Design and Synthesis of New Non-Antibiotic Doxycyclin Derivatives Against α-Synuclein Aggregation with Anti-Inflammatory Properties

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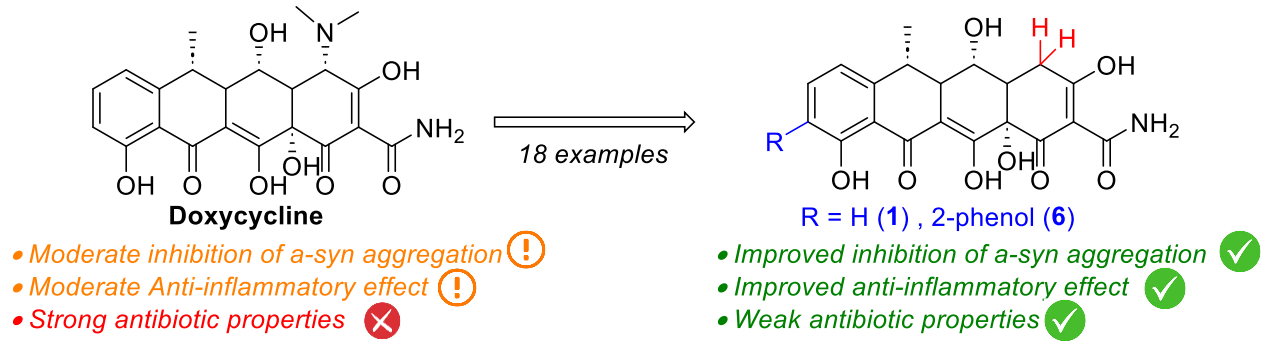


ABSTRACT

Doxycycline is a tetracycline commonly used for its antibiotic properties and capacity to treat acne- and rosacea-like skin lesions. It has also recently demonstrated interesting effects against Parkinson's disease pathomechanisms. Notably, doxycycline was reported to limit amyloid-type aggregation of α-synuclein and curtail neurodegeneration-related inflammatory processes. However, the potential therapeutic interest of doxycycline is limited due to its antibiotic activity. The design of novel doxycycline derivatives was undertaken to generate non-antimicrobial doxycycline derivatives with still neuroprotective properties. Specifically, the dimethyl-amino at C_4 group was reduced to significantly diminish the antibiotic activity, and several coupling reactions were performed at position C_9 of the D ring. Among 18 novel tetracyclines, seven derivatives with reduced antibiotic activity were more efficient than their parent compound in reducing α-synuclein aggregation. Among those, two derivatives exerted better anti-inflammatory effects than doxycycline, at concentrations that are not cytotoxic. Thus, compounds **1** and **6** seem to have a better neuroprotective potential than doxycycline, making them excellent candidates for further pre-clinical investigations.

1. INTRODUCTION

Parkinson's disease is the second most encountered neurodegenerative disease, after Alzheimer's disease, affecting 2% of the elderly population (over 65 years old)[1]. Current treatments are solely symptomatic and thus do not influence disease development. The most prevalent effect in patients affected by Parkinsonism is the loss of dopaminergic neurons in the *Substancia nigra pars compacta*. Even though the exact factors responsible for this neuronal death remain unknown, intracellular deposition of abnormally aggregated α-synuclein (α-Syn) has been invoked.[2] Mitochondrial dysfunction has also been observed in the pathogenesis of Parkinson's disease,[3] and the influence of toxicants such as methyl-phenyl-tetrahydropyridine (MPTP), for instance, has been extensively reported.[4,5] The unmet therapeutic needs for treating Parkinson's disease drive research groups worldwide to identify and develop therapeutic agents to stop or delay the progression of the disease. In this context, Doxycycline (**DOX**), a tetracycline derivative, has been studied and demonstrated excellent neuroprotective effects in experimental models without any significant signs of toxicity.[6] Among these neuroprotective effects, **DOX** was able to prevent the aggregation of α-Syn;[7] reduce oxidative stress, act as an anti-inflammatory agent,[8] induce cellular redistribution of aggregates in an animal model of Parkinson's disease[9] among other activities on inflammatory processes in *ex vitro* and *in cellulo* assays.[7,10–12] These pieces of evidence make **DOX** a good candidate as a novel therapeutic agent of interest for Parkinson's disease treatment but also for Alzheimer disease.[13] Interestingly, in animal models of Parkinson's disease, **DOX** has been found to limit neurodegenerative effects (*e.g.,* neuron death, neuro-inflammation) in hemiparkinsonian mice receiving intra-striatal injections of 6-hydroxydopamine (6-OHDA)[14] and in mice expressing human α-Syn (A53T).[15] Using **DOX** for long-term treatments in Parkinson's disease patients may lead however to potential antibiotic resistance and microbiota disruption. In that context, we became involved in **DOX** derivatives' design, synthesis, and biological study. 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.[16] Recently, we showed that a new tetracycline derivative, a "reduced" demeclocycline derivative called **DDMC**, showed promising neuroprotective activity by interfering with α-Syn amyloid-like aggregation and without exhibiting significant antibiotic properties.[11] Moreover, demeclocycline (**DMC**), **DOX**, and their corresponding reduced analogs **DDMC** and **DDOX** have also shown neuroprotective properties through their ability to chelate iron, preventing oxidative stress.[17] (Figure 1) In continuation to these past studies, and motivated by these encouraging results, a series of original **DOX** derivatives was synthesized to increase the anti-aggregative properties towards α-Syn. At the same time, evaluating their cytotoxicity would determine their ability to protect dopaminergic cells against apoptosis and, consequently, Parkinson's disease. Evaluation of the antibacterial activity of new molecules will serve to be selective in treating Parkinson's disease, avoiding bacterial resistance.

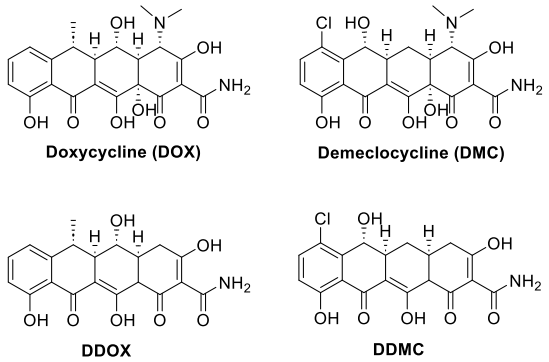


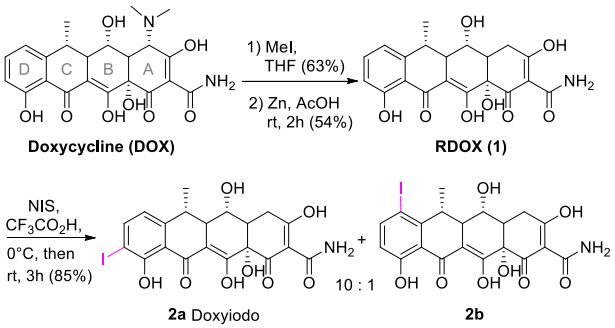
Figure 1. Structures of previously studied tetracyclines: **DOX**, **DMC**, **DDOX** and **DDMC**.

2. RESULTS AND DISCUSSION

2.1. Chemical Synthesis.

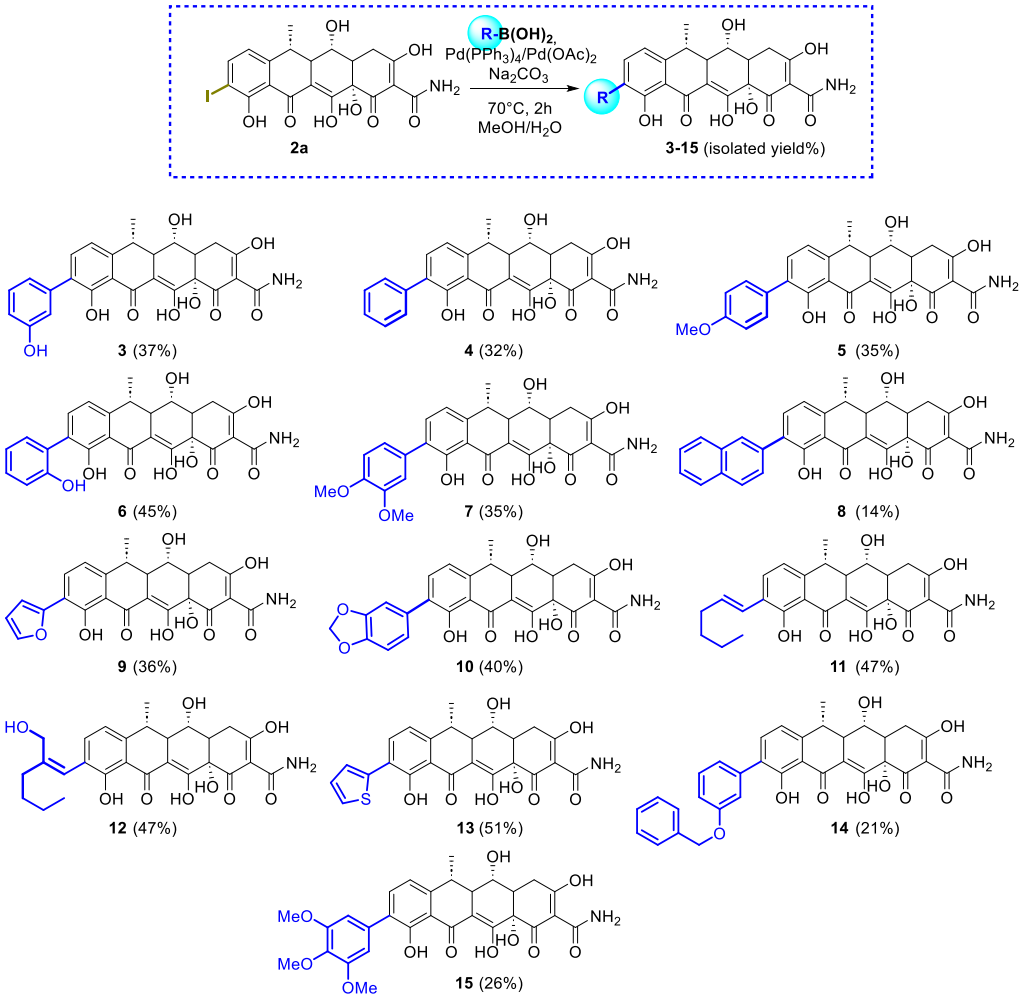
It is well accepted that the *N*-dimethylamino function at C-4 position on the upper half of the tetracycline core structure in ring A is crucial for the antibacterial properties.[18,19] Additionally, previous research has established a strong correlation between function and structure, emphasizing the importance of maintaining a specific structural motif in tetracyclines, which is vital in inhibiting protein amyloid aggregation.[6,20] To prepare new analogs, the aromatic ring D seemed to us the most accessible part to work on due to the capacity of phenol moiety to promote aromatic electrophilic substitution at either position C_9 and/or C_7. Particularly, halogenation of these positions would lead to further cross-coupling reactions.

Thus, we decided to remove the dimethylamino group, as reported in the literature.[18,21–23] The compound 4-des-*N*-dimethylaminodoxycycline **1** (**RDOX**) was 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 significative formation of **DDOX**.[17] In a second step, we focused our attention on the functionalization of the aromatic ring. After several attempts, we found that CF_3COOH and NIS at 0 °C were the best conditions to selectively iodinate **RDOX**[24] on position C_9 (**2a**) over position C_7 (**2b**) with about a 10:1 ratio. Preparative HPLC then separated the two regio-isomers **2a** and **2b**. Attempts towards bromination were unsuccessful, leading to unselective reactions and an inseparable mixture of products. (Scheme 1).



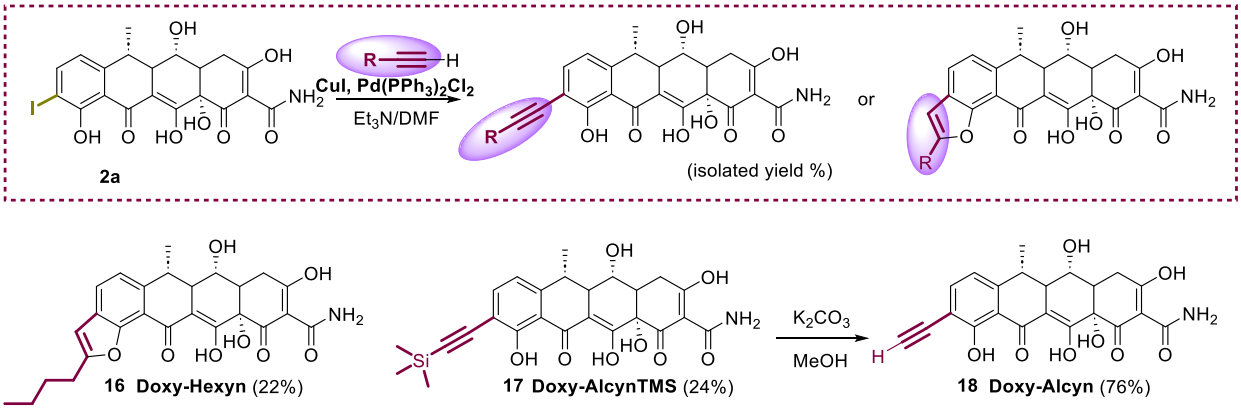
Scheme 1. Synthesis of 4-*des*-N-dimethylaminodoxycycline **1** (**RDOX**) and 9-iododerivative **2a** and **2b.**

A third final step consisted in performing a cross-coupling reaction at C_9 position of D ring to install new functionalities. 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 low toxicity of boronic acids. The use of MeOH as a solvent for this transformation[25,26] 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 doxycycline by Suzuki cross-coupling from 9-iodo-**RDOX 2a**.

The insertion of alkyne derivatives was also investigated through Sonogashira cross-coupling reaction. DMF was crucial to solubilize the tetracycline 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 **18**. (Scheme 3).



Scheme 3. Preparation of benzofuranyl derivative **16** and alkynes derivatives **17** and **18** from a Sonogashira cross-coupling reaction with 9-iodo-**RDOX 2a**.

2.2. *Antibacterial activity.*

Table 1. MIC of synthesized products against several strains of bacteria

Compounds	MIC µM (µg/mL)		
	P. aeruginosa PAO1	*E. coli ATCC 25922*	*S. aureus 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

Because this study aimed to find promising molecules to treat Parkinson's disease and because they might be taken chronically to prevent the development of the disease, a secondary objective was to decrease the antibiotic activity of tetracyclines to not interfere with another medication but also not to develop resistance.[19,27] 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). 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 new compounds against Gram-positive bacteria *S. aureus* was decreased by at least 8-fold and up to 250-fold compared to doxycycline, indicating moderate to low activity. Overall, removing the dimethylamino group dramatically reduces antibiotic activity, highlighting the role of this substituent. (Table 1).

2.3. *Inhibition of α-Synuclein aggregation.*

It has been previously reported that tetracyclines, specifically **DOX**, can avoid or diminish α-Syn amyloid-like aggregation.[6,7,20] 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 α-Syn in the absence or the presence of 20 µM of each tetracycline at 37 °C under orbital agitation. The cross-β structure, which is the hallmark of amyloid aggregation,[28] was monitored by Thioflavin T (ThT) fluorescence emission at 482 nm (λexc 450 nm).[29]

The results showed that seven of the eighteen new tetracyclines (**17**, **16**, **14**, **6**, **12**, **RDOX,** and **4)** decreased the ThT fluorescence intensity compared to the control (Figure 2). Interestingly, the antiaggregant potential of each molecule was not the same, with tetracyclines **17**, **16**, **14,** and **6** reaching the highest level of significance (Figure 2).

A lipophilic substituent at C9 seems favorable to inhibit the aggregation of α-Syn, with compounds **16** and **17** exhibiting the best activities while **18**, having lost the trimethylsilyl group, is ineffective. However, it is worth noting that **RDOX** showed efficient inhibition, while the corresponding iodo-derivative **2a**, with an iodine atom instead of hydrogen at position C-9, completely lost the inhibition of the aggregation of α-Syn. The conformation also seems important because alkenes **11** and **12** were less effective; thus, suppressing conformational rotation adjacent to D rings seems beneficial for compounds **16** and **17**. Another case is *meta*-benzyloxy derivative **14**, one of the most effective compounds, which contrasts with the free *meta*-phenol **3,** leading to a negative

result (*i.e.*, **3** acts as a pro-aggregative molecule). According to previous observations, protection as benzyl ether provides lipophilic properties, and the aromatic ring restrains conformational rotation adjacent to the D ring of tetracycline.

A supplementary hydroxy group adjacent to the phenol of the D ring is also beneficial (compound **6**), improving the antiaggregant activity, which is not unexpected considering it gives additional hydrogen bonding for antiaggregatory properties.

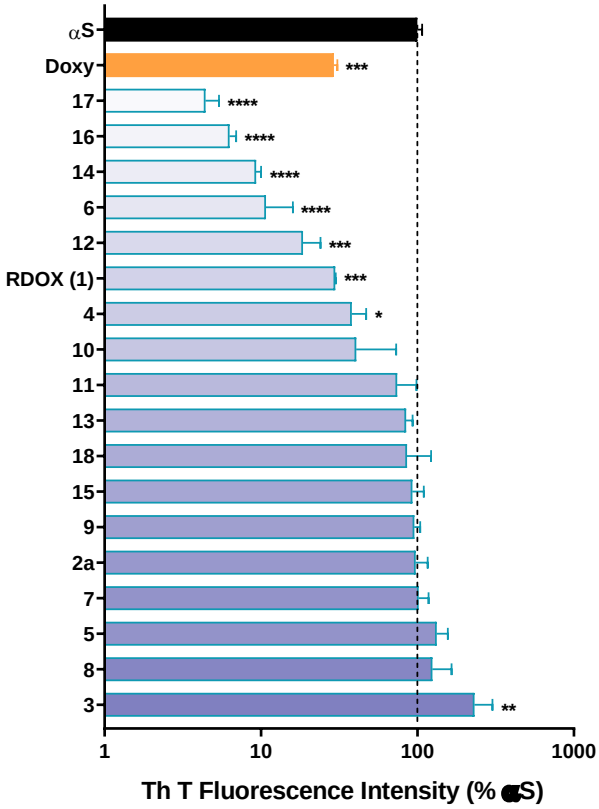


Figure 2. Effect of the new tetracyclines derivatives **1-18** on the α-Syn aggregation as measured by the fluorescence emission intensity of 25 μM ThT in a solution containing 70 μM of α-Syn incubated in the presence of 20 μM of each compound after 120 h of incubation. Bars= mean+SEM. Significant differences are indicated in the figure as follows: ****p<0.0001; ***p<0.001; **p<0.01*p<0.05 vs.α-Syn control ANOVA, Dunnets multiple comparison test.

2.4. *Cellular cytotoxicity.*

Although doxycycline has proven to be clinically safe[30] through its uses against different diseases, here we evaluated the impact of the structural modifications of new tetracyclines on cellular toxicity. Therefore, a lactate dehydrogenase (LDH) assay was performed, which is commonly used as a measure of cytotoxicity by membrane impairment. In this case, microglia cells were treated with 20 μM of the molecules in all cases. We used no-treated cells as the 100% release of physiological and non-pathological LDH. Results evidenced that **6**, **12,** and **RDOX** do not show cytotoxicity at 20 μM, with no significant differences with respect to the control-treated condition. In contrast, the positive control with 1% Triton X-100 displayed high cytotoxicity, as reflected by the 150% increase in the release of LDH into the medium culture according to the control (Figure 3). Moreover, to complement the cytotoxicity assay, we tested the cell viability through the colorimetric MTT metabolic activity after exposure to the tetracyclines derivatives **1**, **4**, **6**, **10**, **12**, **14**, **16**, **17**.[31] Results showed that two tetracycline derivatives, **6** and **RDOX** do not influence cell viability up to 20 μM (See Supplementary information), reinforcing the non-toxicity of both compounds.

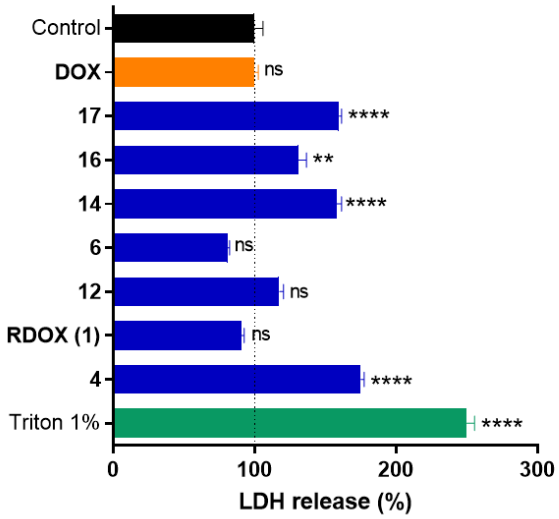


Figure 3. LDH cytotoxicity assay in primary microglia cells after the addition of each tetracycline. Cytotoxicity values were expressed as a % of LDH release relative to the untreated control cells (Control). As a cytotoxic control, Triton 1% was used, which induced complete disruption of the cells. Data represent the mean ± S.E.M (n = 6). One-way ANOVA followed by Holm-Sidak's multiple comparisons tests. Significant differences are indicated in the figure as follows: ****p < 0.0001; **p < 0.01 vs Control.

2.5. *Anti-inflammatory effect.*

Tumor necrosis factor alpha (TNF-α) is a key inflammatory protein produced in response to various inflammatory and pathological conditions in the human body.[32] Acute activation results in tissue repair and protective immune response induction. However, if it becomes chronic, it can be deleterious to the brain, resulting in neurodegeneration. Such activation is observed during viral encephalitis, bacterial meningitis, multiple sclerosis, ischemia, trauma, Parkinson's disease, and similar conditions. Here, we evaluate the anti-inflammatory properties of the most promising products **6** and **RDOX,** regarding anti-aggregation of α-Syn and without cytotoxicity, on lipopolysaccharide (LPS)-activated microglial cells. As expected, LPS triggered the release of TNF-α in treated primary microglia cells, being our inflammogenic control

conditions. In the presence of **6** and **RDOX**, the TNF-α level significantly diminished around 77.5 and 46.9 %, respectively, compared to LPS treatment (Figure 4). Interestingly, both molecules performed better than the precursor **DOX**, where at the concentration tested, this antibiotic cannot decrease the TNF-α release.[8]

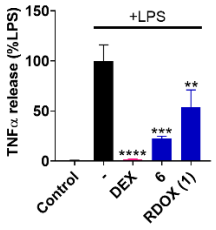


Figure 4. TNFα release from microglial cells upon undergoing (i) no treatment (Control), exposure to (ii) LPS (-) or pre-treated with (iii) 2.5 µM dexamethasone (DEX), (iv) 20 µM **6**; (v) 20 µM **RDOX** (**1**); 4 h before LPS treatment. The bars represent the mean ± S.E.M (n = 3). Significant differences are indicated in the figure as follows: *** $p < 0.001$; ** $p < 0.001$ vs. (-) LPS.

3. CONCLUSIONS

Doxycycline (**DOX**) has shown previously in several *in vitro* and *in vivo* models of Parkinson's disease beneficial effects towards two pathomechanisms involved in the degenerative process of dopaminergic neurons: α-Syn antiaggregant properties and anti-inflammatory activity. We thus designed a chemical library containing eighteen new tetracyclines obtained through Suzuki cross-coupling or Sonogashira cross-coupling reaction of 9-Iodo-**RDOX 2a** with the corresponding nucleophiles. The reduction of the dimethylamino group significantly decreased the antibiotic activity for most of the compounds, enabling the utilization of those novel tetracyclines in chronic prescriptions**.** The identification of new leads for Parkinson's disease treatment was then undertaken. We hypothesized that new chemical entities showing α-Syn antiaggregant properties combined with anti-inflammatory activity could have a disease-modifying effect and thus be promising therapeutic agents. All synthesized compounds were therefore tested on a model of α-Syn anti-aggregation, and eight of them (**17**, **16**, **14**, **6**, **12**, **10**, **4**, and **RDOX)** exhibited a decrease in the ThT fluorescence intensity. It seems that lipophilic properties of the substituent at position C_9 generally induce the α-Syn anti-aggregation but requires a reduced degree of liberty adjacent to the aromatic ring of tetracycline. The positioning of an adjacent hydroxy group, such as with compound **6**, also improves the aggregation by finally adding one successive hydrogen bond possible to the south part of the tetracycline. Among those active compounds, an LDH assay was performed, commonly used to measure the cytotoxicity of microglial cells. The assays showed that only compounds **6** and **RDOX** did not display significant cytotoxicity at 20 µM. Thus, the anti-inflammatory properties of those two hits on LPS-activated microglial cells showed powerful anti-inflammatory properties, unlike doxycycline. The study concludes that compound **6** and **RDOX** showed better abilities to treat Parkinson's disease than doxycycline (**DOX**) on several points of comparison: better anti-aggregating, better cell viability, less cytotoxicity, better anti-inflammatory effect, and weak antibiotic properties. Together, these results emphasize that **6** and **RDOX (1)** are promising drug candidates and should be further studied, for instance, on *in vivo* models of Parkinson's disease, before entering pre-clinical studies.

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 F_{254} (0.25 mm) plates purchased from Merck. Compounds were visualized by exposure to a UV lamp (λ = 254 and 365 nm). All Preparative chromatographies were performed on an Xbridge (Waters) C18 5 µm, [Ø 19 mm x 150 mm or Ø 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.[33,34] ^1H and ^{13}C 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 ($CDCl_3$: δ_C = 77.16 ppm unless notified, residual $CHCl_3$ in $CDCl_3$: δ_H = 7.26 ppm; C_6D_6: δ_C = 128.06 ppm unless notified, residual C_6HD_5 in C_6D_6: δ_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, UDEFT,[35] was used for 1D $^{13}C\{^1H\}$ 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 CH_3I (2.7 mL, 43.2 mmol, 10 eq) was added. The reaction mixture was stirred at 40 °C for 24 h under argon atmosphere. After cooling at room temperature, the solvent was evaporated under reduced pressure. The residue was dissolved in a minimum of MeOH, and Et_2O 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). ^{13}C 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 $C_{23}H_{27}N_2O_8$ $[M]^+$: 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 CH_2Cl_2, washed with HCl (1 M) and brine, dried over $MgSO_4$, filtered off, and concentrated *in vacuo*. Precipitation in EtOAc/*n*-pentane afforded compound **RDOX (1)** as a yellow solid in 54% yield (220 mg). ^{1}H NMR (300 MHz, DMSO-*d*$_6$): δ 15.36 (s, 1H, C_{12}-OH), 11.53 (s, 1H, C_{10}-OH), 8.85, 8.74 (2brs, each 1H, NH_2), 7.51 (t, *J* = 8.0 Hz, 1H, H_8), 6.91 (d, *J* = 8.0 Hz, 1H, H_7), 6.86 (d, *J* = 8.0 Hz, 1H, H_9), 6.75 (brs, 1H, C_{12a}-OH), 5.25 (brd, *J* = 5.4 Hz, 1H, C_5-OH), 3.47 (m, 1H, H_5), 2.98 – 2.75 (m, 2H, H_4), 2.60 (p, *J* = 6.7 Hz, 1H, H_6), 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, H_6-Me) ppm. ^{13}C{^{1}H} 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 $C_{20}H_{20}NO_8$ $[M+H]^+$: 402.1183, found 402.1189.

9-Iodo-RDOX (2a). In a 25mL round-bottom flask, **RDOX** (**1**) (110.0 mg, 2.7 ×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 ×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 $MgSO_4$, 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 H_2O + 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. ^{1}H NMR (300 MHz, Acetone-*d*$_6$): δ 15.12 (s, 1H, C_{12}-OH), 12.60 (s, 1H, C_{10}-OH), 9.00 (brs, 1H, NH_2), 7.99 (d, *J* = 8.2 Hz,1H, H_8), 7.63 (brs, 1H, NH_2), 6.83 (d, 1H, *J* = 8.2 Hz, H_7), 5.77 (s, 1H, C_{12a}-OH), 4.33 (dd, *J* = 8.3 Hz, 1H, C_5-OH), 3.81 (q, *J* = 8.3 Hz, 1H, H_5), 3.06 (dd, *J* = 18.6, 5.5 Hz, 1H, H_4), 2.97 (dd, *J* = 18.6, 3.0 Hz, 1H, H_4), 2.80 (m, 1H, H_6), 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, H_6-Me) ppm. ^{13}C{^{1}H} 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. (CH_3) ppm. HRMS (ESI): calculated for $C_{20}H_{19}INO_8$ $[M+H]^+$: 528.0150, found 528.0157.

General Procedure for Suzuki Coupling. In a 25mL 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 $Pd(PPh_3)_4$ (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 Na_2CO_3 (93.5 mg, 8.8 ×10^{-1} mmol, 3.0 eq) in H_2O (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 $MgSO_4$, filtered off, and evaporated under reduced pressure. The crude product was first purified on a silica gel column (eluent CH_2Cl_2 + 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 H_2O + 0.1% formic acid / ACN, gradient 35 to 60% of ACN over 15 min. ^{1}H NMR (400 MHz, Acetone-*d*$_6$): δ 15.22 (brs, 1H, C_{12}-OH), 12.37 (s, 1H, OH), 9.05 (brs, 1H, OH), 8.32 (brs, 1H, NH_2), 7.63 (brs, 1H, NH2), 7.58 (d, *J* = 8.0 Hz, 1H, H_8), 7.25 (t, *J* = 8.0 Hz, 1H, H_e), 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, H_7), 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, C_5-OH), 3.83 (dd, *J* = 9.8, 7.5 Hz, 1H, H_5), 2.90-3.09 (m, 2H, H_4), 2.85 (m, 1H, H_6), 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, H_6-Me) ppm. ^{13}C{^{1}H} 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 $C_{26}H_{23}NO_8$ $[M+H]^+$: 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 H_2O + 0.1% formic acid / ACN, gradient 50 to 70% of ACN over 15 min. ^{1}H NMR (300 MHz, Acetone-*d*$_6$): δ 15.25 (brs, 1H, C_{12}-OH), 12.36 (s, 1H, C_{10}-OH), 9.04 (brs, 1H, NH_2), 7.64 (brs, 1H, NH_2), 7.63 –7.55 (m, 2H, H_8+H_b), 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, H_7), 5.76 (brs, 1H, OH), 4.34 (brs, 1H, C_5-OH), 3.84 (m, 1H, H_5), 3.12 –3.91 (m, 2H, H_4), 2.85 (m, 1H, H_6), 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 H, H_6-Me) ppm. ^{13}C{^{1}H} 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 $C_{26}H_{23}NO_8$ $[M+H]^+$: 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 H_2O + 0.1% formic acid / ACN, gradient 50 to 70% of ACN over 15 min. ^{1}H NMR (300 MHz, *Acetone-d*$_6$): δ 18.43 (s, 1H, C_{12}-OH), 15.27 (brs, 1H, OH), 12.35 (s, 1H, C_{10}-OH), 9.03 (brs, 1H, NH_2), 7.63 (s, 1H, NH_2), 7.53-7.58 (m, 3H, H_8+H_b), 6.95-7.07 (m, 3H, H_7+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, H_5), 3.07 (dd, *J* = 18.7, 5.5 Hz, 1H, H_4), 2.98 (dd, *J* = 18.7, 3.4 Hz, 1H, H_4), 2.83 (m, 1H, H_6), 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. ^{13}C{^{1}H} 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 $C_{27}H_{26}NO_9$ $[M+H]^+$: 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 H_2O + 0.1% formic acid / ACN, gradient 40 to 60% of ACN over 15 min. ^{1}H NMR (300 MHz, Acetone-*d*$_6$): δ 15.25 (brs, 1H, C_{12}-OH), 12.33 (s, 1H, OH),

9.04 (brs, 1H, NH_2), 7.87 (brs, 1H, OH), 7.69 (brs, 1H, NH_2), 7.54 (d, J = 8.0 Hz, 1H, H_8), 7.15-7.29 (m, 2H, H_d and H_f), 7.04 (dd, J = 8.0, 1.2 Hz, 2H, H_7), 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, C_5-OH), 3.83 (q, J = 8.5 Hz, 1H, H_5), 3.07 (dd, J = 18.7, 5.4 Hz, 1H, H_4), 2.97 (dd, J = 18.7, 3.2 Hz, 1H, H_4), 2.83 (dq, J = 12.6, 6.8 Hz, 1H, H_6), 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, H_6-Me) ppm. $^{13}C\{^1H\}$ 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 $C_{26}H_{23}NO_8$ $[M+H]^+$: 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 H_2O + 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, C_{12}-OH), 12.36 (s, 1H, C_{10}-OH), 9.03 (brs, 1H, NH_2), 7.63 (brs, 1H, NH_2), 7.59 (d, J = 8.1 Hz, 1H, H_8), 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, H_7), 7.00 (d, J = 8.3 Hz, 1H, H_e), 5.72 (brs, 1H, C_{12a}-OH), 4.34 (d, 1H, J = 8.6 Hz, C_5-OH), 3.85 (s, 6H, OMe), 3.81 (m, 1H, H_5), 3.07 (dd, J = 18.6, 5.4 Hz, 1H, H_4), 2.97 (dd, J = 18.6, 3.2 Hz, 1H, H_4), 2.81 (m, 1H, H_6), 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. $^{13}C\{^1H\}$ 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, 126.9i5, 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 $C_{28}H_{28}NO_{10}$ $[M+H]^+$: 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 H_2O + 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, C_3-OH), 15.30 (brs, 1H, C_{12}-OH), 12.43 (s, 1H, C_{10}-OH), 9.04 (brs, 1H, NH_2), 8.12 (s, 1H, H_j), 7.95 (d, J = 8.1 Hz, 1H, H_c), 7.95 – 7.91 (m, 2H, He & H_h), 7.80 (dd, J = 8.6, 1.7 Hz, 1H, H_b), 7.75 (d, J = 8.0 Hz, 1H, H_8), 7.66 (brs, 1H, NH_2), 7.49-7.56 (m, 2H, H_f & H_g), 7.12 (d, J = 8.0 Hz, 1H, H_7), 5.78 (s, 1H, C_{12a}-OH), 4.39 (d, J = 8.5 Hz, 1H, C_5-OH), 3.85 (dt, J = 9.9, 8.5 Hz, 1H, H_5), 3.09 (dd, J = 18.8, 5.4 Hz, 1H, H_4), 2.99 (dd, J = 18.8, 3.2 Hz, 1H, H_4), 2.89 (dq, J = 12.6, 6.7 Hz, 1H, H_6), 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, H_6-Me) ppm. $^{13}C\{^1H\}$ 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 $C_{30}H_{26}NO_8$ $[M+H]^+$: 528.1653, found 528.1660

9-(furan-2-yl)-RDOX (9) **(DoxyFur).** From 120 mg of **2a**, 38.2 mg (36%) of the targeted product was isolated after purification. Eluent H_2O + 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, C_{12}-OH), 12.73 (s, 1H, C_{10}-OH), 9.03 (brs, 1H, NH_2), 8.00 (d, J = 8.2 Hz, 1H, H_8), 7.65 (brs, 1H, NH_2), 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, H_7), 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, C_5-OH), 3.81 (dt, J = 9.9, 7.6 Hz, 1H, H_5), 3.03 (dt, J = 18.7, 5.4 Hz, 1H, H_4), 3.03 (dt, J = 18.7, 3.4 Hz, 1H, H_4), 2.81 (dq, J = 12.6, 6.8 Hz, 1H, H_6), 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, H_6-Me) ppm. $^{13}C\{^1H\}$ 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 $C_{24}H_{22}NO_9$ $[M+H]^+$: 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: H_2O + 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, C_{12}-OH), 12.37 (s, 1H, C_{10}-OH), 9.04 (brs, 1H, NH_2), 7.69 (brs, 1H, NH_2), 7.56 (d, J = 8.1 Hz, 1H, H_8), 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, H_7), 6.03 (s, 2H, O-CH_2-O), 5.82 (brs, 1H, C_{12a}-OH), 4.41 (d, J = 8.2 Hz, 1H, C_5-OH), 3.81 (dt, J = 9.9, 8.2 Hz, 1H, H_5), 3.07 (dd, J = 18.6, 5.0 Hz, 1H, H_4), 2.97 (dd, J = 18.6, 3.1 Hz, 1H, H_4), 2.81 (dq, J = 12.6, 6.5 Hz, 1H, H_6), 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. $^{13}C\{^1H\}$ 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 $C_{27}H_{24}NO_{10}$ $[M+H]^+$: 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 H_2O + 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, C_3-OH), 15.23 (brs, 1H, C_{12}-OH), 12.23 (s, 1H, C_{10}-OH), 9.03 (brs, 1H, NH_2), 7.68 (d, J = 8.1 Hz, OH, 1H), 7.64 (brs, 1H, NH_2), 6.92 (d, 1H, J = 8.1 Hz, H_8), 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, C_5-OH), 3.79 (dt, J = 10.0, 8.1 Hz, 1H, H_5), 3.01 (dd, J = 18.7, 5.5 Hz, 1H, OH, H_4), 2.96 (dd, J = 18.7, 3.2 Hz, 1H, OH, H_4), 2.76 (dq, J = 12.6, 6.7 Hz, 1H, H_6), 2.50 (dd, J = 12.5, 7.5 Hz, 1H, H_4), 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, H_6-Me), 1.47 (m, 2H, H_d), 2.20 (m, 2H, H_e), 0.93 (t, 3H, J = 7.1 Hz, H_f) ppm. $^{13}C\{^1H\}$ 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 $C_{26}H_{30}NO_8$ $[M+H]^+$: 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 H_2O + 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, C_{12}-OH), 12.12 (s, 1H, C_{10}-OH), 9.03 (brs, 1H, NH_2), 7.63 (brs, 1H, NH_2), 7.53 (d, J = 8.0 Hz, 1H, H_8), 7.53 (d, J = 8.0 Hz, 1H, H_7), 6.42 (s, 1H, H_a), 5.72 (s, 1H, OH, C_{12a}-OH), 4.32 (d, J = 8.6 Hz, 1H, C_5-OH), 4.18 (d, J = 4.2 Hz, 2H, H_b-CH_2OH), 3.80 (dt, J = 10.0, 8.6

Hz, 1H, H_5), 3.75 (brt, J = 4.2 Hz, 1H, OH), 3.06 (dd, J = 18.6, 5.7 Hz, 1H, H_4), 2.97 (dd, J = 18.6, 3.4 Hz, 1H, H_4), 2.77 (m, 1H, H_6), 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, H_6-Me), 1.41 (sext, J = 7.3 Hz, 2H, H_e), 0.95 (t, 3H, J = 7.3 Hz, H_f) ppm. $^{13}C\{^1H\}$ 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 $C_{27}H_{30}NO_8$ $[M+H\text{-}H_2O]^+$: 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 H_2O + 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, C_{12}-OH), 12.79 (s, 1H, C_{10}-OH), 9.04 (brs, 1H, NH_2), 7.90 (d, 1H, J = 8.2 Hz, H_8), 7.66 (d, J = 3.4 Hz, 1H, H_d), 7.63 (brs, 1H, NH_2), 7.47 (d, J = 5.4 Hz, 1H, H_b), 7.12 (dd, J = 5.4, 3.4 Hz, 1H, H_c), 1H), 7.01 (d, J = 8.2 Hz, 1H, H_7), 5.74 (brs, 1H, C_{12a}-OH), 4.33 (d, 1H, J = 8.6 Hz, C_5-OH), 3.81 (q, 1H, J = 8.6 Hz), 3.07 (dd, J = 18.6, 5.3 Hz, 1H, H_4), 2.97 (dd, J = 18.6, 3.3 Hz, 1H, H_4), 2.78 (m, 1H, H_6), 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. $^{13}C\{^1H\}$ NMR (75 MHz, Acetone-d_6): 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 $C_{24}H_{22}NO_9S$ $[M+H]^+$: 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 H_2O + 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, C_{12}-OH), 12.36 (s, 1H, C_{10}-OH), 9.04 (brs, 1H, NH_2), 7.60 (brs, 1H, NH_2), 7.58 (d, J = 8.0 Hz, 1H, H_8), 7.50 (d, J = 7.7 Hz, 2H, H_i), 7.41 – 7.28 (m, 5H, H_j+H_k+H_b+H_e), 7.18 (d, J = 7.7 Hz, 1H, H_d), 7.03 (d, J = 8.0 Hz, 1H, H_7), 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, C_5-OH), 3.82 (dt, J = 9.9, 8.5 Hz, 1H, H_5), 3.07 (dd, J = 18.6, 5.5 Hz, 1H, H_4), 2.98 (dd, J = 18.6, 3.4 Hz, 1H, H_4), 2.85 (m, 1H, H_6), 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, H_6-Me) ppm. $^{13}C\{^1H\}$ 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 (CH_3) ppm. HRMS (ESI): calculated for $C_{33}H_{30}NO_9$ $[M+H]^+$: 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 H_2O + 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, C_3-OH), 15.24 (brs, 1H, OH), 12.39 (s, 1H, C_{10}-OH), 9.04 (brs, 1H, NH_2), 7.65 (brs, 1H, NH_2), 7.63 (d, J = 8.0 Hz, 1H, H_8), 7.04 (dd, J = 8.0 1.0 Hz, 1H, H_7), 6.91 (s, 2H, H_b), 5.77 (brs, 1H, C_{12a}-OH), 4.38 (d, 1H, J = 8.5 Hz, C_5-OH), 3.86 (s, 6H, H_c-OMe), 3.79-3.85 (m, 1H, H_5), 3.78 (s, 3H, H_d-OMe), 3.07 (dd, J = 18.6, 5.5 Hz, 1H, H_4), 2.98 (dd, J = 18.6, 3.1 Hz, 1H, H_4), 2.84 (dq, J = 12.5, 6.6 Hz, 1H, H_6), 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, H_6-Me) ppm. $^{13}C\{^1H\}$ 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 $C_{29}H_{30}NO_{11}$ $[M+H]^+$: 568.1813, found 568.1823.

General Procedure for Sonogashira Coupling. In a 25mL two-neck round-bottom flask, 9-iodo-RDOX **2a** (160 mg, 3.0 $\times 10^{-1}$ mmol, 1.0 eq), $Pd(PPh_3)_2Cl_2$ (10.7 mg, 1.5 $\times 10^{-2}$ mmol, 0.05 eq) and CuI (2.9 mg, 1.5 $\times 10^{-2}$ mmol, 0.05 eq) were suspended in NEt_3 (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 µL, 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 × 25 mL), washed with HCl (1M) and brine, dried over $MgSO_4$, filtered off, and evaporated under reduced pressure. The crude material was first purified on a silica gel column (eluent CH_2Cl_2 + 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 H_2O + 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, C_3-OH), 17.25 -15.00 (brs, 1H, C_{12}-OH), 9.12 (brs, 1H, NH_2), 7.73 (d, J = 8.2 Hz, 1H, H_8), 7.67 (brs, 1H, NH_2), 7.34 (d, J = 8.2 Hz, 1H, H_7), 6.57 (t, J = 1.0 Hz, 1H, H_a), 5.47 (brs, 1H, C_{12a}-OH), 4.24 (brs, 1H, C_5-OH), 3.83 (t, J = 8.6 Hz, 1H, H_5), 3.03 (dd, J = 18.5, 5.4 Hz, 1H, H_4), 2.98 (dd, J = 18.5, 4.0 Hz, 1H, H_4), 2.94 – 2.87 (m, 1H, H_6), 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, H_6-Me), 1.46 (h, J = 7.5 Hz, 2H, H_e), 0.96 (t, J = 7.3 Hz, 3H, H_f). $^{13}C\{^1H\}$ NMR (100 MHz, Acetone-d_6): δ 195.83 (C_3), 193.73 (C_{12}), 188.85 (C_{13}), 179.66 (C_{11}), 174.96 ($CONH_2$), 162.02 (C_b), 153.27 (C_9), 143.19 (C_{10}), 130.48 (C_{6a}), 126.09 (C_8), 120.36 (C_7), 115.58 (C_{10a}), 106.81 (C_{11a}), 102.23 (C_a), 100.02 (C_{2a}), 76.73 (C_{12a}), 70.34 (C_5), 47.49 (C_{5a}), 44.60 (C_{4a}), 39.82 (C_6), 31.69 (C_4), 30.53 (C_d), 28.62 (C_c), 22.91 (C_6-Me), 17.24 (H_e), 14.05 (H_f). HRMS (ESI): calculated for $C_{26}H_{28}NO_8$ $[M+H]^+$: 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 H_2O + 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, C_{12}-OH), 12.12 (s, 1H, C_{10}-OH), 9.03 (brs, 1H, NH_2), 7.63 (brs, 1H, NH_2), 7.53 (d, 1H, J = 8.1 Hz, H_8), 6.94 (d, J = 8.1 Hz, 1H, H_7), 5.79 (s, 1H, C_{12a}-OH), 4.36 (brd, J = 8.5 Hz, 1H, C_5-OH), 3.81 (q, J = 8.1 Hz, 1H, H_5), 3.07 (dd, J = 18.6, 5.5 Hz, 1H, H_4), 2.98 (dd, J = 18.6, 3.2 Hz, 1H, H_4), 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, H_6-Me), 0.24 (s, 9H, TMS) ppm. $^{13}C\{^1H\}$ 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 $C_{25}H_{27}NO_8Si$ $[M+H]^+$: 498.1579, found 498.1586.

9-(ethynyl)-RDOX (18). In a 10 mL round-bottom flask, compound **17** (40 mg, 8.0 ×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 µL) 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 × 25 mL), washed with HCl (1M) and brine, dried over $MgSO_4$, filtered off, and evaporated under reduced pressure. Compound **18** was isolated without further purification (25 mg, 74%). ^{1}H NMR (400 MHz, Acetone-d_6): δ 18.43 (s, 1H, C_{12}-OH), 15.17 (brs, 1H, C_3-OH), 12.26 (s, 1H, C_{10}-OH), 9.02 (brs, 1H, NH_2), 7.66 (brs, 1H, NH_2), 7.64 (d, *J* = 8.1 Hz, 1H, H_8), 6.97 (d, *J* = 8.1 Hz, 1H, H_7), 5.78 (s, 1H, C_{12a}-OH), 4.37 (brd, *J* = 6.8 Hz, 1H, C_5-OH), 3.81 (m, 1H, H_5), 3.79 (s, 1H, H_7), 3.07 (dd, *J* = 18.7, 5.4 Hz, 1H, H_4), 2.96 (dd, *J* = 18.7, 3.5 Hz, 1H, H_4), 2.81 (dt, *J* = 12.5, 6.7 Hz, 1H, H_6), 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, H_6-Me) ppm. ^{13}C{^{1}H} 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 $C_{22}H_{20}NO_8$ $[M+H]^+$: 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.[36] 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, pH 7.4. Prior to the aggregation assay, the protein stock solutions were centrifuged for 30 min at 12,000× g to remove microaggregates. Protein concentration was determined by measuring the absorbance at 280 nm using the extinction coefficient ε275 = 5600 cm^{-1} M^{-1}.

Protein Aggregation. Assays The aggregation protocol was adapted from previous studies [10]. The different aggregated species were formed by incubating recombinant α-Syn samples (70 µM) 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 all tetracyclines derivatives at 20 µM.

Thioflavin T (ThT) Fluorescence Assay. Aggregation studies with α-Syn in the absence or presence of DMC or DDMC were performed by measuring the fluorescence emission of ThT at different time points according to LeVine.[37] 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.[17]

Primary Microglial Cell Isolation. Microglial cell isolation was performed as previously described.[38] 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 l0.000 µg/mL streptomycin (P-E). Soon after cells were seeded at a density of two brains per 10 mL of media in a T75 flask and incubated at 37 °C with 5% CO_2. 14 days of incubation, cells were harvested by trypsinization and resuspended in DMEM containing 0,1% FBS and P-E.

Cell Cytotoxicity. To evaluate the safety of the molecules in terms of toxicity, we measured the lactate dehydrogenase (LDH) activity released in the extracellular medium. To assess this evaluation, 4×10^5 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 and no pathological 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.

Detection of TNF-α in Cell Supernatant by -ELISA Assay. 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, 3×10^5 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).[39] Briefly, the Minimal Inhibitory Concentrations (MICs) were determined with an inoculum of 10^5 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.

The Supporting information is available free of charge at :
Copies of ^{1}H and ^{13}C NMR spectra, and HPLC chromatograms; MTT assays (pdf)

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Funding Sources

We thank the Association France Parkinson (GAO 2018, DOXY- PARK) and Carnot institute (CM 098 and 120) for financial support.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

We thank Benjamin Ayela for exploratory research and Blandine Séon-Méniel for technical help and her participation in organizing the tetracycline chemical library. We thank Karine Leblanc and Somia Rhabbati for support with the setup of HPLC conditions for chromatographic separation and Remy Franco for NMR services.

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, thioflavine T; TLC, thin layer chromatography; TMS, trimethylsilyl; TOF, time of flight; UDEFT, uniform driven equilibrium Fourier transform;

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