



HAL
open science

C9-Functionalized Doxycycline Analogs as Drug Candidates to Prevent Pathological α -Synuclein Aggregation and Neuroinflammation in Parkinson's Disease Degeneration

Clémence Rose, Rodrigo Hernán Tomas-grau, Brenda Zabala, Patrick Pierre Michel, Jean-michel Brunel, Rosana Chehín, Rita Raisman-vozari, Laurent Ferrié, Bruno Figadère

► **To cite this version:**

Clémence Rose, Rodrigo Hernán Tomas-grau, Brenda Zabala, Patrick Pierre Michel, Jean-michel Brunel, et al.. C9-Functionalized Doxycycline Analogs as Drug Candidates to Prevent Pathological α -Synuclein Aggregation and Neuroinflammation in Parkinson's Disease Degeneration. *ChemMedChem*, 2024, 19, 10.1002/cmdc.202300597 . hal-04736533

HAL Id: hal-04736533

<https://hal.science/hal-04736533v1>

Submitted on 15 Oct 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Copyright

Accepted Article

Title: C9-Functionalized Doxycycline Analogs as Drug Candidates to Prevent Pathological α -Synuclein Aggregation and Neuroinflammation in Parkinson's Disease Degeneration

Authors: Laurent Ferrié, Bruno Figadère, Clémence Rose, Rodrigo Hernán Tomas-Grau, Rosana Chehín, Brenda Zabala, Patrick Pierre Michel, Rita Raisman-Vozari, and Jean-Michel Brunel

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

To be cited as: *ChemMedChem* **2024**, e202300597

Link to VoR: <https://doi.org/10.1002/cmdc.202300597>

RESEARCH ARTICLE

C9-Functionalized Doxycycline Analogs as Drug Candidates to Prevent Pathological α -Synuclein Aggregation and Neuroinflammation in Parkinson's Disease Degeneration

Clémence Rose,^{#[a]} Rodrigo Hernán Tomas-Grau,^{#[b]} Brenda Zabala,^[c] Patrick Pierre Michel,^[c] Jean-Michel Brunel,^[d] Rosana Chehín,^[b] Rita Raisman-Vozari,^{*[c]} Laurent Ferrié,^{*[a]} Bruno Figadère,^{*[a]}

[a] Dr. C. Rose, Dr. L. Ferrié, Dr. B. Figadère

BioCIS,

CNRS, Université Paris-Saclay

91400 Orsay, France

E-mail: laurent.ferrie@universite-paris-saclay.fr; bruno.figadere@universite-paris-saclay.fr

[b] Dr. R. H. Tomas-Grau, Prof. R. Chehín

IMMCA

CONICET-UNT-SIPROSA

Tucumán 4000, Argentina.

[c] B. Zabala, P. P. Michel, R. Raisman-Vozari

Paris Brain Institute-ICM

Inserm, Sorbonne Université, CNRS, Hôpital Pitié Salpêtrière

75013 Paris, France

E-mail: ritaraisman@gmail.com

[d] Dr. J. M. Brunel

UMR_MD1 Membranes et Cibles Thérapeutiques, U1261 INSERM,

Aix-Marseille Université,

13385 Marseille, France

Equal contribution

Supporting information for this article is given via a link at the end of the document.

Abstract: Doxycycline, a semi-synthetic tetracycline, is a widely used antibiotic for treating mild-to-moderate infections, including skin problems. However, its anti-inflammatory and antioxidant properties, combined with its ability to interfere with α -synuclein aggregation, make it an attractive candidate for repositioning in Parkinson's disease. Nevertheless, the antibiotic activity of doxycycline restricts its potential use for long-term treatment of Parkinsonian patients. In the search for non-antibiotic tetracyclines that could operate against Parkinson's disease pathomechanisms, eighteen novel doxycycline derivatives were designed. Specifically, the dimethyl-amino group at C₄ was reduced, resulting in limited antimicrobial activity, and several coupling reactions were performed at position C₉ of the aromatic D ring, this position being one of the most reactive for introducing substituents. Using the Thioflavin-T assay, we found seven compounds were more effective than doxycycline in inhibiting α -synuclein aggregation. Furthermore, two of these derivatives exhibited better anti-inflammatory effects than doxycycline in a culture system of microglial cells used to model Parkinson's disease neuroinflammatory processes. Overall, through structure-activity relationship studies, we identified two newly designed tetracyclines as promising drug candidates for Parkinson's disease treatment.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder mainly characterized by the loss of *substantia nigra* dopaminergic neurons, resulting in typical motor symptoms.^[1,2] The incidence 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 possible therapeutic target for neuroprotective drug development.^[4,5] Neuroinflammatory processes also appear to contribute crucially to PD neurodegeneration, and their activation may depend for some part on α -Syn aggregation.^[6]

Tetracyclines have a long history of clinical use owing to their antibiotic, antifungal, and antineoplastic properties. Additionally, they have also been shown to operate as potential matrix metalloproteinase inhibitors and exhibit antioxidant and anti-inflammatory properties.^[7,8] Furthermore, a retrospective study demonstrated a positive association between the use of tetracycline and a decreased risk of developing PD. For instance, doxycycline (DOX) has demonstrated excellent neuroprotective effects in different experimental models without significant toxicity signs.^[9–11] In particular, DOX was able to prevent or modulate the aggregation process of α -Syn,^[12,13] reduce oxidative stress, repress neuroinflammatory processes,^[13,14] in either *ex vitro* and

RESEARCH ARTICLE

in vitro assays as well as *in vivo* models that mimic PD neurodegenerative events.^[12,15–17] These pieces of evidence, along with DOX's ability to cross the blood-brain barrier^[18] and its safe toxicological profile make this tetracycline a good candidate for neuroprotection in PD but also for Alzheimer's disease,^[19,20] Huntington's Disease^[11] and other neurodegenerative diseases.^[10,17,21] However, using DOX for long-term treatment in PD patients may lead to potential antibiotic resistance and microbiota disruption.^[22] Recently, we reported that a non-antibiotic tetracycline derivative of demeclocycline (DMC) called DDMC can interfere with α -Syn aggregation.^[16] Moreover, DDMC and a non-antibiotic derivative of DOX, DDOX, have also shown neuroprotective properties for vulnerable dopaminergic neurons through their ability to prevent oxidative stress-mediated insults (Figure 1).^[23]

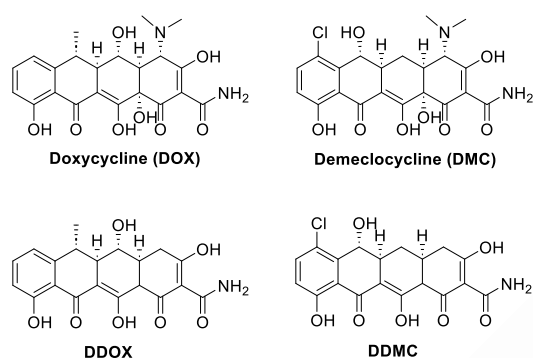


Figure 1. Structures of the reduced tetracyclines DDMC and DDOX and their respective parent compounds DMC and DOX.

Due to the aromatic ring's well-established significance as an excellent site for chemical modifications in structure-activity relationship (SAR) studies of the tetracycline family,^[24] we sought to explore the impact of substituting the C₉ position of the D ring of doxycycline (DOX) on α -Syn aggregation. Moreover, the loss of antibiotic activity was targeted to potentiate the neuroprotective action of our novel compounds.

In the present study, eighteen novel non-antibiotic DOX derivatives were synthesized, and our goal would be identifying promising compounds, among those not showing non-antimicrobial properties, that exhibit enhanced anti-aggregative properties against α -Syn and diminished neuroinflammation compared to parent DOX compound. This study would culminate in SAR data on the neuroprotective potential of C₉-substituted DOX derivatives, enabling the selection of compounds worthy of a further *in-vivo* investigation. Ultimately, our overarching goal is to contribute to the advancement of strategies aimed at halting or slowing down the progression of Parkinson's disease.

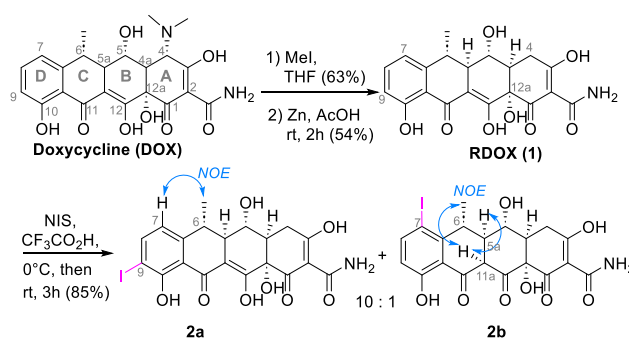
Results and Discussion

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 of tetracyclines.^[25,26] We confirmed ourselves that the loss of this functional group reduces the antibiotic activity of newly designed tetracyclines.^[16,23] Additionally, our previous research on the subject pointed to the importance of maintaining a specific structural motif in tetracyclines, which is crucial to interact with cross- β structures characteristic of amyloid aggregates of α -Syn.¹⁹ The substitution at the C₉ position was already studied in the tetracycline class to modulate their antibiotic activity;^[28–31] however, nothing was reported about 4-*des-N*-dimethylamino tetracyclines with substituents at the C₉ position. Indeed, to prepare these new analogs, the aromatic D ring appears to be well suited to achieve aromatic electrophilic substitutions at either positions C₉ or C₇. Specifically, halogenation of these positions would lead to further cross-coupling reactions.

Thus, we first performed the dimethylamino group reduction using previously reported methods.^[25,28,32,33] The compound 4-*des-N*-dimethylaminodoxycycline RDOX (**1**) 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.^[23] In a second step, we focused our attention on the functionalization of the aromatic ring. After several attempts, we found that NIS in CF₃CO₂H and at 0 °C were the best conditions to selectively iodinate RDOX^[34] on position C₉ (**2a**) over position C₇ (**2b**), with about a 10:1 ratio. Preparative HPLC then separated the two regio-isomers **2a** and **2b**. Structural assignment of **2a** was unambiguous with the help of NOESY 2D NMR, showing a cross-peak between H₇ and C₆-Me, which does not exist in **2b**. Surprisingly, isomer **2b** showed it exists predominantly as a tautomer being in 11,12-diketo form rather than the usual keto-enol disposition; H_{11a} is oriented below, supported by NOE correlation with C₆-Me and H_{5a}. Attempts towards bromination of RDOX were unsuccessful in our hands, leading to unselective reactions and/or an inseparable mixture of products. (Scheme 1).



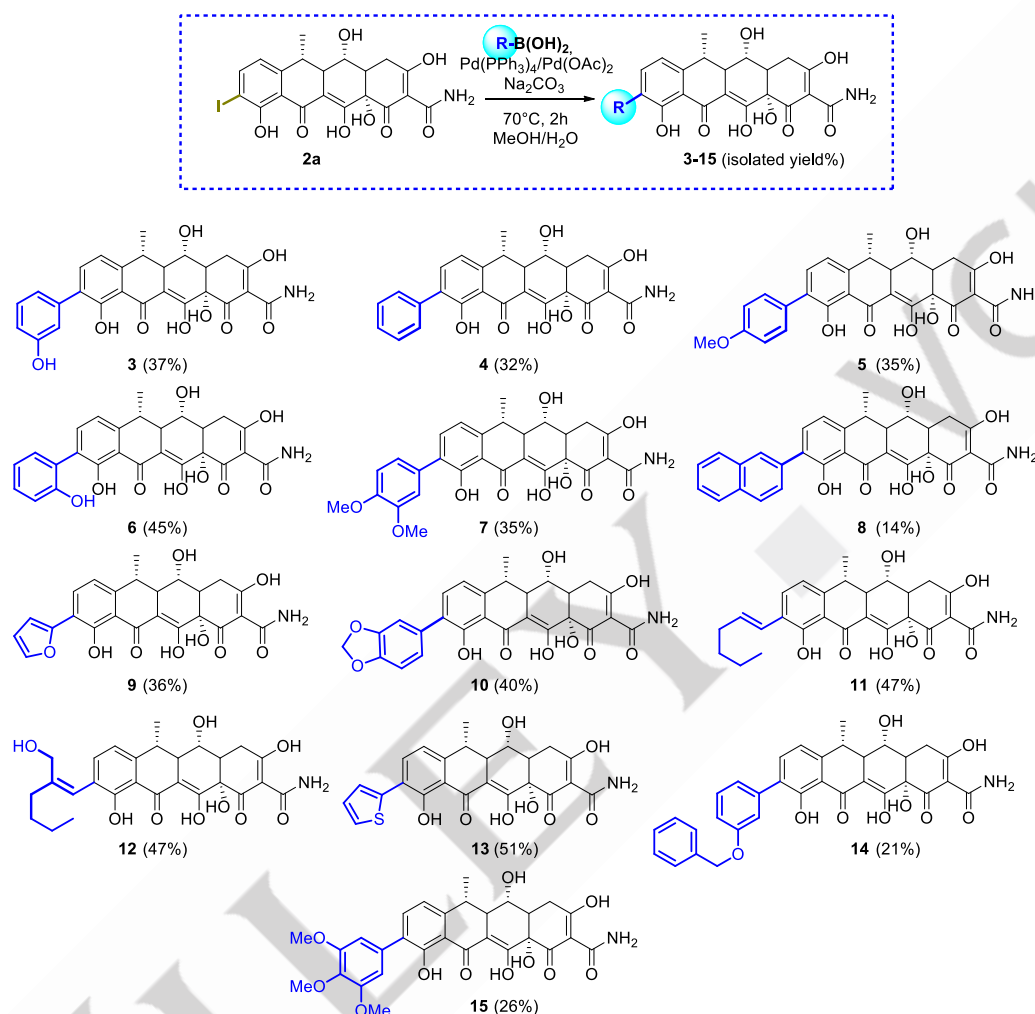
Scheme 1. Synthesis of 4-*des-N*-dimethyl-aminodoxycycline RDOX (**1**) and 9-iodo derivative **2a**. Key NOE-2D NMR correlations (in blue) are shown for structural assignment of **2a** and **2b**.

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

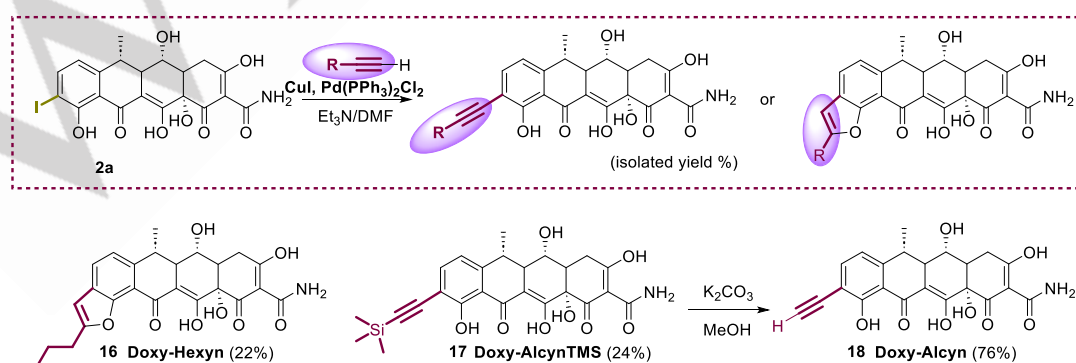
RESEARCH ARTICLE

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^[30,35] 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 satisfied to achieve the insertion of alkene derivatives (**11-12**). (Scheme 2)



Scheme 2. Preparation of 9-substituted 4-des-N-dimethylaminodoxycycline derivatives by Suzuki cross-coupling from **2a**.



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

RESEARCH ARTICLE

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 on the steric and electronic properties of the alkyne. Hexyne afforded only the product of a subsequent cycloisomerization with hydroxyl at C₁₀ (**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).

Antibacterial activity

One of the study's main objectives was to suppress the antibiotic activity of tetracyclines as a requirement of chronic administration in neurodegenerative diseases, to avoid the development of antibiotic resistance and perturbations in gut microbiota.^[24,26] Thus, all synthesized compounds were evaluated for their antibacterial activity against several Gram-negative (*i.e.*, *Pseudomonas aeruginosa* PA01 and *E. coli* ATCC25922) and a Gram-positive strains (*Staphylococcus aureus* ATCC25923), and their minimal inhibitory concentrations (MIC) were determined. Interestingly, newly synthesized tetracyclines demonstrated no antibacterial activity below 200 μM against Gram-negative *P. aeruginosa* and *E. coli*, except compound 10, which was effective against *E. coli* with an efficacy comparable to that of DOX. The antibiotic activity of our novel tetracycline 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. Altogether, these results further emphasize that the antimicrobial activity of tetracyclines depends on the presence of a dimethylamino group at C₄. Meanwhile, the nature of the substituent at C₉ does not seem to modulate the antibacterial activity significantly. Indeed, 9-*t*Bu-DOX, which possesses both a *tert*-butyl group at C₉ and a dimethylamino group at C₄, demonstrated higher MIC values than DOX (>200, 50, 6.25 μM against *P. aeruginosa*, *E. coli*, and *S. aureus*, respectively),^[36] meaning that the substitution at C₉ may also decrease the antibacterial activity *per se* (Table 1).

Table 1. Minimal inhibitory concentrations (MIC) (μM) of newly synthesized tetracyclines **1-18** against Gram⁺ and Gram⁻ bacteria

Compounds	<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
9- <i>t</i> Bu-DOX	>200	50	6.25

Inhibition of α -synuclein aggregation.

It has been previously reported that some tetracyclines, specifically DOX, can inhibit α -Syn aggregation.^[9,12,27] To analyze the capacity of all the novel synthesized tetracyclines 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 at 600 rpm for 120 hours. The cross- β structure, which is the hallmark of amyloid aggregation,^[37] was monitored by Thioflavin T (ThT) fluorescence emission at 482 nm (λ_{exc} 450 nm).^[38]

The results indicate that seven of the eighteen new tetracyclines tested decreased ThT fluorescence intensity compared to the control condition. This anti-aggregant potential varied among these seven molecules. Compound **17** was the most effective, as it reduced the ThT signal by approximately 95.6% compared to the control condition. Compounds **16**, **14**, **6**, **12**, RDOX, and **4**, were also quite efficacious as they reduced the ThT fluorescent signal by 93.7%, 90.7%, 89.3%, 81.3%, 70.25%, and 62%, respectively (Figure 2).

The SAR study among our series of tetracycline compounds reveals some interesting information. Firstly, it can be observed that the loss of the dimethylamino group at C₄ does not influence α -Syn aggregation by comparing DOX and RDOX, which both exhibit about 70% efficacy in the ThT assay. Secondly, C₉ substitution predominantly provides no improvement compared to the parent compound DOX (-70.3%); this is for instance the case for **4** (-62%), **10** (-59.4%), **13** (-15.4%), **15** (-7.2%), **9** (-4.7%), **2a** (+2.6%), **7** (+1.6%), **5** (+32.7%), **8** (+24.4%). However, a couple of compounds demonstrate better inhibitory activity than DOX. In the case of compound **14**, the presence of free hydroxy substituents on the aromatic D ring seems to favor the anti-aggregant potential with a decrease of 89.3% of the ThT fluorescent signal. This result is not unexpected, as the ortho position provides hydrogen bonding with phenol at the C₁₀ position. A similar observation could be deduced for compound **12**, albeit with slightly lower inhibitory activity (-81.5%), resulting presumably from a longer distance between phenol at C₁₀ and the hydroxy group on the side chain. In contrast, the *meta*-phenol in

RESEARCH ARTICLE

compound **3** appears to exert a pro-aggregative effect with an augmentation of 131% of the ThT signal. This phenomenon could be attributed to the *meta* position, which does not enable H-bonding with the C₁₀ position.

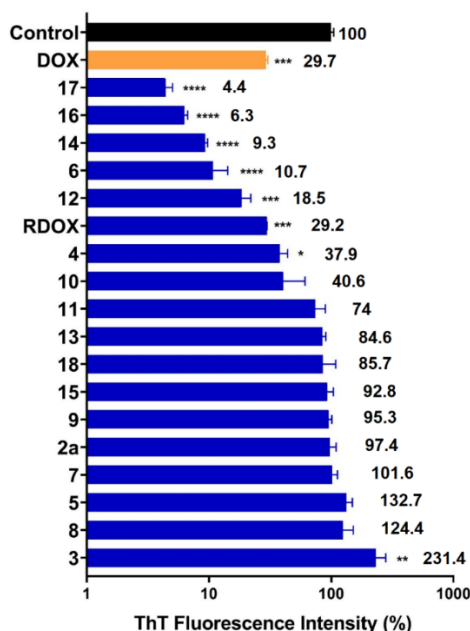


Figure 2. Effect of the new tetracyclines derivatives RDOX, 2a and 3-18 on α -Syn aggregation. The fluorescence emission intensity of 25 μ M Thioflavin T (ThT) was measured in a solution containing 70 μ M α -Syn alone (Control) and in the presence of 20 μ M of DOX or each tetracycline compound (RDOX, 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 condition. Subsequently, the percentages obtained for each independent set of samples were averaged. The data are presented as the mean \pm SEM from three independent experiments ($n = 3$). Numbers above bars represent the % of the ThT signal after treatment. Statistical analysis was performed using One-way ANOVA followed by Dunnett's test. The figure indicates significant differences: **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ vs. Control.

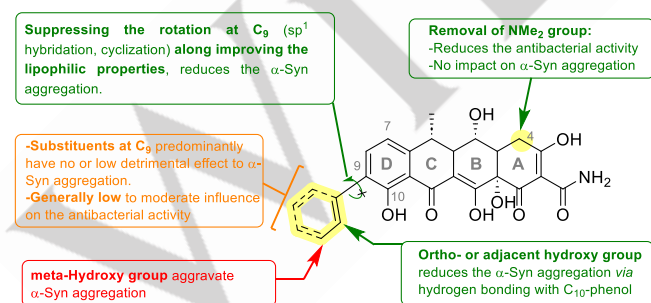


Figure 3. Summary of the SAR study concerning chemical modifications at C₄ and C₉ positions of doxycycline toward the antimicrobial and anti-aggregant properties of newly designed derivatives.

Another critical factor appears to be the conformational rotation at C₉, which interferes with the aggregation process of α -Syn. Cyclic structures, such as in compound **16** and sp¹ hybridization, such as in compound **17**, demonstrated the best inhibitory effects against α -Syn, with reductions of –93.7% and –

95.6% compared to the control, respectively. Interestingly, removing the lipophilic trimethylsilyl (TMS) group in compound **17** to give compound **18** suppressed the inhibitory activity against α -Syn aggregation. Thus, the reduction of conformational rotation must be associated with a pronounced lipophilic character, such as that of the *n*-butyl group in compound **16** or the TMS group in compound **17**. Indeed, compound **11**, although bearing a fatty chain, has reduced anti-aggregation properties compared to **16**. Compound **14**, with two consecutive aromatic rings, also strongly reduces α -Syn aggregation (–89.7%). Despite the free conformational rotation at C₉, it can be hypothesized that π -stacking might lead to anti-aggregative properties, such as in the polyphenol compound resveratrol.^[39]

Analysis of cytotoxic effects

The appropriate safety profile of DOX has allowed its administration for decades.^[40] However, new chemical modifications could potentially impact the cytotoxicity of newly designed compounds. Consequently, we performed a lactate dehydrogenase (LDH) assay, commonly used to measure cytotoxicity as it reflects cell plasma membrane disruption. We used cultivated microglia as a model system for this purpose. Untreated microglial cells were used to determine LDH values under control conditions (100%), whereas optimal membrane leakage was obtained by lysing the cells with 1% Triton X-100. Under these conditions, LDH release was increased by 150% compared to the control (Figure 4). In addition, microglial cells were also treated with 20 μ M of the new tetracycline derivatives, demonstrating an effect against 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) assay^[41] after exposure to the tetracyclines derivatives RDOX, **4**, **6**, **12**, **14**, **16**, **17**. Results showed that two tetracycline derivatives, **6**, **12** and RDOX, did not affect cell viability up to 20 μ M, reinforcing the idea that these two compounds are not toxic (Figure S-1, see supporting information).

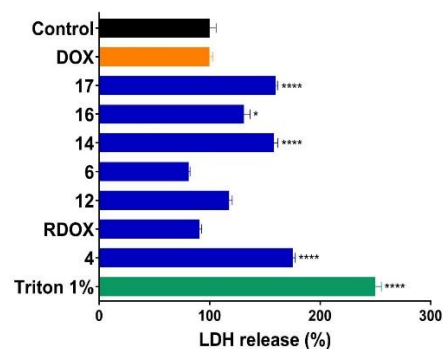


Figure 4. Cytotoxicity of anti-aggregative tetracyclines of α -Syn. LDH release in microglial cell cultures in the absence (Control) or after adding 20 μ M of each tetracycline compound (DOX, **17**, **16**, **14**, **6**, **12**, RDOX or **4**). Bars represent the mean values expressed in percentage, 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 test. Significant differences are indicated in the figure: * $p < 0.05$, **** $p < 0.0001$ vs. control.

RESEARCH ARTICLE

Anti-inflammatory effect

Tumor necrosis factor alpha (TNF- α), a cytokine that plays a crucial role in response to various inflammatory insults, may also take part in a detrimental role in PD neurodegeneration.^[42] This study evaluated the potential of the non-cytotoxic compounds **6** and RDOX in preventing TNF- α release in primary microglial cells activated by lipopolysaccharide (LPS) using the parent compound DOX as a reference drug. As previously reported,^[13] DOX becomes effective at 50 μ M in the present experimental setting. In contrast, when cells were pre-treated with either **6** or RDOX at 20 μ M, LPS-induced TNF- α release was significantly reduced by 77 and 47 %, respectively, compared to cultures without tetracycline treatment (Figure 5). These results show that **6** or RDOX are intrinsically more effective than DOX in inhibiting TNF- α release induced by LPS stimulation in primary microglia cells.

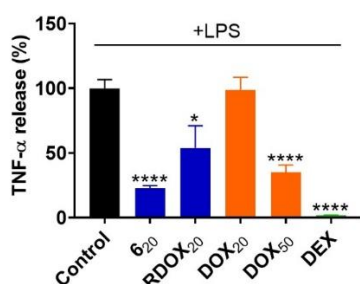


Figure 5. Anti-inflammatory effect of anti-aggregant and non-toxic tetracyclines compounds. TNF- α release in primary microglial cell cultures pre-treated for 4 h with DOX, **6**, or RDOX at 20 μ M, DOX at 50 μ M or dexamethasone (DEX) at 2.5 μ M, and then stimulated with 10 ng/ml of LPS for 24 h. The bars represent the mean \pm S.E.M (n = 3). Statistical analysis was performed using One-way ANOVA followed by Dunnett's test. Significant differences are indicated in the figure: * p < 0.05, **** p < 0.0001 compared to the LPS.

Conclusions

Previous studies have demonstrated that DOX has beneficial effects in several *ex vitro*, *in vitro* and *in vivo* models of PD by targeting key pathomechanisms involved in the degenerative process of this disorder, such as α -Syn aggregation and neuroinflammation.^[12,13,40,43,44] However, its potential use as a PD drug is limited by its antibiotic properties, which could lead to antimicrobial resistance and perturbations of gut microbiota.

Therefore, we have designed eighteen new tetracyclines intending to overcome this issue and improve the anti-aggregative properties of DOX. Reduction of the dimethylamino group at position C4 and substitution of the accessible position C9 of the D ring, which was never studied for this aim, was carried out. Sonogashira cross-coupling reactions of 9-iodo-derivatives **2a** with different coupling partners were applied, yielding the new C₉-substituted RDOX derivatives. Reducing the dimethylamino group led to a significant decrease in the antibiotic activity for most compounds, making these novel tetracyclines of potential interest. Eight compounds [**17**, **16**, **14**, **6**, **12**, **10**, **4**, and RDOX] were equally or more active than their parent DOX in an *ex vitro* ThT fluorescence assay that allows monitoring α -Syn aggregation. From this screening step, the SAR analysis showed that the NMe₂ group at the C₄ position does not contribute to this inhibitory activity. Noticeably, an adjacent hydroxy group at position C₉ positively enhanced the inhibitory capacity against α -Syn

aggregation (compounds **6** and **12**) by adding a hydrogen bonding to the existing hydroxy-carbonyl sequence of tetracyclines, which plays a crucial role in the inhibition of protein amyloid aggregation.^[27,45] The suppression of the degree of liberty adjacent to the aromatic ring of the tetracycline and the improvement of the lipophilic properties are also required (compounds **16** and **17**) to observe a significant decrease in α -Syn aggregation. Improving π -stacking effects with substituents at C₉, such as in compound **14**, also reduces α -Syn aggregation, although this route must be further explored. The best compounds for future *in-vivo* studies were determined by LDH assay to ensure their absence of cytotoxicity.

Consequently, only compounds **6** and RDOX showed no cytotoxicity at 20 μ M. Most interestingly, compounds **6** and RDOX showed greater anti-aggregating and anti-inflammatory properties than DOX (**1**), as they were effective at lower concentrations. Thus, this study concludes with the potential of C₉ modification to enhance the anti-aggregation properties of tetracyclines against α -Syn. These findings highlight the promising drug candidacy of **6** and RDOX. Protein and cell seeding assays,^[16] as well as midbrain cell culture in a spontaneous death model^[23] should give us more insights into the neuroprotective potential of these compounds before launching further *in vivo* studies in PD models. These results will be reported in due course.

Experimental Section

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 monohydrate was purchased from Alfa Aesar (25 g, about €207), 9-*t*-Bu-DOX from Echelon Biosciences, Salt Lake City, USA (100 mg, about \$161). 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 μ m, [\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.^[46,47] 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: δ _C = 77.16 ppm unless notified, residual CHCl₃ in CDCl₃: δ _H = 7.26 ppm; C₆D₆: δ _C = 128.06 ppm unless notified, residual C₆HD₅ in C₆D₆: 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, called UDEFT,^[48] 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. The purity of each purified compound was evaluated by HPLC, with UV detection at 254 nm. Chromatograms of each purified compound are provided in supporting information, and the purity is greater than 90%, up to 100%, except for compounds **8** and **9**, with 88 and 86% purity, respectively.

RESEARCH ARTICLE

RDOX (1). In a 100 mL 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 50 mL 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 25 mL round-bottom flask, RDOX (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 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 10:1 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. NOE correlation between C₆-Me and one CH aromatic could confirm the 9-iodo regioisomer. 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 C₂₀H₁₉NO₈ [M+H]⁺: 528.0150, found 528.0157.

7-Iodo-RDOX (2b). A sample of **2b** could be isolated. The leak of NOE correlation between C₆-Me and one CH aromatic could confirm the 7-iodo regioisomer. It predominantly exists as the tautomer having CH in position C_{11a}. ¹H NMR (300 MHz, Acetone-*d*₆) δ 11.63 (s, 1H), 8.79 (brs, 1H), 7.93 (d, *J* = 8.6 Hz, 1H), 7.68 (brs, 1H), 6.65 (d, *J* = 8.8 Hz, 1H), 5.55 (s, 1H), 4.97 (d, *J* = 7.8 Hz, 1H), 4.31 (d, *J* = 6.9 Hz, 1H), 3.94 (qd, *J* = 7.1, 2.1 Hz, 1H), 3.59 (q, *J* = 10.9, 7.2 Hz, 1H), 2.99 (dd, *J* = 18.3, 5.1 Hz, 1H), 2.90 (dd, *J* = 18.3, 2.2 Hz, 1H), 2.65 (td, *J* = 10.9, 7.2, 2.1 Hz, 1H), 2.34 (q, *J* = 10.8, 5.0, 2.2 Hz, 1H), 1.29 (d, *J* = 7.4 Hz, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 199.26, 197.07, 196.66, 194.64, 174.64, 163.49, 150.15, 148.10, 119.13, 117.90, 100.17, 87.57, 81.68, 67.57, 57.08, 48.88, 47.36, 39.04, 30.74, 19.61. HRMS (ESI): calculated for C₂₀H₁₉IINO₈ [M+H]⁺: 528.0150, found 528.0159; calculated for C₂₀H₁₈IINNaO₈ [M+Na]⁺: 549.9969, found 549.9976.

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, H₉), 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+H₉), 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,

RESEARCH ARTICLE

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 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 $C_{27}H_{26}NO_9$ [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 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_a and H_i), 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_a), 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 $C_{26}H_{23}NO_8$ [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 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_i), 7.02 (dd, *J* = 8.1, 0.9 Hz, 1H, H₇), 7.00 (d, *J* = 8.3 Hz, 1H, H_a), 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 $C_{28}H_{28}NO_{10}$ [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 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_i), 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_r & 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 $C_{30}H_{26}NO_8$ [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 $C_{24}H_{22}NO_9$ [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: 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_i), 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 $C_{27}H_{24}NO_{10}$ [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 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

RESEARCH ARTICLE

Hz, H₆-Me), 1.47 (m, 2H, H₆), 2.20 (m, 2H, H₆), 0.93 (t, 3H, *J* = 7.1 Hz, H₁) 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, H₁) 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), 1H), 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): 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_l+H_m), 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 584.1922.

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_d-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 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 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_e), 0.96 (t, *J* = 7.3 Hz, 3H, H₁) ppm. 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 (H₁). 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),

RESEARCH ARTICLE

0.24 (s, 9H, TMS) ppm. $^{13}\text{C}\{^1\text{H}\}$ 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 $\text{C}_{25}\text{H}_{27}\text{NO}_8\text{Si}$ $[\text{M}+\text{H}]^+$: 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 \times 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%). ^1H NMR (400 MHz, Acetone- d_6): δ 18.43 (s, 1H, $\text{C}_{12}\text{-OH}$), 15.17 (brs, 1H, $\text{C}_3\text{-OH}$), 12.26 (s, 1H, $\text{C}_{10}\text{-OH}$), 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, $\text{C}_{12a}\text{-OH}$), 4.37 (brd, $J = 6.8$ Hz, 1H, $\text{C}_5\text{-OH}$), 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, $\text{H}_6\text{-Me}$) ppm. $^{13}\text{C}\{^1\text{H}\}$ 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 $\text{C}_{22}\text{H}_{20}\text{NO}_8$ $[\text{M}+\text{H}]^+$: 426.1183, found 426.1193.

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.^[49] 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 12,000x g to remove microaggregates. Protein concentration was determined by measuring the absorbance at 280 nm using the extinction coefficient $\epsilon_{275} = 5600 \text{ cm}^{-1} \text{ M}^{-1}$.

In response to the potential neurotoxicity risks associated with α -Syn and their aggregates, we implemented stringent safety protocols to ensure the safety of our team and minimize biological hazards. These measures include strict adherence to standard operating procedures, the use of comprehensive personal protective equipment, dedicated containment spaces, expert training, advanced containment equipment like Biological Safety Cabinets, specialized tools for aerosol control, thorough decontamination procedures, access control, and meticulous documentation.^[50–52] These precautions collectively prioritize the well-being of our team and safeguard the integrity of our research, demonstrating our unwavering commitment to responsible scientific exploration.

Protein Aggregation Assay The aggregation protocol was adapted from previous studies.^[12] 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 for 120 h in the absence or presence of each tetracycline derivatives at 20 μM . Operationally, powdered tetracyclines were first dissolved in DMSO to create a 50 mM stock solution (100% solubility in all cases). Subsequently, each

sample was diluted in PBS 1X to obtain an aqueous working solution at 500 μM , which was prepared just before use. Strict quality control measures were implemented to guarantee consistent and replicable results.^[52]

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 according to LeVine.^[53] Changes in the emission fluorescence spectra were monitored at an excitation wavelength of 450 nm using a Fluoromax-4 spectrofluorometer. Each molecule employed in this study underwent biophysical characterization using conventional methods of absorbance and fluorescence. Those experiments were performed to guarantee that the spectral changes observed in ThT resulted exclusively from conformational alterations in the protein and to confirm that these molecules did not display fluorescence that could potentially overlap with the ThT probe. Additionally, all compounds were individually tested to confirm their ThT-negative status.

Orthogonal Assays

To assess the inhibition of α -Syn amyloid aggregation by lead compounds RDOX and **6**, two orthogonal experiments were conducted.

Congo Red Absorbance Spectroscopy: a 10 mM Congo red working solution was prepared by diluting a stock solution in 10 mM PBS, pH 7.4. Samples were obtained following the incubation of 70 μM α -Syn with RDOX or **6**. Absorbance measurements, following Tomas-Grau et al.'s protocol,^[16] involved mixing each sample with PBS and CR solution to achieve a final dye concentration of 20 μM . Prepared samples were vortexed at 23 °C and 300 rpm for 30 min before being read in a TECAN Infinite M200 microplate reader, recording absorption from 400–700 nm.

Bis-ANS Fluorescent Assay: Samples, acquired after incubating 70 μM α -Syn with either RDOX or **6**, were combined with bis (1-Anilino-naphthalene-8-Sulfonic Acid), known as Bis-ANS, to achieve a final concentration of 5 μM . Immediately after, the samples were gently mixed (avoiding vigorous mixing), and each one was excited at 395 nm. Fluorescence emission was recorded from 410 to 610 nm using a Fluoromax-4 spectrofluorometer.^[19] Additional discussion, and UV-absorbance and fluorescence spectra are available in supporting information.

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.^[23]

Primary Microglial Cell Isolation. Microglial cell isolation was performed as previously described^[54] using the whole brains of C57BL/6J mouse pups (Janvier LABS, Le Genest St Isles, France). A suspension of cells obtained by mechanically trituration of the brain tissue was plated in polyethyleneimine pre-coated T75 flasks containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) and a cocktail of penicillin, and streptomycin. The isolation occurred spontaneously over a period of 14–16 days. Isolated cells were then harvested by trypsinization to produce subcultures in 96-well multiwell plates.

RESEARCH ARTICLE

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. For that, 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 μ M. 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 . To evaluate the anti-inflammatory properties of test molecules on LPS-activated primary microglia, we used an ELISA kit assay (ThermoFisher; #BMS607-3). Precisely, 3×10^5 cells/well were seeded in 96 well plates and after 24 h, cells were pre-treated with compound **6** or RDOX to a final concentration of 20 μ M. LPS was then added 4 h later at a final concentration of 10 ng/mL. Dexamethasone (2.5 μ M) was used as reference immunosuppressive drug. 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).^[55] Briefly, the Minimal Inhibitory Concentrations (MICs) were determined with an inoculum of 10^5 CFU in 200 μ L 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.

Supporting Information

Copies of ^1H and ^{13}C NMR spectra, and HPLC chromatograms; MTT assays; Raw data from Figures 2,3 and 4. Orthogonal UV-absorbance and fluorescence experiment for ThT fluorescence assays. The authors have cited additional references within the Supporting Information.^[56–62]

Acknowledgments

We thank the French Association France Parkinson (GAO 2018, DOXY- PARK) and Carnot Institute (CM 098 and 120) for financial support. Also, this research was funded by grants from the Scientific and Technological Promotion National Agency—Argentina PICT-2020-SERIEA-02706 and PICT-2020-SERIEA-02255-Raíces (III) and Tucumán National University grant PIUNT-D759.

Conflict of Interests

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Abbreviations

α -Syn, α -synuclein; ACN, Acetonitrile; ANOVA, analysis of variance; DEX, dexamethasone; TNF- α , Tumor necrosis factor- α ; 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; PD, Parkinson disease; 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.

Keywords: Parkinson disease • tetracycline • alpha-synuclein • aggregation • anti-inflammation

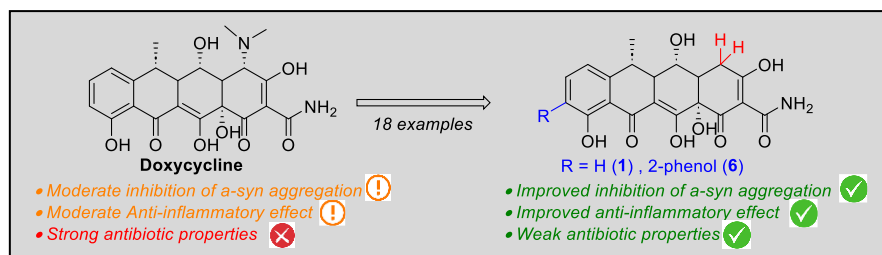
- [1] J. Parkinson, *J. Neuropsychiatry Clin. Neurosci.* **2002**, *14*, 223–236.
- [2] P. Damier, E. C. Hirsch, Y. Agid, A. M. Graybiel, *Brain* **1999**, *122*, 1437–1448.
- [3] E. R. Dorsey, B. R. Bloem, *JAMA Neurol.* **2018**, *75*, 9–10.
- [4] A. L. Fink, *Acc. Chem. Res.* **2006**, *39*, 628–634.
- [5] M. G. Spillantini, M. L. Schmidt, V. M.-Y. Lee, J. Q. Trojanowski, R. Jakes, M. Goedert, *Nature* **1997**, *388*, 839–840.
- [6] A. Gustot, J. I. Gallea, R. Sarroukh, M. S. Celej, J.-M. Ruyschaert, V. Raussens, *Biochem. J.* **2015**, *471*, 323–333.
- [7] T. Stoilova, L. Colombo, G. Forloni, F. Tagliavini, M. Salmona, *J. Med. Chem.* **2013**, *56*, 5987–6006.
- [8] M. O. Griffin, E. Fricovsky, G. Ceballos, F. Villarreal, *Am. J. Physiol.-Cell Physiol.* **2010**, *299*, C539–C548.
- [9] M. Bortolanza, G. C. Nascimento, S. B. Socias, D. Ploper, R. N. Chehin, R. Raisman-Vozari, E. Del-Bel, *J. Neural Transm.* **2018**, *125*, 1403–1415.
- [10] J. Yrjänheikki, R. Keinänen, M. Pellikka, T. Hökfelt, J. Koistinaho, *Proc. Natl. Acad. Sci.* **1998**, *95*, 15769–15774.
- [11] E. Paldino, C. Balducci, P. La Vitola, L. Artioli, V. D'Angelo, C. Giampà, V. Artuso, G. Forloni, F. R. Fusco, *Mol. Neurobiol.* **2020**, *57*, 1889–1903.
- [12] F. González-Lizárraga, S. B. Socias, C. L. Ávila, C. M. Torres-Bugeau, L. R. S. Barbosa, A. Binolfi, J. E. Sepúlveda-Díaz, E. Del-Bel, C. O. Fernandez, D. Papy-Garcia, R. Itri, R. Raisman-Vozari, R. N. Chehin, *Sci. Rep.* **2017**, *7*, 1–13.
- [13] F. V. Santa-Cecília, B. Socias, M. O. Ouidja, J. E. Sepúlveda-Díaz, L. Acuña, R. L. Silva, P. P. Michel, E. Del-Bel, T. M. Cunha, R. Raisman-Vozari, *Neurotox. Res.* **2016**, *29*, 447–459.
- [14] A. Dominguez-Meijide, V. Parrales, E. Vasili, F. González-Lizárraga, A. König, D. F. Lázaro, A. Lannuzel, S. Haik, E. Del Bel, R. Chehin, R. Raisman-Vozari, P. P. Michel, N. Bizat, T. F. Outeiro, *Neurobiol. Dis.* **2021**, *151*, 105256.
- [15] N. C. Ferreira Junior, M. dos Santos Pereira, N. Francis, P. Ramirez, P. Martorell, F. González-Lizárraga, B. Figadère, R. Chehin, E. Del Bel, R. Raisman-Vozari, P. P. Michel, *Cells* **2021**, *10*, 2163.
- [16] R. Tomas-Grau, F. González-Lizárraga, D. Ploper, C. L. Avila, S. B. Socias, P. Besnault, A. Tourville, R. M. Mella, P. Villacé, C. Salado, C. Rose, B. Seon-Ménier, J.-M. Brunel, L. Ferrié, R. Raisman-Vozari, P. P. Michel, B. Figadère, R. Chehin, *Cells* **2022**, *11*, 2759.
- [17] M. Rahmani, S. E. Negro Álvarez, E. B. Hernández, *Eur. J. Pharm. Sci.* **2022**, *175*, 106237.
- [18] J. Lucchetti, C. Fracasso, C. Balducci, A. Passoni, G. Forloni, M. Salmona, M. Gobbi, *J. Pharmacol. Exp. Ther.* **2019**, *368*, 32–40.
- [19] L. Medina, F. González-Lizárraga, A. Dominguez-Meijide, D. Ploper, V. Parrales, S. Sequeira, M.-S. Cima-Omori, M. Zweckstetter, E. Del Bel,

RESEARCH ARTICLE

- P. P. Michel, T. F. Outeiro, R. Raisman-Vozari, R. Chehín, S. B. Socias, [20] *Front. Aging Neurosci.* **2021**, *13*.
- C. Balducci, G. Santamaria, P. La Vitola, E. Brandi, F. Grandi, A. R. [21] Viscomi, M. Beeg, M. Gobbi, M. Salmona, S. Ottonello, G. Forloni, *Neurobiol. Aging* **2018**, *70*, 128–139.
- A. Minagar, J. S. Alexander, R. N. Schwendimann, R. E. Kelley, E. [22] Gonzalez-Toledo, J. J. Jimenez, L. Mauro, W. Jy, S. J. Smith, *Arch. Neurol.* **2008**, *65*, 199–204.
- I. Markulin, M. Matasin, V. E. Turk, M. Salković-Petrisic, *J. Neural [23] Transm.* **2022**, *129*, 773–804.
- A. Tourville, S. Viguier, F. González-Lizárraga, R. H. Tomas-Grau, P. [24] Ramirez, J.-M. Brunel, M. Dos Santos Pereira, E. Del-Bel, R. Chehin, L. Ferrié, R. Raisman-Vozari, B. Figadère, P. P. Michel, *Antioxidants* **2023**, *12*, 575.
- D. Fuoco, *Antibiotics* **2012**, *1*, 1–13.
- Z.-Y. Zhou, H.-T. Wang, X.-W. Wang, R.-P. Wang, Y. Du, J.-Y. Liu, J. [25] *Chin. Pharm. Sci.* **2004**, *13*, 217.
- I. Chopra, M. Roberts, *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 232–260.
- S. B. Socias, F. González-Lizárraga, C. L. Avila, C. Vera, L. Acuña, J. [26] E. Sepulveda-Diaz, E. Del-Bel, R. Raisman-Vozari, R. N. Chehin, *Prog. Neurobiol.* **2018**, *162*, 17–36.
- J. Hlavka, R. J. Ablin, *Tetracycline Derivatives and Methods of Use [27] Thereof*, **2003**, WO2003030819A2.
- D. J. Koza, Y. A. Nsiah, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2163–2165.
- M. L. Nelson, M. Y. Ismail, L. McIntyre, B. Bhatia, P. Viski, P. Hawkins, [28] G. Rennie, D. Andorsky, D. Messersmith, K. Stapleton, J. Dumornay, P. Sheahan, A. K. Verma, T. Warchol, S. B. Levy, *J. Org. Chem.* **2003**, *68*, 5838–5851.
- C. Berens, S. Lochner, S. Löber, I. Usai, A. Schmidt, L. Drueppel, W. [29] Hillen, P. Gmeiner, *ChemBioChem* **2006**, *7*, 1320–1324.
- A. Angusti, S. T. Hou, X. S. Jiang, H. Komatsu, Y. Konishi, T. Kubo, J. [30] Lertvorachon, G. Roman, *Tetracyclines and Their Use as Calpain Inhibitors*, **2005**, WO2005082860A1.
- P. Abato, T. Bowser, P. Higgins, A. K. Verma, J. Zhang-Hoover, [31] *Tetracycline Compounds for the Treatment of Rheumatoid Arthritis and Related Method of Treatment*, **2010**, WO2010033939A1.
- M. Bergström, G. Suresh, V. R. Naidu, C. R. Unelius, *Eur. J. Org. Chem.* [32] **2017**, *2017*, 3234–3239.
- T. Bowser, P. Abato, *7-Disubstituted-Phenyl Tetracycline Derivatives*, [33] **2013**, WO2013181391A2.
- M. Draveny, C. Rose, A. Pinet, L. Ferrié, B. Figadère, J.-M. Brunel, M. [34] Masi, *Molecules* **2023**, *28*, 4262.
- M. Vilar, H.-T. Chou, T. Lührs, S. K. Maji, D. Riek-Loher, R. Verel, G. [35] Manning, H. Stahlberg, R. Riek, *Proc. Natl. Acad. Sci.* **2008**, *105*, 8637–8642.
- A. Lau, R. W. L. So, H. H. C. Lau, J. C. Sang, A. Ruiz-Riquelme, S. C. [36] Fleck, E. Stuart, S. Menon, N. P. Visanji, G. Meisl, R. Faidi, M. M. Marano, C. Schmitt-Ulms, Z. Wang, P. E. Fraser, A. Tandon, B. T. Hyman, H. Wille, M. Ingelsson, D. Klenerman, J. C. Watts, *Nat. Neurosci.* **2020**, *23*, 21–31.
- A. R. A. Ladiwala, J. C. Lin, S. S. Bale, A. M. Marcelino-Cruz, M. [37] Bhattacharya, J. S. Dordick, P. M. Tessier, *J. Biol. Chem.* **2010**, *285*, 24228–24237.
- C. Balducci, G. Forloni, *Front. Pharmacol.* **2019**, *10*.
- T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55–63.
- C. K. Glass, K. Saijo, B. Winner, M. C. Marchetto, F. H. Gage, *Cell* **2010**, [38] *140*, 918–934.
- M. Lazzarini, S. Martin, M. Mitkovski, R. R. Vozari, W. Stühmer, E. D. [39] Bel, *Glia* **2013**, *61*, 1084–1100.
- P. L. Vitola, L. Artioli, M. Cerovic, C. Poletto, L. Dacomo, S. Leva, C. [40] Balducci, G. Forloni, *Parkinsonism Relat. Disord.* **2023**, *106*, 105229.
- M. Bortolanza, G. C. do Nascimento, R. Raisman-Vozari, E. Del-Bel, [41] *Br. J. Pharmacol.* **2021**, *178*, 2595–2616.
- J. Fenneteau, S. Vallerotto, L. Ferrié, B. Figadère, *Tetrahedron Lett.* [42] **2015**, *56*, 3758–3761.
- L. Ferrié, I. Ciss, J. Fenneteau, S. Vallerotto, M. Seck, B. Figadère, *J. [43] Org. Chem.* **2022**, *87*, 1110–1123.
- M. Pliot, M. Bourdonneau, K. Elbayed, J.-M. Wieruszkeski, G. Lippens, [44] *Magn. Reson. Chem.* **2006**, *44*, 943–947.
- W. Hoyer, T. Antony, D. Cherny, G. Heim, T. M. Jovin, V. Subramaniam, [45] *J. Mol. Biol.* **2002**, *322*, 383–393.
- A. Fenyi, A. Coens, T. Bellande, R. Melki, L. Bousset, *Sci. Rep.* **2018**, [46] *8*, 10788.
- L. Bousset, P. Brundin, A. Böckmann, B. Meier, R. Melki, *J. Park. Dis.* [47] **2016**, *6*, 143–151.
- N. K. Polinski, L. A. Volpicelli-Daley, C. E. Sortwell, K. C. Luk, N. [48] Cremades, L. M. Gottler, J. Froula, M. F. Duffy, V. M. Y. Lee, T. N. Martinez, K. D. Dave, *J. Park. Dis.* **2018**, *8*, 303–322.
- H. Levine III, *Protein Sci.* **1993**, *2*, 404–410.
- J. E. Sepulveda-Diaz, M. O. Ouidja, S. B. Socias, S. Hamadat, S. [49] Guerreiro, R. Raisman-Vozari, P. P. Michel, *Glia* **2016**, *64*, 1912–1924.
- G. Carret, J. D. Cavallo, H. Chardon, C. Chidiac, P. Choutet, P. [50] Courvalin, H. Dabernat, L. Drugeon, F. Dubreuil, V. Goldstein, V. Jarlier, R. Leclercq, M. H. Nicolas-Chanoine, A. Philippon, C. Quentin-Noury, B. Rouveix, J. Sirot, C. J. Soussy, *Int. J. Antimicrob. Agents* **2003**, *21*, 364–391.
- K. A. Murray, C. J. Hu, H. Pan, J. Lu, R. Abskharon, J. T. Bowler, G. M. [51] Rosenberg, C. K. Williams, G. Elezi, M. Balbirnie, K. F. Faull, H. V. Vinters, P. M. Seidler, D. S. Eisenberg, *Proc. Natl. Acad. Sci.* **2023**, *120*, e2217835120.
- Š. Korat, N. S. R. Bidesi, F. Bonanno, A. Di Nanni, A. N. N. Hoàng, K. [52] Herfert, A. Maurer, U. M. Battisti, G. D. Bowden, D. Thonon, D. Vugts, A. D. Windhorst, M. M. Herth, *Pharmaceuticals* **2021**, *14*, 847.
- J. Estaun-Panzano, M.-L. Arotcarena, E. Bezar, *Neurobiol. Dis.* **2023**, [53] *176*, 105966.
- K. A. Conway, S.-J. Lee, J.-C. Rochet, T. T. Ding, R. E. Williamson, P. [54] T. Lansbury, *Proc. Natl. Acad. Sci.* **2000**, *97*, 571–576.
- A. R. Braun, E. E. Liao, M. Horvath, P. Kalra, K. Acosta, M. C. Young, [55] N. N. Kochen, C. H. Lo, R. Brown, M. D. Evans, W. C. K. Pomerantz, E. Rhoades, K. Luk, R. L. Cornea, D. D. Thomas, J. N. Sachs, *Npj Park. Dis.* **2021**, *7*, 1–17.
- A. A. Reinke, J. E. Gestwicki, *Chem. Biol. Drug Des.* **2011**, *77*, 399–411.
- K. A. Conway, S. J. Lee, J. C. Rochet, T. T. Ding, J. D. Harper, R. E. [56] Williamson, P. T. Lansbury, *Ann. N. Y. Acad. Sci.* **2000**, *920*, 42–45.

RESEARCH ARTICLE

Entry for the Table of Contents



Modified doxycycline derivatives were synthesized to treat Parkinson's disease. Improvement was reached with some C9-substitution and removal of dimethyl amino-group, leading to improved inhibition of α -synuclein aggregation and anti-inflammatory effect and reduced antibiotic activity. These findings pave the way to promising *in-vivo* studies.

Institute and/or researcher Twitter usernames: @BioCIS, @FerrieLaurent, @InstitutCerveau, @ImmcaLab, @Rodrigo_HTG