5a}), 2.24 (dm, 1H, *J* = 11.3 Hz, H_{4a}), 1.44 (d, 3H, *J* = 6.7 Hz, H₆-Me) ppm. ¹³C{¹H} NMR (75 MHz, DMSO-*d*₆): δ 194.94, 192.47, 192.02, 176.83, 173.34, 161.06, 148.04, 136.47, 115.80, 115.62, 115.53, 106.62, 98.08, 74.57, 67.64, 62.21, 45.91, 43.04, 29.27, 15.86 ppm. HRMS (ESI): calculated for C₂₀H₂₀NO₈ [M+H]⁺: 402.1183, found 402.1189.

9-Iodo-RDOX (2a). In a 25mL round-bottom flask, **RDOX (1)** (110.0 mg, 2.7 $\times 10^{-1}$ mmol, 1.0 eq) was dissolved in trifluoroacetic acid (2.9 mL), and the solution was put into an ice bath. *N*-Iodosuccinimide (67.9 mg, 3.0 $\times 10^{-1}$ mmol, 1.1 eq) was portion-wise added at 0°C, and the reaction was stirred at room temperature for 3 hours. TFA was evaporated under reduced pressure, and then the organic phase was extracted with EtOAc, washed with HCl_(aq) (1M) and brine, dried over MgSO₄, filtered off, and concentrated under reduced pressure. Precipitation in EtOAc/*n*-pentane afforded iodinated compounds **2a** and **2b** in 88% yield (126 mg) as a 1:10 mixture of isomers (position 7: position 9). Further purification by preparative HPLC (eluent H₂O + 0.1% formic acid / ACN, gradient 45 to 70% of ACN over 15 min) afforded **2a** contaminated by about 6% of 7,9-diodo-RDOX. ¹H NMR (300 MHz, Acetone-*d*₆): δ 15.12 (s, 1H, C₁₂-OH),

12.60 (s, 1H, C₁₀-OH), 9.00 (brs, 1H, NH₂), 7.99 (d, *J* = 8.2 Hz, 1H, H₈), 7.63 (brs, 1H, NH₂), 6.83 (d, 1H, *J* = 8.2 Hz, H₇), 5.77 (s, 1H, C_{12a}-OH), 4.33 (dd, *J* = 8.3 Hz, 1H, C₅-OH), 3.81 (q, *J* = 8.3 Hz, 1H, H₅), 3.06 (dd, *J* = 18.6, 5.5 Hz, 1H, H₄), 2.97 (dd, *J* = 18.6, 3.0 Hz, 1H, H₄), 2.80 (m, 1H, H₆), 2.55 (dd, *J* = 12.5, 8.5 Hz, 1H, H_{5a}), 2.49 (ddd, *J* = 10.0, 3.6, 2.5 Hz, 1H, H_{4a}), 1.57 (d, *J* = 6.9 Hz, 3H, H₆-Me) ppm. ¹³C{¹H} NMR (100 MHz, Acetone-*d*₆): δ 195.96, 193.91, 193.03, 177.03, 174.99, 161.58, 149.78, 146.39, 118.90, 116.99, 107.32, 99.78, 83.64, 75.89, 69.72, 47.44, 44.50, 39.35, 30.65, 16.38. (CH₃) ppm. HRMS (ESI): calculated for C₂₀H₁₉INO₈ [M+H]⁺: 528.0150, found 528.0157.

General Procedure for Suzuki Coupling. In a 25 mL two-neck round-bottom flask, **2a** (155 mg, 2.95 × 10⁻¹ mmol, 1.0 eq), Pd(OAc)₂ (6.6 mg, 2.95 × 10⁻² mmol, 0.1 eq), and Pd(PPh₃)₄ (34.0 mg, 2.95 × 10⁻² mmol, 0.1 eq) were dissolved in MeOH (11.5 mL), and the resulting mixture was purged under Argon for 10 minutes. A solution of Na₂CO₃ (93.5 mg, 8.8 × 10⁻¹ mmol, 3.0 eq) in H₂O (3.5 mL) was added, followed by the addition of a solution of the aryl boronic acid (5.3 × 10⁻¹ mmol, 1.8 eq) in MeOH (3.5 mL). The reaction mixture was stirred at 70 °C for 2 hours under Argon. After cooling at room temperature, the resulting solution was filtered on a small pad of Celite, and the filtrate was concentrated under reduced pressure. Then, the organic phase was extracted with EtOAc (3 × 25 mL), washed with HCl (1M) and brine, dried over MgSO₄, filtered off, and evaporated under reduced pressure. The crude product was first purified on a silica gel column (eluent CH₂Cl₂ + 1% formic acid) and then by preparative HPLC.

9-(*m*-hydroxyphenyl)-RDOX (3). From 150 mg of **2a**, 52 mg (37%) of the targeted product were isolated after purification. Conditions for preparative HPLC: eluent H₂O + 0.1% formic acid / ACN, gradient 35 to 60% of ACN over 15 min. ¹H NMR (400 MHz, Acetone-*d*₆): δ 15.22 (brs, 1H, C₁₂-OH), 12.37 (s, 1H, OH), 9.05 (brs, 1H, OH), 8.32 (brs, 1H, NH₂), 7.63 (brs, 1H, NH₂), 7.58 (d, *J* = 8.0 Hz, 1H, H₈), 7.25 (t, *J* = 8.0 Hz, 1H, H_c), 7.12 (t, *J* = 1.5 Hz, 1H, H_b), 7.06 (dt, 1H, *J* = 7.5, 1.3 Hz, H_f), 7.04 (dd, 1H, *J* = 7.5, 1.0 Hz, H₇), 6.81 (ddd, *J* = 7.5, 2.3, 1.0 Hz, 1H, H_d), 5.77 (brs, 1H, C_{12a}-OH), 4.36 (brd, *J* = 7.9 Hz, 1H, C₅-OH), 3.83 (dd, *J* = 9.8, 7.5 Hz, 1H, H₅), 2.90-3.09 (m, 2H, H₄), 2.85 (m, 1H, H₆), 2.57 (dd, *J* = 12.3, 7.5 Hz, 1H, H_{5a}), 2.46 (m, 1H, H_{4a}), 1.62 (d, 3H, *J* = 6.8 Hz, H₆-Me) ppm. ¹³C{¹H} NMR (100 MHz, Acetone-*d*₆): δ ¹³C NMR (75 MHz, Acetone) δ 195.93, 195.02, 193.21, 176.21, 175.01, 160.50, 158.02, 148.42, 139.37, 137.93, 137.93, 129.92, 129.49, 121.39, 117.26, 116.62, 115.11, 107.60, 99.80, 75.83, 69.75, 47.78, 44.52, 39.52, 30.61, 16.40 ppm. HRMS (ESI): calculated for C₂₆H₂₃NO₈ [M+H]⁺: 494.1446, found 494.1451.

9-Phenyl-RDOX (4). From 120 mg of **2a**, 35.0 mg (32%) of the targeted product was isolated after purification. Conditions for preparative HPLC: eluent H₂O + 0.1% formic acid / ACN, gradient 50 to 70% of ACN over 15 min. ¹H NMR (300 MHz, Acetone-*d*₆): δ 15.25 (brs, 1H, C₁₂-OH), 12.36 (s, 1H, C₁₀-OH), 9.04 (brs, 1H, NH₂), 7.64 (brs, 1H, NH₂), 7.63–7.55 (m, 2H, H₈+H₆), 7.43 (tt, *J* = 7.3, 1.2 Hz, 2H, H_c), 7.34 (tt, *J* = 7.3, 1.2 Hz, H_d), 7.06 (dd, *J* = 8.0, 1.2 Hz, 1H, H₇), 5.76 (brs, 1H, OH), 4.34 (brs, 1H, C₅-OH), 3.84 (m, 1H, H₅), 3.12–3.91 (m, 2H, H₄), 2.85 (m, 1H, H₆), 2.58 (dd, *J* = 12.5, 7.1 Hz, 1H, H_{5a}), 2.49 (ddd, *J* = 10.2, 5.0, 3.5 Hz, 1H, H_{4a}), 1.61 (d, 3H, *J* = 6.8 Hz, H₆-Me) ppm. ¹³C{¹H} NMR (75 MHz, Acetone-*d*₆): δ 195.93, 195.02, 193.15, 176.15, 174.94, 162.32, 160.36, 148.45, 137.98 (2C), 130.12 (2C), 129.34, 128.90 (2C), 128.01, 116.66, 107.60, 99.68, 75.74, 69.55, 47.65, 44.37, 39.48, 30.45, 16.33 ppm. HRMS (ESI): calculated for C₂₆H₂₃NO₈ [M+H]⁺: 478.1496, found 478.1598.

9-(*p*-methoxyphenyl)-RDOX (5). From 120 mg of **2a**, 41.4 mg (35%) of the targeted product was isolated after purification. Conditions for preparative HPLC: eluent H₂O + 0.1% formic acid / ACN, gradient 50 to 70% of ACN over 15 min. ¹H NMR (300 MHz, Acetone-*d*₆): δ 18.43 (s, 1H, C₁₂-OH), 15.27 (brs, 1H, OH), 12.35 (s, 1H, C₁₀-OH), 9.03 (brs, 1H, NH₂), 7.63 (s, 1H, NH₂), 7.53-7.58 (m, 3H, H₈+H₆), 6.95-7.07 (m, 3H, H₇+H_c), 5.74 (s, 1H, C_{12a}-OH), 4.34 (d, *J* = 8.5 Hz, 1H, OH), 3.84 (s, 3H, OMe), 3.81 (m, 1H, H₅), 3.07 (dd, *J* = 18.7, 5.5 Hz, 1H, H₄), 2.98 (dd, *J* = 18.7, 3.4 Hz, 1H, H₄), 2.83 (m, 1H, H₆), 2.57 (dd, *J* = 12.3, 7.4 Hz, 1H, H_{5a}), 2.50 (ddd, *J* = 10.0, 5.3, 3.5 Hz, 1H, H_{4a}), 1.61 (d, 3H, *J* = 6.8 Hz) ppm. ¹³C{¹H} NMR (75 MHz, Acetone-*d*₆): δ 195.94, 195.10, 193.23, 176.16, 175.02, 160.49, 160.11, 147.92, 137.74, 131.28, 131.23, 130.27, 129.20, 116.84, 116.68, 114.43, 107.61, 99.84, 75.87, 69.79, 55.62, 47.86, 44.56, 39.52, 30.35, 16.43 ppm. HRMS (ESI): calculated for C₂₇H₂₆NO₉ [M+H]⁺: 508.1602, found 508.1609.

9-(*o*-hydroxyphenyl)-RDOX (6). From 110 mg of **2a**, 46.8 mg (45%) of the targeted product were isolated after purification. Conditions for preparative HPLC: eluent H₂O + 0.1% formic acid / ACN, gradient 40 to 60% of ACN over 15 min. ¹H NMR (300 MHz, Acetone-*d*₆): δ 15.25 (brs, 1H, C₁₂-OH), 12.33 (s, 1H, OH), 9.04 (brs, 1H, NH₂), 7.87 (brs, 1H, OH), 7.69 (brs, 1H, NH₂), 7.54 (d, *J* = 8.0 Hz, 1H, H₈), 7.15-7.29 (m, 2H, H_d and H_f), 7.04 (dd, *J* = 8.0, 1.2 Hz, 2H, H₇), 6.96 (dd, *J* = 8.0, 1.2 Hz, 2H, H_c), 6.92 (td, *J* = 7.6, 0.9 Hz, 1H, H_e), 5.82 (brs, 1H, C_{12a}-OH), 4.42 (brd, 1H, *J* = 8.2 Hz, C₅-OH), 3.83 (q, *J* = 8.5 Hz, 1H, H₅), 3.07 (dd, *J* = 18.7, 5.4 Hz, 1H, H₄), 2.97 (dd, *J* = 18.7, 3.2 Hz, 1H, H₄), 2.83 (dq, *J* = 12.6, 6.8 Hz, 1H, H₆), 2.83 (dd, *J* = 12.6, 6.8 Hz, 1H, H_{5a}), 2.50 (ddd, *J* = 10.2, 5.4, 3.2 Hz,

1H, H_{4a}), 1.61 (d, 3H, *J* = 6.8 Hz, H₆-Me) ppm. ¹³C{¹H} NMR (75 MHz, Acetone-*d*₆): δ 195.93, 194.91, 193.17, 176.08, 174.94, 160.46, 155.64, 148.36, 139.43, 132.27, 129.72, 126.95, 125.34, 120.40, 117.11, 116.63, 116.43, 107.49, 99.70, 75.75, 69.64, 47.74, 44.42, 39.49, 30.52, 16.38 ppm. HRMS (ESI): calculated for C₂₆H₂₃NO₈ [M+H]⁺: 494.1446, found 494.1452.

9-(3,4-dimethoxyphenyl)-RDOX (7). From 120 mg of **2a**, 54.2 mg (44%) of the targeted product was isolated after purification. Conditions for preparative HPLC: eluent H₂O + 0.1% formic acid / ACN, gradient 50 to 70% of ACN over 15 min. ¹H NMR (300 MHz, Acetone-*d*₆): δ 15.24 (brs, 1H, C₁₂-OH), 12.36 (s, 1H, C₁₀-OH), 9.03 (brs, 1H, NH₂), 7.63 (brs, 1H, NH₂), 7.59 (d, *J* = 8.1 Hz, 1H, H₈), 7.24 (d, *J* = 2.1 Hz, 1H, H_b), 7.15 (dd, *J* = 8.3, 2.1 Hz, 1H, H_f), 7.02 (dd, *J* = 8.1, 0.9 Hz, 1H, H₇), 7.00 (d, *J* = 8.3 Hz, 1H, H_c), 5.72 (brs, 1H, C_{12a}-OH), 4.34 (d, 1H, *J* = 8.6 Hz, C₅-OH), 3.85 (s, 6H, OMe), 3.81 (m, 1H, H₅), 3.07 (dd, *J* = 18.6, 5.4 Hz, 1H, H₄), 2.97 (dd, *J* = 18.6, 3.2 Hz, 1H, H₄), 2.81 (m, 1H, H₆), 2.55 (dd, *J* = 12.6, 7.6 Hz, 1H, H_{5a}), 2.51 (ddd, 1H, *J* = 9.9, 5.4, 3.2 Hz, 1H, H_{4a}), 1.60 (d, 3H, *J* = 6.7 Hz) ppm. ¹³C{¹H} NMR (75 MHz, Acetone-*d*₆): δ 195.93, 194.91, 193.17, 176.08, 174.94, 160.46, 155.64, 148.36, 139.43, 132.27, 129.72, 126.95, 125.34, 120.40, 117.11, 116.63, 116.43, 107.49, 99.70, 75.75, 69.64, 47.74, 44.42, 39.49, 30.52, 16.38 ppm. HRMS (ESI): calculated for C₂₈H₂₈NO₁₀ [M+H]⁺: 538.1708, found 528.1717.

9-(2-Naphtyl)-RDOX (8). From 140 mg of **2a**, 20.0 mg (14%) of the targeted product was isolated after purification. Conditions for preparative HPLC: eluent H₂O + 0.1% formic acid / ACN, gradient 55 to 75% of ACN over 15 min. ¹H NMR (400 MHz, Acetone-*d*₆): δ 18.46 (s, 1H, C₃-OH), 15.30 (brs, 1H, C₁₂-OH), 12.43 (s, 1H, C₁₀-OH), 9.04 (brs, 1H, NH₂), 8.12 (s, 1H, H_f), 7.95 (d, *J* = 8.1 Hz, 1H, H_c), 7.95 – 7.91 (m, 2H, H_e & H_h), 7.80 (dd, *J* = 8.6, 1.7 Hz, 1H, H_b), 7.75 (d, *J* = 8.0 Hz, 1H, H₈), 7.66 (brs, 1H, NH₂), 7.49–7.56 (m, 2H, H_f & H_g), 7.12 (d, *J* = 8.0 Hz, 1H, H₇), 5.78 (s, 1H, C_{12a}-OH), 4.39 (d, *J* = 8.5 Hz, 1H, C₅-OH), 3.85 (dt, *J* = 9.9, 8.5 Hz, 1H, H₅), 3.09 (dd, *J* = 18.8, 5.4 Hz, 1H, H₄), 2.99 (dd, *J* = 18.8, 3.2 Hz, 1H, H₄), 2.89 (dq, *J* = 12.6, 6.7 Hz, 1H, H₆), 2.60 (dd, *J* = 12.6, 7.6 Hz, 1H, H_{5a}), 2.51 (ddd, 1H, *J* = 9.9, 5.4, 3.2 Hz, 1H, H_{4a}), 1.64 (d, 3H, *J* = 6.7 Hz, H₆-Me) ppm. ¹³C{¹H} NMR (100 MHz, Acetone-*d*₆): δ 195.95, 195.06, 193.19, 176.35, 175.01, 160.64, 148.69, 138.30, 135.71, 134.46, 133.61, 129.32, 129.02, 128.92, 128.54, 128.43, 128.21, 126.97, 126.91, 116.94, 116.86, 107.63, 96.70, 75.85, 69.72, 47.79, 44.52, 39.58, 30.59, 16.42. HRMS (ESI): calculated for C₃₀H₂₆NO₈ [M+H]⁺: 528.1653, found 528.1660

9-(furan-2-yl)-RDOX (9). From 120 mg of **2a**, 38.2 mg (36%) of the targeted product was isolated after purification. Eluent H₂O + 0.1% formic acid / ACN, gradient 50 to 70% of ACN over 15 min. ¹H NMR (400 MHz, Acetone-*d*₆): δ 15.19 (brs, 1H, C₁₂-OH), 12.73 (s, 1H, C₁₀-OH), 9.03 (brs, 1H, NH₂), 8.00 (d, *J* = 8.2 Hz, 1H, H₈), 7.65 (brs, 1H, NH₂), 7.62 (dd, 1H, *J* = 1.8, 0.7 Hz, H_d), 7.08 (dd, *J* = 3.4, 0.7 Hz, 1H, H_b), 7.07 (dd, *J* = 7.7, 1.1 Hz, 1H, H₇), 6.58 (dd, *J* = 3.4, 1.8 Hz, 1H, H_c), 5.77 (s, 1H, C_{12a}-OH), 4.36 (d, *J* = 8.5 Hz, 1H, C₅-OH), 3.81 (dt, *J* = 9.9, 7.6 Hz, 1H, H₅), 3.03 (dt, *J* = 18.7, 5.4 Hz, 1H, H₄), 3.03 (dt, *J* = 18.7, 3.4 Hz, 1H, H₄), 2.81 (dq, *J* = 12.6, 6.8 Hz, 1H, H₆), 2.55 (dd, *J* = 12.6, 7.6 Hz, 1H, H_{5a}), 2.49 (ddd, *J* = 9.9, 5.4, 3.4 Hz, 1H, H_{4a}), 1.60 (d, *J* = 6.8 Hz, 3H, H₆-Me) ppm. ¹³C{¹H} NMR (100 MHz, Acetone-*d*₆): δ 195.95, 194.96, 193.12, 176.40, 174.98, 158.91, 149.87, 147.83, 142.53, 132.49, 119.10, 116.83, 116.72, 112.72, 111.20, 107.54, 99.77, 75.83, 69.71, 47.64, 44.47, 39.44, 30.62, 16.37 ppm. HRMS (ESI): calculated for C₂₄H₂₂NO₉ [M+H]⁺: 468.1289, found 468.1295.

9-(3,4-methylenedioxy-phenyl)-RDOX (10). From 100 mg of **2a**, 40.0 mg (40%) of the targeted product was isolated after purification. Conditions for preparative HPLC eluent: H₂O + 0.1% formic acid / ACN, gradient 40 to 80% of ACN over 15 min. ¹H NMR (300 MHz, Acetone-*d*₆): δ 15.24 (brs, 1H, C₁₂-OH), 12.37 (s, 1H, C₁₀-OH), 9.04 (brs, 1H, NH₂), 7.69 (brs, 1H, NH₂), 7.56 (d, *J* = 8.1 Hz, 1H, H₈), 7.13 (d, *J* = 1.6 Hz, 1H, H_f), 7.05 (dd, *J* = 8.1, 1.6 Hz, 1H, H_b), 7.01 (dd, *J* = 8.1, 0.8 Hz, 1H, H_c), 6.90 (d, 1H, *J* = 8.1 Hz, H₇), 6.03 (s, 2H, O-CH₂-O), 5.82 (brs, 1H, C_{12a}-OH), 4.41 (d, *J* = 8.2 Hz, 1H, C₅-OH), 3.81 (dt, *J* = 9.9, 8.2 Hz, 1H, H₅), 3.07 (dd, *J* = 18.6, 5.0 Hz, 1H, H₄), 2.97 (dd, *J* = 18.6, 3.1 Hz, 1H, H₄), 2.81 (dq, *J* = 12.6, 6.5 Hz, 1H, H₆), 2.53 (dd, *J* = 12.6, 7.5 Hz, 1H, H_{5a}), 2.47 (ddd, *J* = 9.9, 5.0, 3.1 Hz, H_{4a}), 1.59 (d, 3H, *J* = 6.8 Hz) ppm. ¹³C{¹H} NMR (75 MHz, Acetone-*d*₆): δ 195.93, 195.02, 193.16, 176.10, 174.95, 160.28, 148.30, 148.11, 147.82, 137.78, 131.77, 128.99, 123.57, 116.76, 116.59, 110.60, 108.78, 107.58, 102.06, 99.69, 75.75, 69.57, 47.66, 44.38, 39.45, 30.48, 16.33 ppm. HRMS (ESI): calculated for C₂₇H₂₄NO₁₀ [M+H]⁺: 522.1395, found 522.1400.

9-(1-(E)-hexenyl)-RDOX (11). From 80 mg of **2a**, 20.9 mg (29%) of the targeted product was isolated after purification. Conditions for preparative HPLC: eluent H₂O + 0.1% formic acid / ACN, gradient 65 to 85% of ACN over 15 min. ¹H NMR (400 MHz, Acetone-*d*₆): δ 18.44 (s, 1H, C₃-OH), 15.23 (brs, 1H, C₁₂-OH), 12.23 (s, 1H, C₁₀-OH), 9.03 (brs, 1H, NH₂), 7.68 (d, *J* = 8.1 Hz, OH, 1H), 7.64 (brs, 1H, NH₂), 6.92 (d, 1H, *J* = 8.1 Hz, H₈), 6.69 (d, *J* = 16.1 Hz, 1H, H_a), 6.35 (dt, *J* = 16.1, 7.1 Hz, 1H, H_b), 5.74 (s, 1H, C_{12a}-OH), 4.33 (d, 1H, *J* = 8.5 Hz, C₅-OH), 3.79 (dt, *J* = 10.0, 8.1 Hz, 1H, H₅), 3.01

(dd, $J = 18.7, 5.5$ Hz, 1H, OH, H₄), 2.96 (dd, $J = 18.7, 3.2$ Hz, 1H, OH, H₄), 2.76 (dq, $J = 12.6, 6.7$ Hz, 1H, H₆), 2.50 (dd, $J = 12.5, 7.5$ Hz, 1H, H₄), 2.47 (ddd, $J = 10.0, 5.5, 3.2$ Hz, 1H, H_{4a}), 2.25 (qd, $J = 7.1, 1.5$ Hz, 2H, H_c), 1.56 (d, 3H, $J = 6.7$ Hz, H₆-Me), 1.47 (m, 2H, H_d), 2.20 (m, 2H, H_e), 0.93 (t, 3H, $J = 7.1$ Hz, H_f) ppm. ¹³C{¹H} NMR (75 MHz, Acetone-*d*₆): δ 195.92, 194.96, 193.18, 175.98, 174.97, 159.98, 147.55, 133.71, 132.80, 125.96, 123.87, 116.55, 116.45, 107.47, 99.77, 75.77, 69.72, 47.78, 44.48, 39.43, 33.85, 32.39, 30.62, 22.93, 16.36, 14.22 ppm. HRMS (ESI): calculated for C₂₆H₃₀NO₈ [M+H]⁺: 484.1966, found 484.1972.

9-(2-(hydroxymethyl)-(Z)-hex-1-en-1-yl)-RDOX (12).

From 98 mg of **2a**, 43.4 mg (47%) of the targeted product were isolated after purification. Conditions for preparative HPLC: eluent H₂O + 0.1% formic acid / ACN, gradient 50 to 70% of ACN over 15 min. ¹H NMR (300 MHz, Acetone-*d*₆): δ 15.25 (brs, 1H, C₁₂-OH), 12.12 (s, 1H, C₁₀-OH), 9.03 (brs, 1H, NH₂), 7.63 (brs, 1H, NH₂), 7.53 (d, $J = 8.0$ Hz, 1H, H₈), 7.53 (d, $J = 8.0$ Hz, 1H, H₇), 6.42 (s, 1H, H_a), 5.72 (s, 1H, OH, C_{12a}-OH), 4.32 (d, $J = 8.6$ Hz, 1H, C₅-OH), 4.18 (d, $J = 4.2$ Hz, 2H, H_b-CH₂OH), 3.80 (dt, $J = 10.0, 8.6$ Hz, 1H, H₅), 3.75 (brt, $J = 4.2$ Hz, 1H, OH), 3.06 (dd, $J = 18.6, 5.7$ Hz, 1H, H₄), 2.97 (dd, $J = 18.6, 3.4$ Hz, 1H, H₄), 2.77 (m, 1H, H₆), 2.52 (dd, $J = 12.7, 7.5$ Hz, 1H, H_{5a}), 2.47 (ddd, $J = 10.0, 5.7, 3.4$ Hz, 1H, H_{4a}), 2.37 (t, 2H, $J = 7.8$ Hz, H_c), 1.53-1.63 (m, 2H, H_d), 1.58 (d, $J = 6.7$ Hz, 3H, H₆-Me), 1.41 (sext, $J = 7.3$ Hz, 2H, H_e), 0.95 (t, 3H, $J = 7.3$ Hz, H_f) ppm. ¹³C{¹H} NMR (75 MHz, Acetone-*d*₆): δ 195.92, 194.87, 193.17, 175.95, 174.93, 160.79, 147.52, 144.27, 137.85, 125.74, 121.52, 116.19, 115.81, 107.45, 99.69, 75.71, 69.60, 60.78, 47.73, 44.40, 39.42, 35.79, 31.15, 30.51, 23.22, 16.31, 14.32 ppm. HRMS (ESI): calculated for C₂₇H₃₀NO₈ [M+H-H₂O]⁺: 496.1966, found 496.1972.

9-(thiophen-2-yl)-RDOX (13).

From 120 mg of **2a**, 52.3 mg (51%) of the targeted product were isolated after purification. Eluent H₂O + 0.1% formic acid / ACN, gradient 50 to 70% of ACN over 15 min. ¹H NMR (300 MHz, Acetone-*d*₆): δ 15.16 (brs, 1H, C₁₂-OH), 12.79 (s, 1H, C₁₀-OH), 9.04 (brs, 1H, NH₂), 7.90 (d, 1H, $J = 8.2$ Hz, H₈), 7.66 (d, $J = 3.4$ Hz, 1H, H_d), 7.63 (brs, 1H, NH₂), 7.47 (d, $J = 5.4$ Hz, 1H, H_b), 7.12 (dd, $J = 5.4, 3.4$ Hz, 1H, H_c), 7.01 (d, $J = 8.2$ Hz, 1H, H₇), 5.74 (brs, 1H, C_{12a}-OH), 4.33 (d, 1H, $J = 8.6$ Hz, C₅-OH), 3.81 (q, 1H, $J = 8.6$ Hz), 3.07 (dd, $J = 18.6, 5.3$ Hz, 1H, H₄), 2.97 (dd, $J = 18.6, 3.3$ Hz, 1H, H₄), 2.78 (m, 1H, H₆), 2.54 (dd, $J = 12.6, 7.3$ Hz, 1H, H_{5a}), 2.49 (ddd, $J = 9.1, 5.3, 3.3$ Hz, 1H, H_{4a}), $J = 1.58$ (d, 3H, $J = 6.7$ Hz) ppm. ¹³C{¹H} NMR (75 MHz, Acetone-*d*₆): ¹³C NMR (75 MHz, Acetone) δ 195.96, 194.89, 193.13, 176.31, 174.97, 159.21, 148.19, 138.79, 135.31, 127.85, 126.58, 126.52, 122.37, 117.00, 116.88, 107.52, 99.81, 75.85,

69.80, 47.65, 44.49, 39.40, 30.72, 16.37 ppm. HRMS (ESI): calculated for C₂₄H₂₂NO₉S [M+H]⁺: 484.1061, found 484.1070.

9-(3-benzyloxy-phenyl)-RDOX (14).

From 90 mg of **2a**, 20.0 mg (24%) of the targeted product were isolated after purification: eluent H₂O + 0.1% formic acid / ACN, gradient 60 to 80% of ACN over 15 min. ¹H NMR (300 MHz, Acetone-*d*₆): δ 15.23 (brs, 1H, C₁₂-OH), 12.36 (s, 1H, C₁₀-OH), 9.04 (brs, 1H, NH₂), 7.60 (brs, 1H, NH₂), 7.58 (d, $J = 8.0$ Hz, 1H, H₈), 7.50 (d, $J = 7.7$ Hz, 2H, H_i), 7.41 – 7.28 (m, 5H, H_j+H_k+H_b+H_c), 7.18 (d, $J = 7.7$ Hz, 1H, H_d), 7.03 (d, $J = 8.0$ Hz, 1H, H₇), 7.00 (ddd, $J = 8.1, 2.5, 0.8$ Hz, 1H, H_b), 5.73 (s, 1H, C_{12a}-OH), 5.16 (s, 2H, H_g), 4.33 (d, $J = 8.5$ Hz, 1H, C₅-OH), 3.82 (dt, $J = 9.9, 8.5$ Hz, 1H, H₅), 3.07 (dd, $J = 18.6, 5.5$ Hz, 1H, H₄), 2.98 (dd, $J = 18.6, 3.4$ Hz, 1H, H₄), 2.85 (m, 1H, H₆), 2.56 (dd, $J = 12.5, 7.6$ Hz, 1H, H_{5a}), 2.49 (ddd, $J = 9.9, 5.5, 3.4$ Hz, 1H, H_{4a}), 1.60 (d, $J = 6.6$ Hz, 3H, H₆-Me) ppm. ¹³C{¹H} NMR (75 MHz, Acetone-*d*₆): δ 195.9, 195.0, 193.2, 176.1, 175.0, 160.5, 159.6, 148.5, 139.4, 138.5, 138.0, 129.9, 129.3, 129.2, 128.6, 128.5, 122.8, 116.9, 116.8, 116.7, 114.6, 107.6, 99.8, 75.8, 70.6, 69.8, 47.7, 44.5, 39.5, 30.7, 16.4 (CH₃) ppm. HRMS (ESI): calculated for C₃₃H₃₀NO₉ [M+H]⁺: 584.1915, found 568.1922.

9-(3,4,5-trimethoxyphenyl)-RDOX (15).

From 120 mg of **2a**, 34.0 mg (26%) of the targeted product were isolated after purification: eluent H₂O + 0.1% formic acid / ACN, gradient 60 to 80% of ACN over 15 min. ¹H NMR (400 MHz, Acetone-*d*₆): δ 18.45 (brs, 1H, C₃-OH), 15.24 (brs, 1H, OH), 12.39 (s, 1H, C₁₀-OH), 9.04 (brs, 1H, NH₂), 7.65 (brs, 1H, NH₂), 7.63 (d, $J = 8.0$ Hz, 1H, H₈), 7.04 (dd, $J = 8.0, 1.0$ Hz, 1H, H₇), 6.91 (s, 2H, H_b), 5.77 (brs, 1H, C_{12a}-OH), 4.38 (d, 1H, $J = 8.5$ Hz, C₅-OH), 3.86 (s, 6H, H_c-OMe), 3.79-3.85 (m, 1H, H₅), 3.78 (s, 3H, H_a-OMe), 3.07 (dd, $J = 18.6, 5.5$ Hz, 1H, H₄), 2.98 (dd, $J = 18.6, 3.1$ Hz, 1H, H₄), 2.84 (dq, $J = 12.5, 6.6$ Hz, 1H, H₆), 2.56 (dd, $J = 12.5, 7.6$ Hz, 1H, H_{5a}), 2.49 (ddd, $J = 9.9, 5.5, 3.1$ Hz, 1H, H_{4a}), 1.61 (d, $J = 6.8$ Hz, 3H, H₆-Me) ppm. ¹³C{¹H} NMR (100 MHz, Acetone-*d*₆): δ 206.11, 195.94, 195.03, 193.19, 176.26, 175.00, 160.41, 154.06, 148.30, 138.87, 137.94, 133.35, 129.44, 116.84, 116.57, 108.08, 107.59, 99.78, 75.83, 69.69, 60.59, 56.56, 47.78, 44.49, 39.51, 30.59, 16.41 ppm. HRMS (ESI): calculated for C₂₉H₃₀NO₁₁ [M+H]⁺: 568.1813, found 568.1823.

General Procedure for Sonogashira Coupling. In a 25 mL two-neck round-bottom flask, 9-iodo-RDOX **2a** (160 mg, 3.0 × 10⁻¹ mmol, 1.0 eq), Pd(PPh₃)₂Cl₂ (10.7 mg, 1.5 × 10⁻² mmol, 0.05 eq) and CuI (2.9 mg, 1.5 × 10⁻² mmol, 0.05 eq) were suspended in NEt₃ (3.1 mL) then dry DMF (3.1 mL) was added, and the resulting mixture was purged under Argon for 10 minutes. TMS acetylene

(215 μ L, 1.5 mmol, 5.0 eq) was added, and the reaction was stirred at 60°C for 12 hours under Argon. After cooling at room temperature, the reaction was filtered on a small pad of Celite, and the filtrate was concentrated under reduced pressure. Then, the organic phase was extracted with EtOAc (3 \times 25 mL), washed with HCl (1M) and brine, dried over MgSO₄, filtered off, and evaporated under reduced pressure. The crude material was first purified on a silica gel column (eluent CH₂Cl₂ + 1% formic acid) and then by preparative HPLC.

[10,9-*b*](1-butylfuran)-RDOX (16). From 98 mg of **2a**, 20.2 mg (22%) of the targeted product were isolated after purification: eluent H₂O + 0.1% formic acid / ACN, gradient 50 to 90% of ACN over 15 min. ¹H NMR (300 MHz, Acetone-*d*₆): δ 18.50 – 17.40 (brs, 1H, OH, C₃-OH), 17.25 -15.00 (brs, 1H, C₁₂-OH), 9.12 (brs, 1H, NH₂), 7.73 (d, *J* = 8.2 Hz, 1H, H₈), 7.67 (brs, 1H, NH₂), 7.34 (d, *J* = 8.2 Hz, 1H, H₇), 6.57 (t, *J* = 1.0 Hz, 1H, H_a), 5.47 (brs, 1H, C_{12a}-OH), 4.24 (brs, 1H, C₅-OH), 3.83 (t, *J* = 8.6 Hz, 1H, H₅), 3.03 (dd, *J* = 18.5, 5.4 Hz, 1H, H₄), 2.98 (dd, *J* = 18.5, 4.0 Hz, 1H, H₄), 2.94 – 2.87 (m, 1H, H₆), 2.83 (t, *J* = 7.6 Hz, 2H, H_c), 2.59 (dd, *J* = 12.6, 7.8 Hz, 1H, H_{5a}), 2.50 (dt, *J* = 9.6, 5.4, 4.0 Hz, 1H, H_{4a}), 1.76 (p, *J* = 7.5 Hz, 2H, H_d), 1.63 (d, *J* = 6.9 Hz, 3H, H₆-Me), 1.46 (h, *J* = 7.5 Hz, 2H, H_c), 0.96 (t, *J* = 7.3 Hz, 3H, H_f). ¹³C {¹H} NMR (100 MHz, Acetone-*d*₆): δ 195.83 (C₃), 193.73 (C₁₂), 188.85 (C₁₃), 179.66 (C₁₁), 174.96 (CONH₂), 162.02 (C_b), 153.27 (C₉), 143.19 (C₁₀), 130.48 (C_{6a}), 126.09 (C₈), 120.36 (C₇), 115.58 (C_{10a}), 106.81 (C_{11a}), 102.23 (C_a), 100.02 (C_{2a}), 76.73 (C_{12a}), 70.34 (C₅), 47.49 (C_{5a}), 44.60 (C_{4a}), 39.82 (C₆), 31.69 (C₄), 30.53 (C_d), 28.62 (C_c), 22.91 (C₆-Me), 17.24 (H_e), 14.05 (H_f). HRMS (ESI): calculated for C₂₆H₂₈NO₈ [M+H]⁺: 482.1809, found 482.1812.

9-(trimethylsilylethynyl)-RDOX (17). From 96 mg of **2a**, 22.0 mg (21%) of the targeted product were isolated after purification: eluent H₂O + 0.1% formic acid / ACN, gradient 60 to 80% of ACN over 15 min. ¹H NMR (400 MHz, Acetone-*d*₆): δ 15.25 (brs, 1H, C₁₂-OH), 12.12 (s, 1H, C₁₀-OH), 9.03 (brs, 1H, NH₂), 7.63 (brs, 1H, NH₂), 7.53 (d, 1H, *J* = 8.1 Hz, H₈), 6.94 (d, *J* = 8.1 Hz, 1H, H₇), 5.79 (s, 1H, C_{12a}-OH), 4.36 (brd, *J* = 8.5 Hz, 1H, C₅-OH), 3.81 (q, *J* = 8.1 Hz, 1H, H₅), 3.07 (dd, *J* = 18.6, 5.5 Hz, 1H, H₄), 2.98 (dd, *J* = 18.6, 3.2 Hz, 1H, H₄), 2.80 (dq, 1H, *J* = 12.6, 6.8 Hz), 2.53 (dd, *J* = 12.6, 7.5 Hz, 1H, H_{5a}), 2.48 (ddd, *J* = 10.0, 5.5, 3.2 Hz, 1H, H_{4a}), 1.57 (d, *J* = 6.6 Hz, 3H, H₆-Me), 0.24 (s, 9H, TMS) ppm. ¹³C {¹H} NMR (100 MHz, Acetone-*d*₆): δ 195.94, 194.24, 193.04, 176.82, 174.95, 163.84, 150.00, 140.64, 116.68, 116.60, 111.92, 107.38, 100.94, 99.73, 99.44, 75.82, 69.68, 47.40, 44.46, 39.56, 30.61, 16.32, 0.09 ppm. HRMS (ESI): calculated for C₂₅H₂₇NO₈Si [M+H]⁺: 498.1579, found 498.1586.

9-(ethynyl)-RDOX (18). In a 10 mL round-bottom flask, compound **17** (40 mg, 8.0 \times 10⁻² mmol, 1.0 eq) was dissolved in a mixture of MeOH/THF (1:1, v:v) (1.8 mL) and an aqueous solution of KOH (1M) (240 μ L) was added dropwise. The reaction mixture was stirred at room temperature for 3 hours under Argon. Then, solvents were evaporated under reduced pressure, and the organic phase was extracted with EtOAc (3 \times 25 mL), washed with HCl (1M) and brine, dried over MgSO₄, filtered off, and evaporated under reduced pressure. Compound **18** was isolated without further purification (25 mg, 74%). ¹H NMR (400 MHz, Acetone-*d*₆): δ 18.43 (s, 1H, C₁₂-OH), 15.17 (brs, 1H, C₃-OH), 12.26 (s, 1H, C₁₀-OH), 9.02 (brs, 1H, NH₂), 7.66 (brs, 1H, NH₂), 7.64 (d, *J* = 8.1 Hz, 1H, H₈), 6.97 (d, *J* = 8.1 Hz, 1H, H₇), 5.78 (s, 1H, C_{12a}-OH), 4.37 (brd, *J* = 6.8 Hz, 1H, C₅-OH), 3.81 (m, 1H, H₅), 3.79 (s, 1H, H₇), 3.07 (dd, *J* = 18.7, 5.4 Hz, 1H, H₄), 2.96 (dd, *J* = 18.7, 3.5 Hz, 1H, H₄), 2.81 (dt, *J* = 12.5, 6.7 Hz, 1H, H₆), 2.54 (dd, *J* = 12.5, 7.6 Hz, 1H, H_{5a}), 2.49 (ddd, *J* = 10.0, 5.4, 3.5 Hz, 1H H_{4a}), 1.57 (s, 3H, H₆-Me) ppm. ¹³C {¹H} NMR (100 MHz, Acetone-*d*₆): δ 195.95, 194.24, 193.04, 176.82, 174.94, 164.02, 150.08, 140.80, 116.70, 116.63, 111.09, 107.39, 99.73, 83.81, 79.31, 75.82, 69.68, 47.38, 44.45, 39.53, 30.60, 16.32 ppm. HRMS (ESI): calculated for C₂₂H₂₀NO₈ [M+H]⁺: 426.1183, found 426.1193.

4.2. Biological Assays

Expression and Purification of Human Recombinant α -Syn. Recombinant wild-type human α -Syn was expressed in Escherichia coli using the pT7-7 plasmid encoding for the protein sequence. Purification was performed as previously described.³⁹ Protein purity was assessed using electrophoresis in polyacrylamide gels under denaturing conditions (SDS-PAGE). The stock solution of α -Syn was prepared in 20 mM HEPES, 150 mM NaCl, and pH 7.4. Prior to the aggregation assay, the protein stock solutions were centrifuged for 30 min at 12,000 \times g to remove microaggregates. Protein concentration was determined by measuring the absorbance at 280 nm using the extinction coefficient $\epsilon_{275} = 5600 \text{ cm}^{-1} \text{ M}^{-1}$.

Protein Aggregation Assay The aggregation protocol was adapted from previous studies.¹⁰ The different aggregated species were formed by incubating recombinant α -Syn samples (70 μ M) in 10 mM PBS, pH 7.4, in a Thermomixer Comfort[®] (Eppendorf, Germany) at 37 °C under orbital agitation at 600 rpm in the absence or presence of each tetracyclines derivatives at 20 μ M.

Thioflavin T (ThT) Fluorescence Assay. Aggregation studies with α -Syn in the absence or presence of the

different non-antibiotic tetracyclines were performed by measuring the fluorescence emission of ThT at different time points according to LeVine.⁴⁰ Changes in the emission fluorescence spectra were monitored at an excitation wavelength of 450 nm using a Fluoromax-4 spectrofluorometer.

Ethic Statement. Mice used were housed, handled, and cared for in strict accordance with the European Union Council Directives (2010/63/EU). The Committee on the Ethics of Animal Experiments Charles Darwin no. 5 approved experimental protocols under authorization number Ce5/2017/005.¹⁹

Primary Microglial Cell Isolation. Microglial cell isolation was performed as previously described.⁴¹ Briefly, hole brains were harvested, and the cells were mechanically disaggregated and resuspended in Dulbecco's Modified Eagle Medium (DMEM) with 10 % heat-inactivated fetal bovine serum (FBS), 10.000 U/mL penicillin, and 10.000 µg/mL streptomycin (P-E). Next, the cells were seeded at a density of two brains per 10 mL of media in a polyethyleneimine (PEI) pre-coated T75 flask and incubated at 37 °C with 5% CO₂. Fourteen days after incubation, microglial cell isolation was completed, and as required, the cells were harvested by trypsinization and resuspended in DMEM containing 0,1% FBS and P-E for plating specific to each experiment.

Cell Cytotoxicity. To evaluate the safety of the molecules in terms of cytotoxicity, we measured the lactate dehydrogenase (LDH) activity released in the extracellular medium. To assess this evaluation, 4×10⁵ cells/well were seeded into 96 well plate. After 24 h, cells were pre-treated with tetracycline derivatives at a final concentration of 20 µM. Control groups consisted of i) non-treated cells, which correspond to physiological release of LDH and ii) cells treated with Triton 1%, which correlates with the maximum level of LDH as a positive control of toxicity. Twenty-four hours after incubation, supernatants were transferred to a new plate, LDH reagents were added according to the manufacturer's instructions (Roche, Lot #11644793001), and the absorbance was read at 490 nm. All experiments were performed in quadruplicate, and the relative cell cytotoxicity (%) was expressed as a percentage relative to the untreated control cells.

Detection of TNF-α released. To evaluate the anti-inflammatory properties of the molecules on LPS-activated primary microglia, the TNF-α released in the culture medium was measured using the ELISA kit (Lot #BMS607-3) from Thermo Fisher Scientific. To this end, 3×10⁵ cells/well were seeded in 96 well plates. After 24h, cells were pre-treated with the tetracyclines

derivatives **6** and **RDOX** to a final concentration of 20 µM. Four hours later, an inflammatory environment was induced by adding LPS at a final concentration of 10 ng/mL. For this experiment, the control groups were Dexamethasone 2.5 µM, LPS only, and the untreated group (Control), in which case only fresh media was added. The absorbance of each sample was measured according to the manufacturer's instructions using a spectrophotometer SpectraMax M4 (Molecular Devices, Sunnyvale, CA, USA).

Antimicrobial assay. The susceptibility of bacterial strains *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 25923) to antibiotics and compounds was determined in microplates using the standard broth dilution method according to the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM).⁴² Briefly, the Minimal Inhibitory Concentrations (MICs) were determined with an inoculum of 10⁵ CFU in 200 µL of MHII containing two-fold serial dilutions of each drug. The MIC was defined as the lowest concentration of the drug that completely inhibited visible growth after incubation for 18 h at 37 °C. To determine all MICs, the measurements were independently repeated in triplicate.

ASSOCIATED CONTENT

Supporting Information.

Copies of ¹H and ¹³C NMR spectra, and HPLC chromatograms; Data from figures 2-4; MTT assays (pdf)

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Notes

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ABBREVIATIONS

α -Syn, α -synuclein; ACN, Acetonitrile; ANOVA, analysis of variance; DEX, dexamethasone; TNF- α , Tumor necrosis factor-alpha; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; DOX, doxycycline; ESI, Electrospray ionization; HPLC, high performance chromatography; LPS, lipopolysaccharide; MIC, Minimal inhibitory concentration; MPTP, methyl-phenyl-tetrahydropyridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NIS, *N*-iodosuccinimide; NMR, nuclear magnetic resonance; 6-OHDA, 6-hydroxydopamine; QTOF, quadrupole time of life; S.E.M., standard error of the mean; ThT, thioflavin T; TLC, thin layer chromatography; TMS, trimethylsilyl; TOF, time of flight; UDEFT, uniform driven equilibrium Fourier transform;

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