



Exploring the reactivity of bicyclic α -iminophosphonates to access new imidazoline I₂ receptor ligands

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ARTICLE INFO

Keywords:

Imidazoline I₂ receptors
imidazoline I₂ receptor ligands
Neuroprotection
Neuroinflammation
Bicyclic α -aminophosphonate
Bicyclic α -phosphoprolines
Alzheimer's disease
Parkinsonism
Caenorhabditis elegans

ABSTRACT

Recent studies pointed out the modulation of imidazoline I₂ receptors (I₂-IR) by selective ligands as a putative strategy to face neurodegenerative diseases. Foregoing the classical 2-imidazoline/imidazole-containing I₂-IR ligands, we report a family of bicyclic α -iminophosphonates endowed with high affinity and selectivity upon I₂-IR and we advanced a representative compound B06 in preclinical phases. In this paper, we describe the synthetic possibilities of bicyclic α -iminophosphonates by exploring its ambivalent reactivity, leading to unprecedented molecules that showed promising activities as I₂-IR ligands in human brain tissues and good BBB permeation capabilities. After *in silico* ADME prediction studies, we assessed the neuroprotective properties of selected compounds and beneficial effect in an *in vitro* model of Alzheimer's and Parkinson's disease. Along with their neuroprotective effect, compounds showed a potent anti-inflammatory response when evaluated in a neuroinflammation cellular model. Moreover, this is the first time that the neuroprotective effects of imidazoline I₂-IR ligands in a transgenic Alzheimer's disease *Caenorhabditis elegans* strain are investigated. Using a thrashing assay, we found a significant cognition improvement in this *in vivo* model after treatment with the new bicyclic α -phosphoprolines. Therefore, our results confirmed the need of exploring structurally new I₂-IR ligands and their potential for therapeutic strategies in neurodegeneration.

1. Introduction

Imidazoline I₂ receptors (I₂-IR) are nonadrenergic binding sites that can be found in the central nervous system (CNS) and many other organs such as the heart and the liver [1–3]. The current consensus is that I₂-IR are not a specified molecular identity but heterogenous proteins that are recognized by the radiolabeled ligand [³H]idazoxan and, with much lower affinity, by the [³H]clonidine and the [³H]p-aminoclonidine [4,5]. The modulation of I₂-IR by standard ligands evidenced their role in

different physiological functions such as analgesia [6], inflammation [7], and human brain disorders [8] encompassing glial tumors [9], depression [10], Alzheimer's disease (AD) [11] and Parkinson's disease (PD) [12,13], amongst others. The chemical structure of I₂-IR ligands [14] is restricted to 2-heterocyclic-2-imidazolines in the standards idazoxan, trazoline, BU224, 2-BFI and BU99008 or the N1-imidazole heterocyclic scaffold in CR4056 (Fig. 1). I₂-IR are emerging as new therapeutic targets, which is endorsed by ongoing clinical trials of CR4056 for the osteoarthritis (phase II) [15] and ¹¹C-BU99008 PET for

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<https://doi.org/10.1016/j.bioorg.2023.106935>

Received 2 July 2023; Received in revised form 19 October 2023; Accepted 21 October 2023

Available online 24 October 2023

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the diagnostics of AD (phase I) [16].

We have contributed to disclosing the pharmacological role of I₂-IR by assessing their modulation with original I₂-IR ligands and by observing *in vivo* physiological responses and modifications of molecular AD-biomarkers in treated murine animal models. We reported the outstanding binding affinity and selectivity for I₂-IR [17] of (2-imidazolin-4-yl)phosphonates [18] synthesized by a microwave assisted multicomponent reaction (Fig. 2A) [19]. The treatment of a murine model of age-related cognitive decline model, the senescence-accelerate mouse prone 8 (SAMP8) with a representative compound, diethyl [(1-(3-chloro-4-fluorobenzyl)-5,5-dimethyl-4-phenyl-4,5-dihydro-1H-imidazol-4-yl)]phosphonate (named as MCR5) resulted in a cognitive improvement and amelioration of AD hallmarks, including the behavioral [20] and psychological symptoms of dementia [21]. This work was the first experimental evidence that brought forward I₂-IR as a new therapeutic target for neurodegeneration. In addition, MCR5 also improved *in vivo* age-related endothelial dysfunction [22]. We described the potential of [(2-benzofuran-2-yl)-1H-imidazole] (named as LSL60101) as a disease-modifying single therapy [23] for the treatment of AD. We also synthesized and pharmacologically characterized a family of congeners embodying the benzofuranyl-2-imidazole skeleton (Fig. 2B) [24]. Moving further with the above-mentioned structures, we described the first I₂-IR ligand that did not feature an imidazoline/imidazole ring. Particularly, we reported a family of bicyclic α -iminophosphonates [25] as a source of high affinity I₂-IR molecules that permitted to carry out a three-dimensional quantitative structure–activity relationship (3D-QSAR). This study revealed key structural parameters that allowed the proposal of a putative pharmacophore for these I₂-IR ligands. We selected the representative compound diethyl (1*RS*,3*aSR*,6*aSR*)-5-(3-chloro-4-fluorophenyl)-4,6-dioxo-1-phenyl-1,3*a*,4,5,6,6*a*-hexahydropyrrolo[3,4-*c*]pyrrole-1-phosphonate (named as B06), with excellent activity and selectivity, for the targeted receptors. B06 ameliorated the cognitive decline and improved the behavior of two murine models, SAMP8 [26] and the familial Alzheimer's disease (5xFAD) [27]. Furthermore, B06 showed de-risked *in vitro* ADME-Tox properties and neuroprotective/anti-inflammatory properties as well as neuroprotective effects in an *in vitro* model of PD [28]. Further and more advanced preclinical development studies of B06 in AD and PD brain disorders is underway (Fig. 2C).

Encouraged by the excellent above-mentioned results obtained when we uncovered the novel I₂-IR ligands' chemical structure of I₂-IR ligands, herein we report the synthetic exploitation of the bicyclic α -iminophosphonates by considering the ambivalent properties (C imine- electrophile/C-3*a*- nucleophile) of the right-inferior quadrant of the molecule leading to the general bicyclic α - phosphoproline structures depicted in Fig. 2C. α -Aminophosphonates, the ubiquitous functional group commonly present in our new compounds, are considered

phosphonic analogues of α -aminoesters, which can be applied as an antibiotic, anticancer and antifungal therapies, and are also enzyme inhibitors due to their tetrahedral structure, able to mimic transition states. α -Aminoesters have been used as scaffolds to synthesize peptidomimetic structures and also to study the mode of action of aminoacids in biomedical transformations [29–33]. In this paper we provide a new synthetic strategy to access diversely substituted bicyclic pyrrolidine-2-phosphonates (phosphoprolines, the phosphonic counterpart of proline) [34–36] and evaluate their anti-AD properties.

Diversely substituted (octahydropyrrolo[3,4-*c*]pyrrole-1-yl)phosphonates were synthesized from previously described bicyclic α -iminophosphonates [27] by reduction of the imine group or nucleophilic addition to the C-imine functional group. We assessed the pharmacological profile of selected new compounds in human tissues and their selectivity versus the related target α_2 -adrenergic receptor (α_2 -AR) by competitive binding assays against the selective I₂-IR radioligand [³H]2-BFI, and competitive studies using the selective radioligand [³H]RX821002 (2-methoxydazoxan), respectively. We carried out *in vitro* PAMPA-BBB assay for the new molecules and evaluated three selected new compounds in a dopaminergic neurodegeneration cellular model. Furthermore, since most neurodegenerative diseases (AD, PD) are characterized by the presence of an exacerbated and sustained neuro-inflammatory process, which is partly responsible for the development of the disease, we completed the study of the *in vitro* biological activity using a cellular neuroinflammation model. Furthermore, since most neurodegenerative diseases (AD, PD) are characterized by the presence of an exacerbated and sustained neuroinflammatory process, which is partly responsible for the development of the disease, we have completed the study of the *in vitro* biological activity using a cellular neuroinflammation model. The positive results motivated us to take the challenge to carry out the first study of the effects after treatment with I₂-IR ligands in the *in vivo* AD model organism *Caenorhabditis elegans* [37]. To this purpose, we used the transgenic strain CL2006 to evaluate the neuronal behavioral phenotype effects of the selected compounds on a thrashing assay. CL2006 expresses human A β 1-42 under control of a muscle-specific promoter and responds to A β 1-42 aggregation with progressive adult-onset paralysis. Due to the novelty of the proposal, we included the standard I₂-IR ligand 2-BFI (Fig. 1) endowed with neuroprotective properties [38–40], and three new compounds with optimal affinity upon these receptors.

Finally, exploring further synthetic transformations, tetradecahydrobipyrrrolo[3,4-*c*]pyrrole-1,1'-diphosphonates were obtained by self-reaction of ambivalent bicyclic α -iminophosphonates in acid conditions after the nucleophilic attack of the C3*a* to the imine functional group to other congener.

All new compounds were characterized and described from a stereochemical point of view by X-ray crystallographic analysis as well as

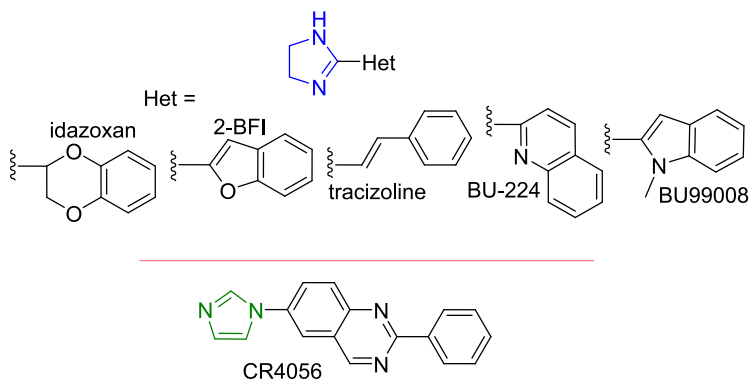


Fig. 1. Structure of the known I₂-IR ligands, imidazoline 2-heterocyclic substituted (idazoxan, 2-BFI, trazoline and BU224), and clinical candidates (BU99008 and CR4056). The imidazoline nucleus is highlighted in blue and the imidazole nucleus in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

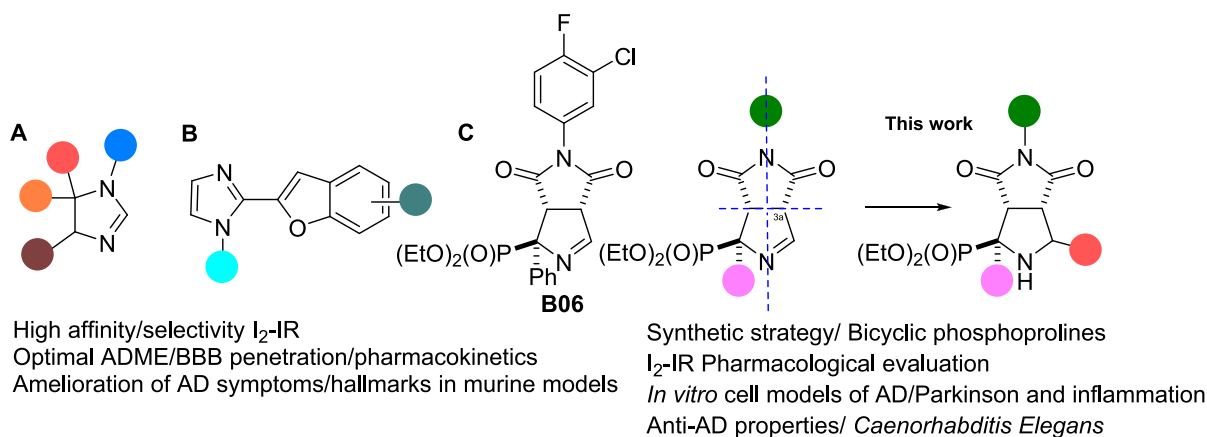


Fig. 2. General structures of the families of I₂-IR ligands reported by our group: A. (2-Imidazolin-4-yl)phosphonates; B. Benzofuranyl-2-imidazoles and C. Chemical structure of B06. Bicyclic α-iminophosphonates and general structure resulting from this work and outlines. Coloured balls indicate a wide-range of substituents.

¹H and ¹³C RMN comparative studies. ADME *in silico* predictions of the new molecules were also included.

2. Results and discussion

2.1. Chemistry

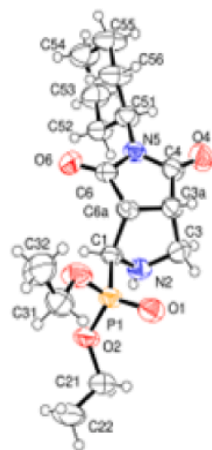
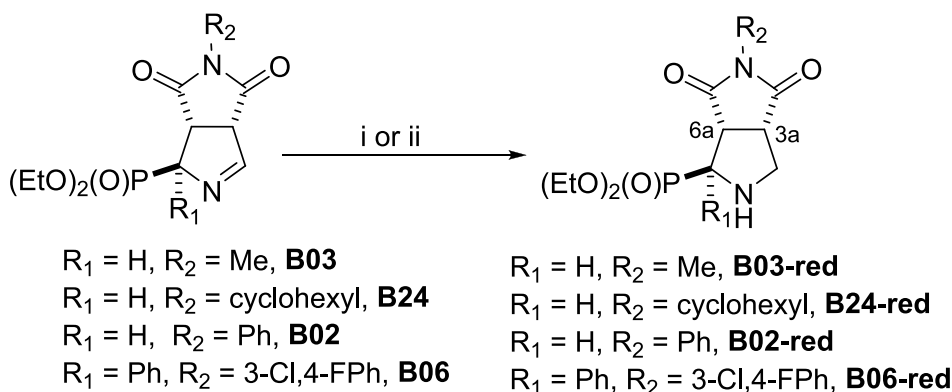
2.1.1. Diastereoselective [3 + 2]cycloaddition reaction

In order to access enough quantities of starting bicycles (Schemes 1 and 2), we undertook their synthesis by following our reported

procedure involving a diastereoselective [3 + 2]cycloaddition reaction [27].

2.1.2. Reduction of the imine functional group

The conversion of the diethyl (3,4-dihydro-2*H*-pyrrol-2-yl)phosphonate moiety of bicyclic derivatives to diethyl (pyrrolidine-2-yl) phosphonate required the reduction of the imine functional group. This transformation was already explored though the hydrogenation of compounds **B03**, **B24** and **B02** at atmospheric pressure in order to give access to **B03-red**, **B24-red**, and **B02-red** in 84, 90 and 87 % yield,



Scheme 1. Reagents and conditions for the reduction of **B03**, **B24**, and **B02**. (i) H₂, Pd/C 5 %, methanol, rt, 24 h, 84 %, **B03-red**; 90 %, **B24-red**, and 87 %, **B02-red** and for **B06** (ii) NaBH₃CN, acetonitrile, water and acetic acid, rt, 1 h, 84 %, **B06-red**. HCl. X-Ray crystallographic structure of **B24-red**.

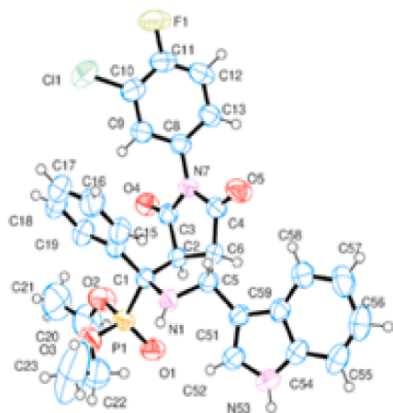
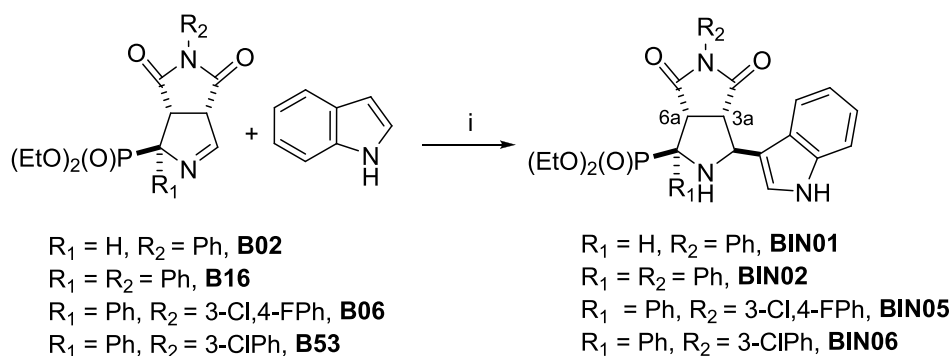


Table 1Significant ^1H and ^{13}C NMR data, chemical shift, multiplicity and coupling constants (Hz) of **BIN01**, **BIN02**, **BIN05** and **BIN06**.

Comp	H3	H3a	H6a	C1	C3	C3a	C6a	C4	C6
BIN01	4.59	3.68	3.96	57.0	61.4	52.6	48.6	175.1	175.3
	d	dd	ddd	d	d	d	d		
BIN02	8.0	9.5, 8.0	18.0, 9.5, 7.0	165.5	17.0	7.5	2.0		
	5.27	3.88	4.47	70.5	58.2	55.9	56.8	172.9	176.2
BIN05	m	ddd	dd	d	d	d			
		9.5, 5.5, 1.0	15.0, 9.5	156.5	15.0	10.0			
BIN06	5.29	3.83	4.44	70.6	58.3	55.8	56.8	172.5	175.9
	d	ddd	dd	d	d	d			
BIN06	6.5	9.5, 5.5, 1.0	15.0, 9.5	156.5	15.0	10.0			
	5.29	3.85	4.45	70.4	58.2	55.7	56.7	172.4	175.8
BIN06	d	ddd	dd	d	d	d			
	5.5	9.5, 5.5, 1.0	15.0, 9.5	156.5	15.0	10.0			

m = multiplet, ddd = doublet of doublet of doublets, dd = doublet of doublets, d = doublet.

phosphorous atom with a value of 17 Hz for **BIN01** and 15.0 Hz for the α -phenyl substituted derivatives. The interaction of the C3a-P led to coupling constants of 7.5 Hz (**BIN01**) and 10.0 Hz (**BIN02**, **BIN05**, **BIN06**) (Fig. 3).

To further explore the reactivity of the prepared compound, the opening of the imide containing ring was carried out. To this end, the treatment of **BIN02** with sodium hydroxide at room temperature during 2.5 h led to an unexpected monocyclic compound, lacking the phosphonate ester functional group and with an 80 % yield. The analysis of the ^1H and ^{13}C NMR spectra and the X-ray crystallography led to the unequivocal description of the new structure **PIP-BIN02** (Scheme 3).

The formation of **PIP-BIN02** could be accounted by considering the attack of the hydroxide anion to the C6, which underwent the opening of the imide function and the generation of an intermediate. The latter decarboxylated and extruded the phosphonate ester to lead the trisubstituted 1-pyrroline **PIP-BIN02** (Scheme 4).

In this scenario, the phosphonate functional group works as an auxiliary group that allowed the initial diastereoselective [3 + 2] cyclocondensation to occur (by increasing the acidity of the α -proton). Furthermore, this phosphonate functional group orientated the diastereoselective entry of the indole substituent and, finally, it was displaced in the basic treatment to afford **PIP-BIN02**.

2.1.4. The bridgehead position of bicyclic α -iminophosphonates as a nucleophile

The addition of nucleophiles to the carbon of the imine function increased the structural complexity of the new molecules (section 2.1.3.), which led us to consider the ambivalent nature of the starting bicyclic α -iminophosphonates. Thus, in the same molecule there was a nucleophilic position, such as C3a (α -carbonyl of an amide and the α -imine position), and an electrophilic position C3 (carbon of the imine). The amphiphilic characteristic in the two different positions of the same molecule was also observed in the unique carbon atom of the isocyanide group of the starting materials, PhosMIC or α -PhPhosMIC, employed in the [3 + 2]cycloaddition reaction (section 2.1.1.) that could react either with nucleophiles or electrophiles [46].

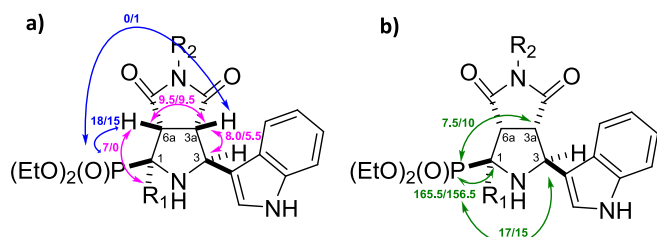
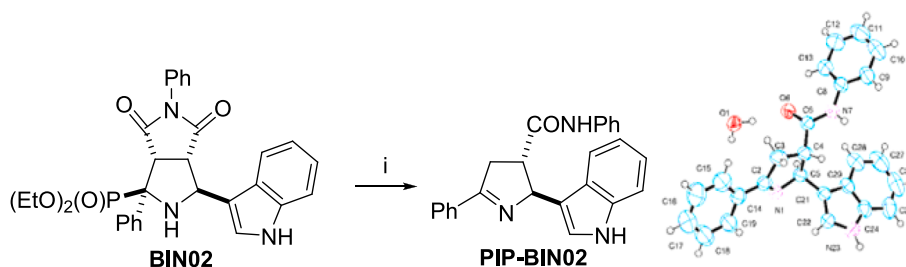


Fig. 3. Representative coupling constants: a) ^1H - ^1H (pink), ^1H -P (blue) and ^{13}C -P (green) for **BIN01/BIN02**, **BIN05**, **BIN06**. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

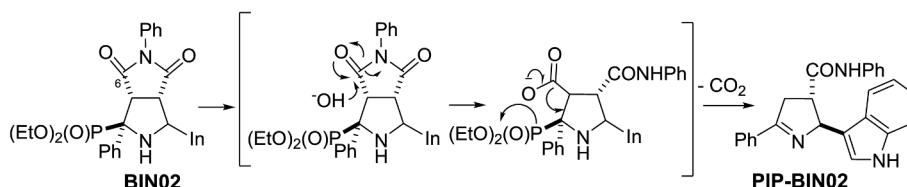
Therefore, we took the challenge of accessing “dimeric diphosphonate bicycles” depicted in Scheme 5. To this end, we treated **B02** with 3 equivalents of trimethylsilyl chloride (TMSCl) in dichloromethane at room temperature during 48 h. The crude mixture was purified leading to the identification of two compounds with the same molecular mass (diastereoisomers) in a 1:2 proportion (GC/MS determination) and a 59 % yield, which named **BxB03/BxB04** (Table 2, entry 1). Increasing TMSCl to 6 equivalents led to a mixture of 1:1.6 ratio of **BxB03/BxB04** in a 45 % yield (Table 2, entry 2). The use of microwave irradiation at 80 °C during 30 min gave a 68 % yield of a 1:1.7 **BxB03/BxB04** mixture (Table 2, entry 3) and 16 % of starting material. Conducting the reaction by using the *N*-methyl derivative **B03** under conditions of entry 2, led to a 64 % yield of a 1:2.2 mixture of **BxB01/BxB02** (Table 2, entry 6). Analogously to conditions in entry 3, **B03** rendered a 58 % mixture 1:1.4 of **BxB01/BxB02** (Table 2, entry 7) and 3 % of starting material. Inspired by the work of M. Noguchi [45], we used the milder acid pyridinium *p*-toluenesulphonate (PPTS), which triggered the dimerization of **B02** after treatment with 1.5 equivalents at reflux temperature during 15 h providing a 57 % yield of a 1:0.8 mixture of **BxB03/BxB04** (Table 2, entry 4). The microwave irradiation at 80 °C for 40 min slightly decreased the reaction yield (46 %) but it maintained the proportion of the final products (Table 2, entry 5). The conditions depicted in entry 4 led to a 1:0.9 mixture of **BxB01/BxB02** in a 57 % yield when **B03** was used as starting material (Table 2, entry 8). Similarly, to the results described in entry 5 when applying to **B03**, an equitable mixture of **BxB01/BxB02**, was isolated in a 43 % yield (Table 2, entry 9). The use of the two different acids (TMSCl and PPTS) led to similar results with both **B02** and **B03** as starting bicycles with a ratio of final dimers ~ 1:1.4–2 with TMSCl and 1:0.8–1 PPTS. Therefore, the substituent in the nitrogen atom, *N*-Me (**B02**) or *N*-Ph (**B03**), does not affect the course of the “dimerization” reaction.

Next, we examined the influence that an α -additional substituent could play in the results of the reaction results compared to the presence of a hydrogen atom. To this end, compound **B16**, that embodies a α -phenyl substituent, was heated at 80 °C with 6 equivalents of TMSCl in a microwave oven for 30 min. The expected dimers **BxB05/BxB06** were isolated in a 1:1.5 ratio and a 68 % yield, along with a 15 % of **B16**. Consequently, the studied reaction took place in a similar fashion, regardless the presence or not of substituents in the α -position.

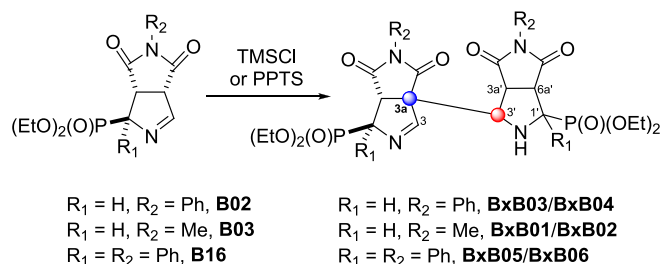
In the dimerization reaction, a new bond, a quaternary stereocenter in the bridged position (C3a) and a stereocenter in the carbon atom contiguous to the nitrogen (C3') were generated. The configurations of the C1, C6a and C3a (and C1', C6a' and C3a') were fixed, due to the stereochemical result of the diastereoselective [3 + 2]cycloaddition reaction of the *N*-methyl, or *N*-phenylmaleimide, with PhosMIC or α -PhPhosMIC (Scheme 5). Considering that the starting **B02**, **B03** and **B16** were racemic mixtures, the dimerization reaction could proceed either between the same enantiomer or between different enantiomers. Therefore, although only two pairs of enantiomers were isolated, eight possible stereoisomers (four pairs of enantiomers) should be considered



Scheme 3. Reagents and conditions for the synthesis of PIP-BIN02 (i) NaOH 0.05 M, tetrahydrofuran: water (2:1), r.t., 2.5 h, 80 % yield. X-Ray crystallographic structure of PIP-BIN02.



Scheme 4. Putative mechanism for the formation of PIP-BIN02. In = indole.



Scheme 5. Reaction of B02, B03 and B16 with TMSCl or PPTS and general structure of the dimers BxB03/BxB04, BxB01/BxB02 and BxB05/BxB06. New quaternary stereocenter highlighted in blue, and new stereocenter highlighted in red.

Table 2

Reaction conditions and yield (ratio) to access dimeric (**BxB01/BxB02**, **BxB03/BxB04**).

Entry	Starting material Reaction conditions	Yield (%)
	B02	BxB03/BxB04
1	TMSCl 3 eq, r.t., CH ₂ Cl ₂ , 48 h	59 (1:2)
2	TMSCl, 6 eq, r.t. CH ₂ Cl ₂ , 48 h	45 (1:1.6)
3	TMSCl, 6 eq, MW, 80 °C, 30 min	68 (1:1.7)
4	PPTS, 1.5 eq, Δ, MeOH, 15 h	57 (1:0.8)
5	PPTS, 1.5 eq, MW, MeOH, 80 °C, 40 min	46 (1:0.8)
	B03	BxB01/BxB02
6	TMSCl 6 eq, r.t., CH ₂ Cl ₂ , 48 h	64 (1:2.2)
7	TMSCl, 6 eq, MW, 80 °C, 30 min	58 (1:1.4)
8	PPTS, 1.5 eq, Δ, MeOH, 15 h	57 (1:0.9)
9	PPTS, 1.5 eq, MW, MeOH, 80 °C, 40 min	43 (1:1)

TMSCl: trimethylsilyl chloride; PPTS: pyridinium p-toluensulphonate; MW: irradiation in a microwave oven.

depending on the face (*exo/endo*) where the reaction took place (Scheme 5).

In general, the addition of nucleophiles to bicyclic systems is more favored through the *exo* face. To confirm this hypothesis, the stereochemistry of **BxB05/BxB06** was indisputably determined by X-ray crystallography analysis (Fig. 4).

Compound **BxB05** results from the *exo* addition of **B16** to the same enantiomer and compound **BxB06** from the *exo* addition to the convex

face to the other enantiomer of **B16**.

Aiming to determine the relative stereochemistry in the three pairs of diastereoisomers, we compared the ¹H and ¹³C NMR spectra of **BxB03/BxB04** and **BxB01/BxB02** with the relative configuration confirmed for **BxB05/BxB06** (Tables 3 and 4).

The chemical shift multiplicity and coupling constants of the different protons and carbons were in concordance with the stereochemistry assigned to the pair of compounds **BxB05/BxB06**. Specifically, the difference in the shield between de protons and carbons at the same position was preserved within the three pairs of dimeric compounds (Scheme 5). Thus, the *exo* addition of **B02** to its enantiomer led to **BxB03** and, in the case of **B03**, it led to **BxB01**. The *exo* self-addition of **B02** and **B03** rendered **BxB04** and **BxB02**, respectively.

In detail, in compounds **BxB01-BxB04**, the H1 hydrogen displayed a coupling constant with the phosphorous atom of ~ 22 Hz, and two minors with the H6a and H3 (2.5–4.0 Hz). The bridged H6a' in **BxB01**, **BxB03** and **BxB04** showed a peak with a multiplicity of ddd due to the coupling constants 17.5–18.0 (H6a'-P), 9.0–10.0 (H6a'-H3a') and 7.0–8.0 Hz (H6a'-H1').

It is noteworthy that in the ¹³C spectra C1/C1' and C3/C3' were doublets due to the presence of the phosphorous atom with significant coupling constants of C1 (159.5–161.5 Hz)/C1' (168.0–175.0 Hz) and C3 (13.0–14.0 Hz)/C3' (14.0–16.5 Hz), respectively. The difference between the C3a within the two members of each pair is conserved (3.5–3.8 ppm) (Fig. 5).

2.2. In silico prediction of physicochemical and ADME pharmacokinetic properties of new compounds

With the goal of describing the potential of the new compounds as drug candidates (Scheme 1, 2 and 5), the favorable drug-likeness features were predicted by the validated medchem program molinspiration software [44]. Molinspiration was used to calculate molecular descriptor properties of Lipinski's rule for the new compounds, i.e., molecular weight, number of H-bond donors & acceptors, topological polar surface area (TPSA), log P, and number of rotatable bonds (nRotB). The Lipinski rule states that the permeation of an orally administered compound is more likely to be absorbed better if the properties of the molecule are the following: molecular weight < 500, log P < 5, number of hydrogen bond acceptors < 10, and number of hydrogen bond donors < 5. The expansion of Lipinski's rules considers several rotatable bonds < 10 and polar surface area ≤ 140 Å. Compounds depicted in Scheme 1

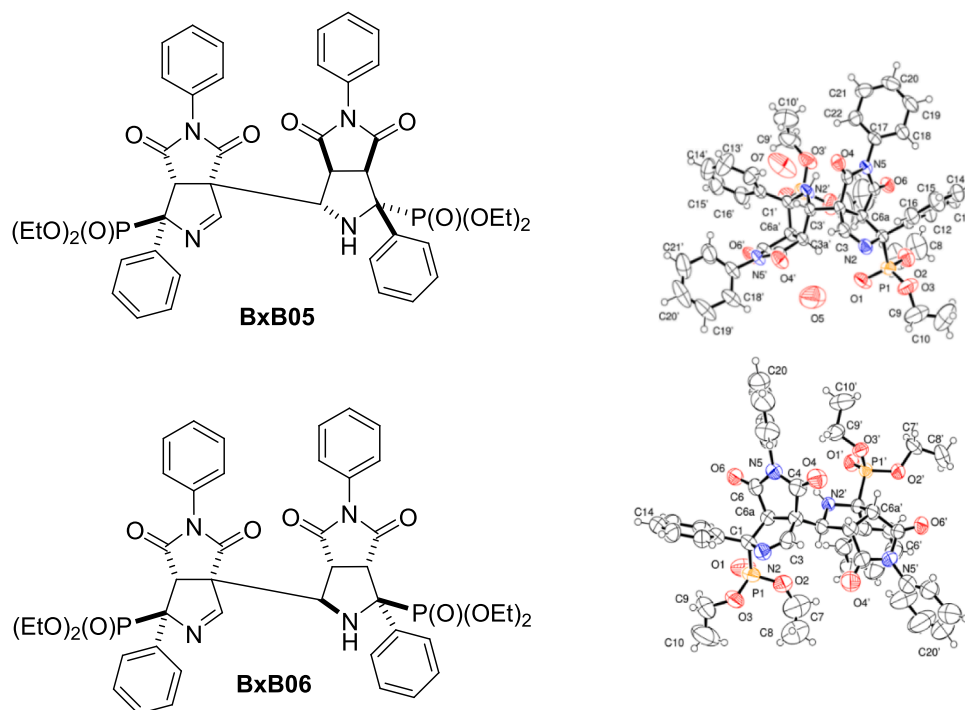


Fig. 4. Chemical structure of BxB05 and BxB06 and their X-ray structures.

Table 3

Significant ^1H data of BxB03/ BxB04, BxB01/ BxB02 and BxB05/ BxB06.

Comp.	H1	H3	H6a	H1'	H3'	H3a'	H6a'
BxB01	4.64	7.69	3.91	3.46	4.33	3.97	3.61
	dt	m	dd	m	m	dd	ddd
BxB02	22.5, 3.5		18.5, 4.0			9.0, 7.0	17.5, 9.0, 8.0
	4.72	7.92	4.02	3.40	4.17–4.33	3.54–3.67	3.54–3.67
BxB03	ddd	dd	ddd	ddd	c.s.	c.s.	c.s.
	22.0, 3.5, 2.5	4.5, 2.5	18.0, 3.5	8.0, 5.0, 3.5			
BxB04	4.82	7.81	4.10–4.41	3.69	4.62	4.10–4.41	3.86
	ddd	dd	c.s.	m	t	c.s.	ddd
BxB05	22.5, 4.0, 3.0	4.0, 3.0			6.5		18.5, 9.5, 7.5
	4.89	8.04	4.14	3.62	3.92	4.57	3.81
BxB06	dt	dd	m	m	dd	dd	ddd
	22.0, 3.0	4.5, 3.0			7.0, 5.0	9.5, 7.5	18.0, 10.0, 8.0
BxB05	–	8.16	4.25–4.43	–	5.28	4.25–4.43	4.25–4.43
		d	c.s.		m	c.s.	c.s.
BxB06	–	3.5					
		8.32	4.30–4.43	–	4.50	4.70	4.30–4.43
		d	c.s.		t	dd	c.s.
		4.0			5.5	9.5, 5.0	

m = multiplet, t = triplet, ddd = doublet of doublet of doublets, dd = doublet of doublets, dt = doublet of triplets, c.s = complex signal.

obeyed Lipinski's rule, and therefore showed good bioavailability. The molecular weights of **BIN02**, **BIN05** and **BIN06** were over 9 %, 19 % and 15 % respectively, lying within the recommended limit for a good bioavailability. The log P of **BIN05** and **BIN06**, were 5.12 and 5.0 respectively, slightly over the < 5 desired value, therefore, good permeability across the cell membrane was expected. **PIP-BIN02** was in full compliance with Lipinski's rules. Dimers depicted in Scheme 5 were discarded for further biological assays due to their high molecular weight (852.82 for **BxB05** and **BxB06**) and their high number of bond acceptors (14), involving possible insufficient oral drug-likeness characteristics (see supporting information). The pharmacokinetics of the new compounds were predicted using Swiss ADME web-based tool [47]. All compounds showed values classified as very soluble (ESOL and Ali classes) and soluble (Silicos), and exhibited optimal high gastrointestinal absorption, which is a requirement for oral drugs. The prepared molecules were not predicted to be substrates of the cytochromes

(CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4). No PAINS alerts were shown, and the general predicted parameters matched with a good drug likeness. The results of ADME prediction are detailed in supporting information.

2.3. Radioligand I_2 -IR binding assays

The pharmacological profile of seven representative compounds with general structures in Schemes 1–3, selected based on comparison with previous reported structures [27], were evaluated by competitive binding studies against the selective I_2 -IR radioligand [^3H]2-BFI and the selective α_2 -AR radioligand [^3H]RX821002. The studies were performed in membranes from *post-mortem* human frontal cortex, a brain area that shows an important density of I_2 -IR and α_2 -AR [48]. Idazoxan, a compound with well-established affinity for I_2 -IR ($\text{pK}_i = 7.41 \pm 0.63$) and α_2 -AR ($\text{pK}_i = 8.35 \pm 0.16$), was used for reference [49]. The inhibition

Table 4
Significant ^{13}C NMR data of **BxB03**/ **BxB04**, **BxB01**/ **BxB02** and **BxB05**/ **BxB06**.

Comp.	C1	C3	C3a	C6a	C1'	C3'	C3a'	C6a'
BxB01	73.3	164.8	74.7	45.2	56.5	61.5	47.9	47.3
	d	d			d	d	d	
BxB02	160.0	13.5			174.5	15.5	7.5	
	74.1	164.4	71.1	47.4	56.2	63.2	47.3	47.5
BxB03	d	d		d	d	d	d	
	160.5	13.5		9.5	174.0	16.0	8.0	
BxB04	73.8	164.8	74.7	48.1	57.0	62.5	45.3	47.5
	d	d		d	d	d	d	
BxB05	159.5	14.0		7.0	175.0	14.5		
	74.7	164.4	70.9	47.5	56.6	63.8	47.4	46.8
BxB06	d	d			d	d	d	
	160.0	13.0			173.5	16.5		
BxB05	85.5	164.4	76.0	51.6	67.0	60.6	49.1	53.8
	d	d			d	d	d	
BxB06	161.5	14.0			172.0	14.0	9.5	
	86.6	164.1	72.5	53.9	69.0	61.7	49.5	53.3
BxB06	d	d			d	d	d	
	160.5	13.5			168.0	15.0	10.5	2.5

d = doublet.

constant (K_i) for each compound was obtained and was expressed as the corresponding $\text{p}K_i$. The selectivity for these two receptors was expressed by the I_2/α_2 index, calculated as the antilogarithm of the ratio between $\text{p}K_i$ values for I_2 -IR and $\text{p}K_i$ values for α_2 -AR. Competitive experiments against [^3H]2-BFI were biphasic for some of the compounds (Table 5).

Basing ourselves results, we compared the $\text{p}K_i$ I_2 affinity displayed by **B24** [27] of 5.74 with its reduced analogous **B24-red**, which depicted a negligible affinity upon these receptors ($\text{p}K_i = 3.32$). As previously reported; **B06**, the *N*-3Cl,4F-phenyl and the α -phenylsubstituted analogue fitted significantly better into a two-site binding model, with a high $\text{p}K_i$ I_2 8.61 ($K_{\text{IH}} = 2.45$ nM) and a low $\text{p}K_i$ I_2 4.29 ($K_{\text{IL}} = 51.2$ μM), with the high-affinity site accounting for a calculated 37 % of the specific binding of the [^3H]2-BFI at 2 nM concentration. Its reduced congener **B06-red** showed an outstanding value fitted into two sites with a $\text{p}K_{\text{IH}}$ of 9.81 and a 50 % occupancy, but a loss of selectivity versus α_2 -AR. Therefore, the reduction of the imine functional group did not represent an improvement in the affinity values of the two structures considered. Next, we undertook the pharmacological evaluation of the compounds resulting from the addition of an indole to the C-imine. **BIN01** presented an I_2 -IR affinity of $\text{p}K_i = 7.46$, in a similar value range to its partner **B02** ($\text{p}K_i = 7.73$). Neither compound showed selectivity upon α_2 -AR. It was worth noting that when comparing non α_2 -AR selective **B16** ($\text{p}K_i = 10.28$) with **BIN02**, a decrease in the I_2 -IR affinity occurred (7.31), which was still in the range of non-selective idazoxan ($\text{p}K_i = 7.41$), but exhibiting a remarkable selectivity index (5012). The affinity of **BIN05** fitted into a two-fold curve with values within the same range ($\text{p}K_{\text{IH}}$ 8.18 and $\text{p}K_{\text{IL}}$ 3.56) of the affinity value for **B06**, but with a lower occupancy of the high-affine site (21 % vs 37 % for **B06**) and a lack of selectivity upon α_2 -AR. **B53** lacked I_2 -IR activity and its 3-indol substituted partner **BIN06** showed $\text{p}K_{\text{IH}} = 7.77$ activity but with only a 17 % of high-affinity site-occupancy. Therefore, the introduction of an indol substituent to

position 3 of the bicyclic system did not have beneficial outcomes in the affinity values.

Finally, the trisubstituted pyrrolidine **PIP-BIN02** did not reveal interesting biological properties as I_2 -IR ligand.

2.4. BBB permeation assay

Considering that the I_2 -IR are in the CNS, ease to cross the BBB is an essential requirement for I_2 -IR ligands in development with potential therapeutic applications in the neuroprotective field. Therefore, the *in vitro* permeability (P_e) of representative compounds from the novel bicyclic families was determined by PAMPA-BBB permeability assay (Table 6).

Seeing the limits established by Di et al. for BBB permeation [50], the compounds **B24-red**, **B06-red**, **BIN02** and **BIN06** were well above the threshold established for high BBB permeation ($\text{P}_e > 5.198 \times 10^{-6}$ cm s^{-1}). Compounds **BIN01**, **BIN05** and **PIP-BIN02** were predicted to have an uncertain BBB permeation ($\text{CNS } +/ -$): $5.198 > \text{P}_e (10^{-6} \text{ cm s}^{-1}) > 2.054$ but they were considered suitable to undergo further *in vitro* and *in vivo* studies to gain more in-depth insights about the pharmacological profile of the new family of I_2 -IR ligands.

2.5. Effects of BIN02, BIN05, and B06-red on preclinical models of neurodegeneration *in vitro*

2.5.1. Neuroprotective role on Alzheimer's and Parkinson's cellular models

Neurons and glial cells (astrocytes and microglia) cultivated *in vitro* in static devices, such as *trans*-well systems, are useful tools for basic linear kinetic studies during drug discovery. The HT-22 mouse hippocampal neuronal cell line is currently recognized as a valuable experimental model for the study of glutamate-induced neurotoxicity associated with AD [51]. Since human neuronal dopaminergic cell line SH-SY5Y shares many characteristics with substantia nigra neurons, it has been established as an appropriate system for simulating the properties of dopaminergic neurons *in vitro* [52]. To assess the possible cytotoxic effect of the compounds, *per se*, we exposed cells to various concentrations of these compounds, ranging from 200 nM to 20 μM . None of the concentrations applied had harmful effects on the cells (Fig. 6).

We then assessed whether the compounds were able to prevent neuronal death caused by exposure to cytotoxic agents, glutamate for HT-22 hippocampal cells, and 6-OHDA for SH-SY5Y dopaminergic neurons (Fig. 7A). Two well-known I_2 -IR ligands, idazoxan and 2-BFI (Fig. 1), were considered standard treatments for reference purposes [53]. A cytotoxicity analysis and the effective dose of idazoxan and 2-BFI were previously reported [27]. Qualitatively, our findings reveal that these compounds showed a potent neuroprotective effect in both neuronal *in vitro* models, being able to highly prevent toxin-induced neuronal death.

2.5.2. *In vitro* evaluation of the anti-inflammatory effect

The extensive and protracted neuroinflammatory process that the CNS experiences as a result of the disease is one of the key causes of

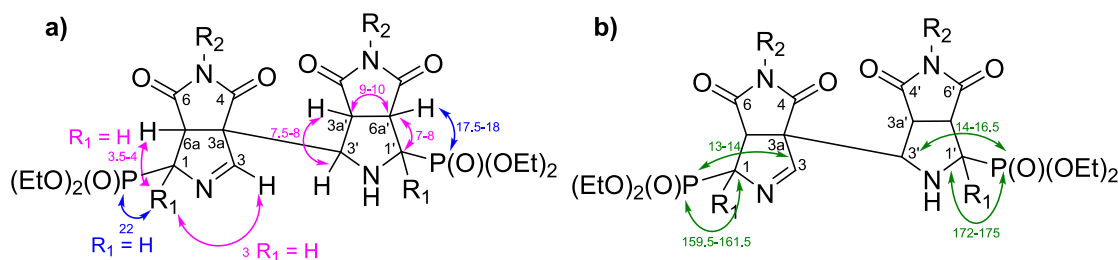


Fig. 5. Representative coupling constants: a) ^1H - ^1H (pink), ^1H -P (blue), and b) ^{13}C -P (green) for **BxB03**/ **BxB04**, **BxB01**/ **BxB02** and **BxB05**/ **BxB06**. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5

I₂-IR and α_2 -AR Binding Affinities (pK_i) of compound idazoxan, previously reported **B24**, **B06**, **B02**, **B16**, **B53** and new compounds **B24-red**, **B06-red**, **BIN01**, **BIN02**, **BIN05**, **BIN06** and **PIP-BIN02**.

Compound	^a [³ H] 2-BFI I ₂ pK _i (one site) ^b [³ H] -2-BFI I ₂ pK _i (two sites: pK _{iH} /K _{iL}) High affinity site %	[³ H]-RX821002 α_2 pK _i	Selectivity I ₂ / α_2
Idazoxan	7.41 ± 0.63	8.35 ± 0.16	
^cB24	5.74 ± 0.51	5.02 ± 0.58	–
B24-red	3.32 ± 0.67	5.38 ± 0.33	–
^cB06	8.56 ± 0.32 8.61 ± 0.28/4.29 ± 0.20 37 ± 4	6.27 ± 0.56	195
B06-red	6.88 ± 0.29 9.81 ± 0.21/<3 50 ± 4	6.66 ± 0.49	1.7
^cB02	7.73 ± 0.19	8.49 ± 0.36	–
BIN01	7.46 ± 0.41	7.87 ± 0.57	–
^cB16	10.28 ± 0.37	10.38 ± 0.22	–
BIN02	7.31 ± 0.39	3.61 ± 0.32	5012
BIN05	3.89 ± 0.19 8.18 ± 0.43/3.56 ± 0.22 21 ± 3	5.51 ± 0.24	–
^cB53	<3	–	–
BIN06	5.89 ± 0.30 7.77 ± 0.36/<3 17 ± 2	6.19 ± 0.26	–
PIP-BIN02	3.59 ± 0.26 8.05 ± 0.29/<3 9 ± 2	6.62 ± 0.22	–

^a Selectivity I₂-IR/ α_2 -AR expressed as the antilog (pK_i I₂-IR – pK_i α_2 -AR). ^b The best fit of the data for most of the compounds was a two-site binding model with high pK_i (pK_{iH}) and low pK_i (pK_{iL}) affinities for both binding sites, respectively. ^c Values published by our group [27].

Table 6

Permeability results (Pe 10^{–6} cm s^{–1}) from the PAMPA-BBB assay for newly reported compounds and their prediction of BBB permeation.

Compound	^a Pe 10 ^{–6} cm s ^{–1}	^b Prediction
B24-red	7.1 ± 0.4	CNS+
B06-red	10.2 ± 0.4	CNS+
BIN01	3.1 ± 0.1	CNS+/-
BIN02	8.8 ± 0.4	CNS+
BIN05	5.0 ± 0.2	CNS+/-
BIN06	9.4 ± 0.9	CNS+
PIP-BIN02	4.9 ± 0.3	CNS+/-

^a PBS/EtOH (70:30) was used as solvent. Values are expressed as mean ± SD of at least three independent experiments.

^b The *in vitro* permeability (Pe) of fourteen commercial drugs through lipid extract of porcine brain membrane together with the test compounds were determined (for the commercial drug values see Table in the supporting information).

neurodegenerative diseases progression. Therefore, determining whether the compounds could mitigate inflammation was our next goal. A neurotoxic effect induced by glutamate in HT-22 hippocampal neurons and by 6-OHDA in dopaminergic neurons typically causes strong inflammatory activity, in addition to cytotoxicity. Thus, we next investigated the possible anti-inflammatory effects of the compounds by examining the nitrite generation in the cultures exposed to the neurotoxin (Fig. 7B). The treatment with **BIN02**, **BIN05**, and **B06-red** considerably reversed the increase in nitrite generation evoked by the toxins' treatment, supporting the findings from the cell viability assays and pointing to a clear anti-inflammatory effect of the compounds. Notably, all the studied compounds outperformed other I₂-IR ligands, idazoxan and 2-BFI, in terms of its ability to reduce inflammation.

Since the main regulators of the inflammatory response in nervous

tissue are glial cells, we next evaluated the role of the compounds in an *in vitro* neuroinflammation model. Interestingly, I₂-IR were found throughout the brain, mainly in glial cells [51], suggesting that this receptor is crucial in neuroinflammation. Therefore, we conducted studies in several cell-based assays that partially imitate the neuro-inflammatory process in AD and PD in order to better assess the role of compounds in inflammatory reactions. For this purpose, we used two established glial lines widely used as a cellular model of neuro-inflammation, the murine-derived BV2 (microglia) and C6 (astroglia) cells. To enhance this research, we also used primary cultures of mouse cortex-isolated glial cells. In response to a pro-inflammatory agent, such as a LPS, cells released nitrite into the culture medium (Fig. 8). Then, production of nitrites was used to assess the possible anti-inflammatory action of the indicated compounds. Results indicate that **BIN02**, **BIN05** and **B06-red** exhibit a potent anti-inflammatory effect on all glial cultures evaluated in comparison with LPS-treated cultures. Interestingly, cultures treated with these compounds showed lower amount of nitrites when compared to the I₂-IR standard ligands idazoxan and 2-BFI.

2.6. *In vivo* efficacy of 2-BFI, B06-red, BIN02, BIN05 on transgenic AD C. Elegans model

The pharmacological impact of the well-established I₂-IR ligand 2-BFI and the selected **B06-red**, **BIN02**, and **BIN05** was evaluated by counting the number of thrashes in CL2006 *C. elegans* strain. Interestingly, all treatments showed a higher number of thrashes in recovery to the vehicle group. In the case of 2-BFI and **BIN05** groups, the increase in the number of thrashes was not statistically significant. On the other, both treatments, **BIN02** and **B06-red**, showed higher number of thrashes, being at least 2-fold more compared to the CL2006 Control group (Fig. 9). Thus, to our knowledge this is the first time, we showed cognitive improvement in *C. elegans* after I₂-IR ligands treatment, suggesting the participation of I₂-IR in the pathogenesis of the cognitive deficits presented in AD.

3. Conclusions

As one of the leading causes of disability worldwide, neurodegenerative disorders have enormous medical, social, and economic implications. Different mechanisms have been involved in the pathogenesis of neurodegenerative diseases, such as oxidative stress, protein aggregation, mitochondrial dysfunction, or excitotoxicity. However, one of the most important events affecting both AD and PD is undoubtedly neuroinflammation. Currently, many efforts are being made towards the development of new treatments that protect neurons and mitigate glial activity, reducing the production of proinflammatory agents, which in turn increases neuronal survival. In this context and supported by previous successful experiences in the exploration of structurally new imidazoline I₂-IR ligands, we have proposed I₂-IR as promising therapeutic target to face neurodegenerative diseases. Here, we examine the synthetic possibilities of bicyclic α -iminophosphonates considering the characteristics of the imine group and the carbon C3a, accessing unprecedented structures that were unequivocally described from the stereochemical point of view and characterized by its affinity/selectivity upon I₂-IR in human brain tissues. We selected three representative compounds, **BIN02**, **BIN05** and **B06-red**, and we demonstrate how I₂-IR ligands, are able to protect neurons from cytotoxic damage in two well-known preclinical models of AD and PD. In addition, these compounds markedly reduce the proinflammatory activity resulting from glial cell activation. Note that, we are reporting the first *in vivo* proof-of-concept of the treatment of *C. elegans* with I₂-IR ligands. Our representative compounds rescue the neurodegenerative condition presented by CL2006 strain at the behavioural phenotype level. Consequently, these results may constitute the basis for considering I₂-IR as anti-AD/PD targets and I₂-IR ligands as future therapeutic hits against these neurodegenerative diseases.

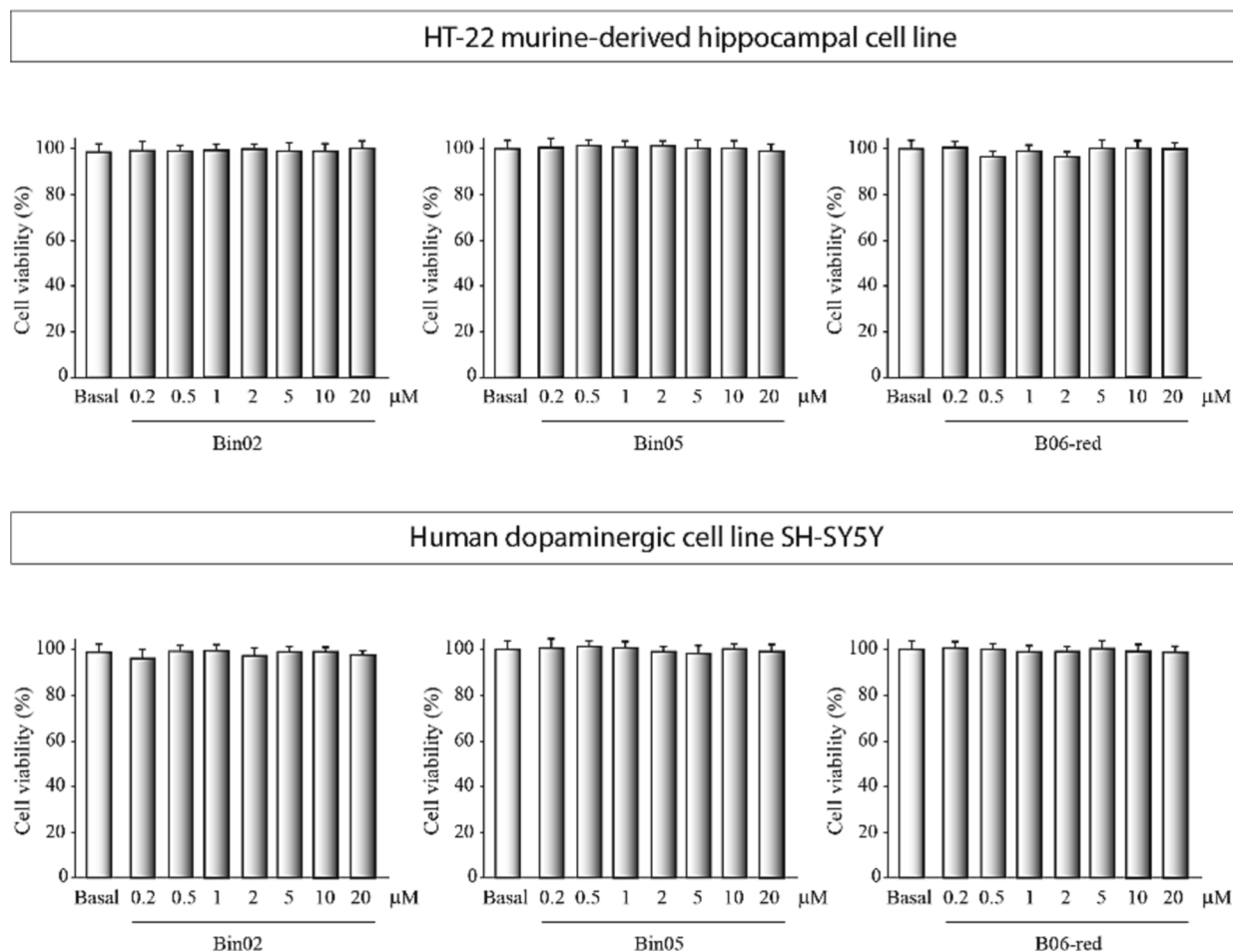


Fig. 6. *In vitro* I₂-IR ligands cytotoxicity analysis. Hippocampal murine-derived HT-22 and SH-SY5Y human dopaminergic cell lines were exposed for 24 h to increasing concentrations of the indicated compounds. A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTT) test was used to evaluate I₂-IR ligands' cytotoxic potential. Values are the mean \pm SD from triplicate determinations repeated at least three times. After confirming the significance of the results with an analysis of variance, a significance level of $p < 0.05$ was applied to the post hoc statistical analyses (Tukey test).

4. Experimental section

4.1. Chemistry

Reagents, solvents and starting products were acquired from commercial sources. The organic layers were dried over MgSO₄. The term "concentration" refers to the vacuum evaporation using a Büchi rotavapor. When indicated, the reaction products were purified by "flash" chromatography on silica gel (35–70 μ m) with the indicated solvent system. IR spectra were performed in a Spectrum Two FT-IR Spectrometer, and only noteworthy IR absorptions (cm⁻¹) are listed. NMR spectra were recorded in CDCl₃ and DMSO-*d*₆ at 400 MHz (¹H) and 100.6 MHz (¹³C), and chemical shifts are reported in δ values downfield from TMS or relative to residual chloroform (7.26 ppm, 77.0 ppm) and DMSO-*d*₆ (2.50 ppm, 39.5 ppm) as an internal standard. Data are reported in the following manner: chemical shift, multiplicity, coupling constant (*J*) in hertz (Hz) and integrated intensity. Multiplicities are reported using the following abbreviations: s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet doublet of doublets; dq, double quadruplet; dt, double of triplet; dtd, double triplet doublet; q, quadruplet; t, triplet; m, multiplet; br s, broad signal, * refers to interchangeable peaks. The accurate mass analyses were carried out using a LC/MSD-TOF spectrophotometer. HPLC-MS (Agilent 1260 Infinity II) analysis was conducted on a Poroshell 120 EC-C15 (4.6 mm \times 50 mm, 2.7 μ m) at 40 °C with mobile phase A (H₂O + 0.05 % formic acid) and B (ACN + 0.05 % formic acid) using a gradient elution and flow rate 0.6

mL/min. The DAD detector was set at 254 nm, the injection volume was 5 μ L, and oven temperature was 40 °C. All tested compounds possess a purity of at least 95 %. The microwave oven used is BI-356006Biotage® Initiator + Microwave System EU, NET INTERLAB S.A.

4.1.1. Diethyl ((1*RS*,3*aRS*,6*aRS*)-5-(3-chloro-4-fluorophenyl)-4,6-dioxo-1-phenyloctahydropyrrolo[3,4-*c*]pyrrol-1-yl)phosphonate hydrochloride (**B06-red.HCl**)

To a solution of CH₃CN (3.7 mL) and H₂O (156 μ L), **B06** (150 mg, 0.31 mmol) was added, and the mixture was stirred for 10 min. Then, NaBH₃CN (39 mg, 0.62 mmol) and AcOH (156 μ L) were added, and the mixture was stirred for 1 h at rt. The solvent was evaporated, EtOAc was added to the residue and the resulting solution was washed with a saturated solution of NaHCO₃. The organic phase was dried and concentrated. The crude was purified by column chromatography (EtOAc) and the residue was suspended in CH₂Cl₂ and a solution of HCl-EtOH 1.25 M to give **B06-red.HCl** (125 mg, 84 %) as a white solid. M.p. 125–127°C (EtOAc). IR (NaCl) 3319, 2983, 2404, 1711, 1499, 1183, 1030, 960, 700 cm⁻¹. ¹H NMR (500 MHz, CDCl₃) δ 1.13 (t, *J* = 7.0 Hz, 3H, CH₂CH₃), 1.31 (t, *J* = 7.0 Hz, 3H, CH₂CH₃), 3.63 (td, *J* = 8.0, 2.5 Hz, 1H, H-3), 3.74 (dd, *J* = 2.5, 10.5 Hz, 1H, H-3), 3.78–3.88 (complex signal, 2H, H-3a and CH₂CH₃), 3.96–4.20 (m, 4H, CH₂CH₃ and H-6a), 6.61 (ddd, *J* = 2.5, 4.0, 9.0 Hz, 1H, ArH), 6.66 (dd, *J* = 6.5, 2.5 Hz, 1H, ArH), 7.04 (t, *J* = 8.5 Hz, 1H, ArH), 7.30–7.41 (m, 3H, ArH), 7.74–7.83 (m, 2H, ArH). ¹³C NMR (126 MHz, CDCl₃) δ 16.2 (d, *J* = 5.5 Hz, CH₂CH₃), 16.4 (d, *J* = 5.5 Hz, CH₂CH₃), 48.1 (d, *J* = 3.5 Hz, C-3), 48.2

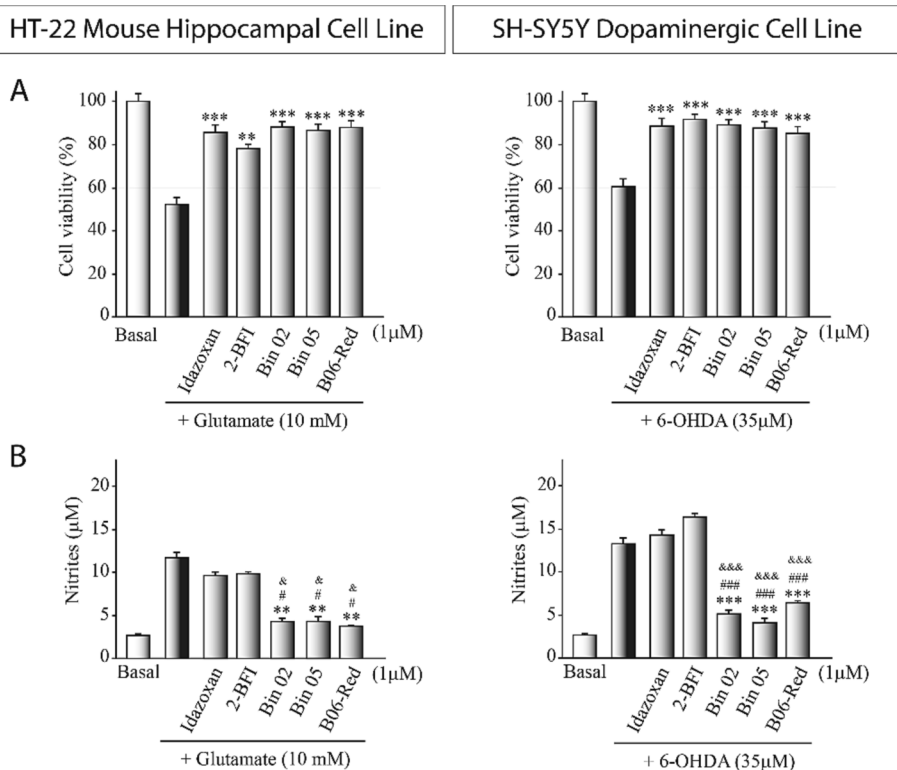


Fig. 7. *In vitro* I₂-IR ligand phenotypic screening: neuroprotective and anti-inflammatory effect. HT-22 hippocampal murine-derived and SH-SY5Y human dopaminergic cell lines were exposed to glutamate (10 μM) and 6-hydroxydopamine (6-OHDA, 35 μM), respectively, for 16 h. Previously, cultures were treated with the tested compounds at 1 μM. A) Cell viability was determined by MTT assay. B) Nitrite production was measured by the Griess reaction in the supernatant of the cells. Values represent the mean ± SD from triplicate determinations repeated at least three times. After confirming the significance of the results with an analysis of variance, a significance level of $p < 0.05$ was applied to the post hoc statistical analyses (Tukey test). ** $p \leq 0.01$, *** $p \leq 0.001$ versus glutamate- or 6-OHDA-treated cultures; # $p \leq 0.05$, ### $p \leq 0.001$ versus idazoxan-treated cultures; & $p \leq 0.5$, && $p \leq 0.001$ versus 2-BFI-treated cultures.

(d, $J = 3.5$ Hz, C-3a), 52.5 (d, $J = 3.5$ Hz, C-6a), 63.5 (d, $J = 8.0$ Hz, CH₂CH₃), 64.2 (d, $J = 7.5$ Hz, CH₂CH₃), 69.8 (d, $J = 149.5$ Hz, C-1), 116.7 (d, $J = 22.5$ Hz, CHAr), 121.4 (d, $J = 19.0$ Hz, C-*ipso*), 126.2 (d, $J = 7.5$ Hz, CHAr), 127.8 (d, $J = 4.0$ Hz, CHAr), 128.0 (d, $J = 2.0$ Hz, 2CHAr), 128.3 (d, $J = 4.5$ Hz, 2CHAr), 128.7 (CHAr), 134.4 (d, $J = 3.5$ Hz, C-*ipso*), 156.6 (C-*ipso*), 158.6 (C-*ipso*), 173.5 (d, $J = 9.0$ Hz, CO), 176.6 (CO). HRMS C₂₂H₂₄ClFN₂O₅P [M + H]⁺ 481.1090; found, 481.1095.

4.1.2. Diethyl ((1*RS*,3*RS*,3*aRS*,6*aRS*)-3-(1*H*-indol-3-yl)-4,6-dioxo-5-phenyloctahydropyrrolo[3,4-*c*]pyrrole-1-yl)-1-phosphonate (**BIN01**)

To a mixture of **B02** (100 mg, 0.29 mmol) and AgBF₄ (83 mg, 0.43 mmol) in anhydrous THF (1.2 mL) under argon atmosphere was added a solution of indole (67 mg, 0.57 mmol) in anhydrous THF (2.6 mL). The mixture was stirred at room temperature for 24 h, and AgBF₄ (111 mg, 0.57 mmol) and indole (67 mg, 0.57 mmol) were added. Then, the reaction mixture was stirred for additional 48 h, filtered through a plug of Celite® with the aid of CH₂Cl₂, and washed with a saturated aqueous solution of NH₄Cl. The organic layer was dried, and the solvent evaporated. The resulting residue was purified by column chromatography (EtOAc/MeOH, 95:5) to afford **BIN01** (74 mg, 56 %) as grey foam. M.p. 156–158°C (EtOAc). IR (NaCl) 3429, 2982, 1716, 1597, 1382 cm⁻¹. ¹H NMR (400 MHz, CDCl₃, COSY, HETCOR) δ 1.37 (t, $J = 7.0$ Hz, 3H, OCH₂CH₃), 1.41 (t, $J = 7.0$ Hz, 3H, OCH₂CH₃), 2.60 (br s, 1H, NH), 3.68 (dd, $J = 9.5, 8.0$ Hz, 1H, H-3a), 3.78 (t, $J = 7.0$ Hz, 1H, H-1), 3.96 (ddd, $J = 18.0, 9.5, 7.0$ Hz, 1H, H-6a), 4.24–4.32 (m, 4H, OCH₂CH₃), 4.59 (d, $J = 8.0$ Hz, 1H, H-3), 7.12 (m, 1H, ArH), 7.17 (m, 1H, ArH), 7.25–7.49 (m, 7H, ArH), 7.89 (d, $J = 6.0$ Hz, 1H, ArH), 8.53 (br s, 1H, NH). ¹³C NMR (101 MHz) δ 16.4 (OCH₂CH₃), 16.5 (OCH₂CH₃), 48.6 (d, $J = 2.0$ Hz, C-6a), 52.6 (d, $J = 7.5$ Hz, C-3a), 57.0 (d, $J = 165.5$ Hz, C-1), 61.4 (d,

$J = 17.0$ Hz, C-3), 63.1 (d, $J = 6.5$ Hz, OCH₂CH₃), 63.4 (d, $J = 6.5$ Hz, OCH₂CH₃), 111.5 (CHIn), 114.0 (C-2aIn), 119.7 (CHIn), 119.8 (CHIn), 122.4 (CHIn), 123.0 (CHIn), 125.6 (C2bIn), 126.4 (2CHAr), 128.7 (CHPh), 129.2 (2CHPh), 131.6 (C-*ipso*), 136.8 (C6aIn), 175.1 (CO), 175.3 (CO). MS-EI m/z 467 M⁺ (23), 358 (24), 329 (100), 294 (15), 183 (33), 156 (34), 175 (11). HRMS C₂₄H₂₇N₃O₅P [M + H]⁺ 468.1684; found, 468.1683.

4.1.3. Diethyl ((1*RS*,3*RS*,3*aRS*,6*aRS*)-3-(1*H*-indol-3-yl)-4,6-dioxo-1,5-diphenyloctahydropyrrolo[3,4-*c*]pyrrole-1-yl)phosphonate (**BIN02**)

To a mixture of **B16** (50 mg, 0.117 mmol) and AgBF₄ (34 mg, 0.176 mmol) in anhydrous THF (2 mL) was added indole (27 mg, 0.23 mmol) under argon atmosphere. The mixture was stirred at room temperature for 48 h. Then, the solvent was evaporated under reduced pressure and the resulting residue was purified by column chromatography (CH₂Cl₂/MeOH, 99:1) to afford **BIN02** (54 mg, 85 %) as white solid. M.p. 123–125 °C (EtOAc). IR (NaCl) 3303, 2923, 1711, 1375, 1178, 1016, 970, 740, 702 690 cm⁻¹. ¹H NMR (400 MHz, CDCl₃, COSY, HETCOR) δ 1.15 (t, $J = 7.0$ Hz, 3H, OCH₂CH₃), 1.39 (t, $J = 7.0$ Hz, 3H, OCH₂CH₃), 3.57 (br s, 1H, NH), 3.88 (ddd, $J = 9.5, 5.5, 1.0$ Hz, 1H, H-3a), 3.94–4.18 (m, 2H, OCH₂CH₃), 4.31 (q, 2H, OCH₂CH₃), 4.47 (dd, $J = 15.0, 9.5$ Hz, 1H, H-6a), 5.27 (m, 1H, H-3), 6.54–6.76 (m, 2H, ArH), 7.16–7.26 (m, 2H, ArH), 7.28–7.34 (m, 4H, ArH), 7.34–7.46 (m, 4H, ArH), 7.78–7.95 (m, 2H, ArH), 8.01 (d, $J = 8.5$ Hz, 1H, ArH), 8.47 (br s, 1H, NH). ¹³C NMR (101 MHz) δ 16.3 (d, $J = 6.0$ Hz, OCH₂CH₃), 16.6 (d, $J = 6.0$ Hz, OCH₂CH₃), 55.9 (d, $J = 10.0$ Hz, C-3a), 56.8 (C-6a), 58.2 (d, $J = 15.0$ Hz, C-3), 63.6 (d, $J = 6.5$ Hz, OCH₂CH₃), 64.6 (d, $J = 7.5$ Hz, OCH₂CH₃), 70.5 (d, $J = 156.5$ Hz, C-1), 111.8 (CHAr), 116.0 (C-*ipso*), 119.5 (CHAr), 120.2 (2CHAr), 122.4 (CHAr), 122.8 (CHAr), 125.4 (C-*ipso*), 126.7 (2CHAr), 128.2 (d, $J = 4.0$ Hz, 2CHAr), 128.4 (d, $J = 2.0$ Hz, CHAr),

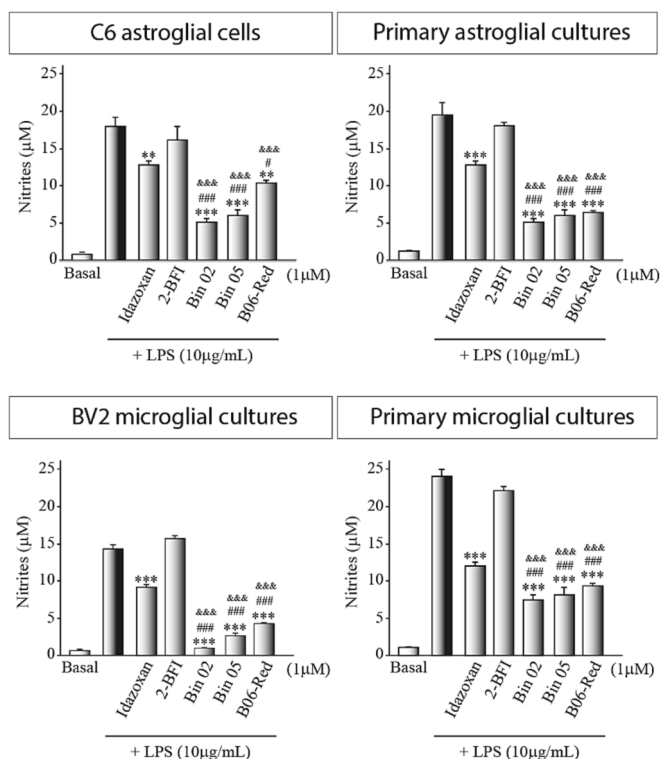


Fig. 8. *In vitro* anti-inflammatory effect of BIN02, BIN05 and B06-red. The Griess reaction was used to assess nitrite generation in the supernatant of glial cells after exposure during 24 h to lipopolysaccharide (LPS, 10 µg/mL) in the presence of the indicated compounds at 1 µM. Values represent the mean \pm SD from triplicate determinations repeated at least three times. After confirming the significance of the results with an analysis of variance, a significance level of $p < 0.05$ was applied to the post hoc statistical analyses (Tukey test). ** $p \leq 0.01$, *** $p \leq 0.001$ versus LPS-treated cultures; # $p \leq 0.05$, ## $p \leq 0.001$ versus idazoxan-treated cultures; &&& $p \leq 0.001$ versus 2-BFI-treated cultures.

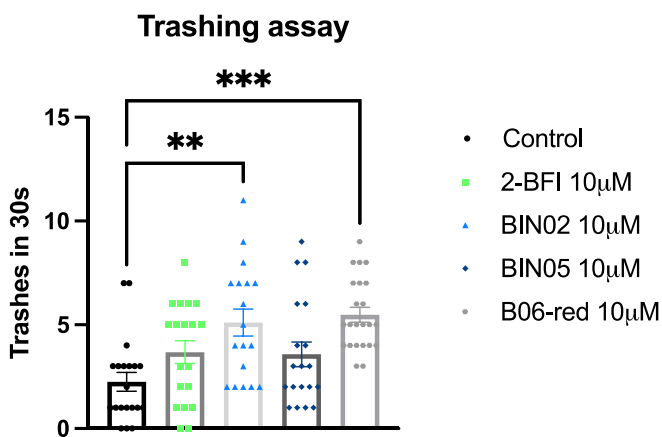


Fig. 9. Study of motility as estimated by thrashing assay ($n = 20$ – 30 worms/group; One-Way ANOVA and post-hoc Tukey's test: ** $p < 0.01$; *** $p < 0.001$). Values represented are mean \pm SEM.

128.8 (2CHAR), 129.1 (2CHAR), 131.5 (C-*ipso*), 134.9 (C-*ipso*), 137.1 (C-*ipso*), 172.9 (CO), 176.2 (CO). HRMS $C_{30}H_{31}N_3O_5P$ [M + H]⁺ 544.1996; found, 544.2006. Purity 96.41 % ($t_R = 4.01$).

4.1.4. Diethyl ((1*RS*,3*aRS*,6*aRS*)-5-(3-chloro-4-fluorophenyl)-3-(1*H*-indol-3-yl)-4,6-dioxo-1-phenyloctahydropyrrolo[3,4-*c*]pyrrol-1-yl)phosphonate (BIN05)

To a mixture of B06 (100 mg, 0.21 mmol) and AgBF₄ (61 mg, 0.31

mmol) in anhydrous THF (5 mL) under argon atmosphere was added indole (49 mg, 0.42 mmol). The mixture was stirred at room temperature for 48 h. Then, the solvent was evaporated under reduced pressure and the resulting residue was purified by column chromatography (CH₂Cl₂/MeOH, 99:1) to afford BIN05 (80 mg, 64 %) as white solid. M. p. 144–147°C (EtOAc). IR (ATR) 2989, 2925, 1713, 1496, 1377, 1237, 1168, 1018, 968, 742 cm⁻¹. ¹H NMR (400 MHz, CDCl₃) δ 1.15 (t, $J = 7.0$ Hz, 3H, OCH₂CH₃), 1.38 (t, $J = 6.7$ Hz, 3H, OCH₂CH₃), 3.56 (br s, 1H, NH), 3.83 (ddd, $J = 9.5, 5.5, 1.0$ Hz, 1H, H-3a), 3.97–4.12 (m, 2H, OCH₂CH₃), 4.23–4.36 (m, 2H, OCH₂CH₃), 4.44 (dd, $J = 14.5, 9.5$ Hz, 1H, H-6a), 5.29 (d, $J = 6.5$ Hz, 1H, H-3), 6.54–6.63 (m, 2H, ArH), 7.06 (t, $J = 8.5$ Hz, 1H, ArH), 7.16–7.28 (m, 2H, ArH), 7.30 (d, $J = 2.5$ Hz, 1H, ArH), 7.34–7.44 (m, 4H, ArH), 7.83–7.90 (m, 2H, ArH), 7.97 (d, $J = 8.0$ Hz, 1H, ArH), 8.51 (s, 1H, NH). ¹³C NMR (101 MHz, CDCl₃) δ 16.3 (d, $J = 6.0$ Hz, OCH₂CH₃), 16.6 (d, $J = 6.0$ Hz, OCH₂CH₃), 55.8 (d, $J = 10.0$ Hz, C-3a), 56.8 (C-6a), 58.3 (d, $J = 15.0$ Hz, C-3), 63.6 (d, $J = 6.5$ Hz, OCH₂CH₃), 64.6 (d, $J = 7.5$ Hz, OCH₂CH₃), 70.6 (d, $J = 156.5$ Hz, C-1), 111.8 (CHAR), 115.9 (C-*ipso*), 116.8 (d, $J = 22.5$ Hz, CHAR), 119.4 (CHAR), 120.2 (CHAR), 121.5 (d, $J = 19.0$ Hz, C-*ipso*), 122.4 (CHAR), 122.8 (CHAR), 125.2 (C-*ipso*), 126.7 (d, $J = 7.5$ Hz, CHAR), 127.8 (d, $J = 4.0$ Hz, CHAR), 128.1 (d, $J = 5.0$ Hz, CHAR), 128.6 (d, $J = 2.5$ Hz, CHAR), 128.9 (d, $J = 2.0$ Hz, 2CHAR), 129.2 (CHAR), 134.9 (C-*ipso*), 137.2 (C-*ipso*), 156.7 (C-*ipso*), 159.2 (C-*ipso*), 172.5 (CO), 175.9 (CO). HRMS $C_{30}H_{29}ClN_3O_5P$ [M + H]⁺ 596.1512; found, 596.1525. Purity 98.21 % ($t_R = 4.30$).

4.1.5. Diethyl ((1*RS*,3*aRS*,6*aRS*)-5-(3-chlorophenyl)-3-(1*H*-indol-3-yl)-4,6-dioxo-1-phenyloctahydropyrrolo[3,4-*c*]pyrrol-1-yl)phosphonate (BIN06)

To a mixture of B53 (100 mg, 0.22 mmol) and AgBF₄ (63 mg, 0.33 mmol) in anhydrous THF (5 mL) under argon atmosphere was added indole (51.5 mg, 0.44 mmol). The mixture was stirred at room temperature for 48 h. Then, the solvent was evaporated under reduced pressure and the resulting residue was purified by column chromatography (CH₂Cl₂/MeOH, 99:1) to afford BIN06 (75 mg, 59 %) as white solid. M.p. 159–161°C (EtOAc). IR (ATR) 2973, 2925, 1713, 1372, 1230, 1163, 1016, 971, 744 cm⁻¹. ¹H NMR (400 MHz, CDCl₃) δ 1.15 (t, $J = 7.0$ Hz, 3H, OCH₂CH₃), 1.38 (t, $J = 7.0$ Hz, 3H, OCH₂CH₃), 3.58 (br s, 1H, NH), 3.85 (ddd, $J = 9.5, 5.5, 1.0$ Hz, 1H, H-3a), 4.00–4.11 (m, 2H, OCH₂CH₃), 4.26–4.36 (m, 2H, OCH₂CH₃), 4.45 (dd, $J = 15.0, 9.5$ Hz, 1H, H-6a), 5.29 (d, $J = 5.5$ Hz, 1H, H-3), 6.56–6.58 (m, 1H, ArH), 6.60 (dt, $J = 7.5, 2.0$ Hz, 1H, ArH), 7.16–7.29 (m, 4H, ArH), 7.31 (d, $J = 2.5$ Hz, 1H, ArH), 7.35–7.45 (m, 4H, ArH), 7.84–7.91 (m, 2H, ArH), 7.99 (d, $J = 8.5$ Hz, 1H, ArH), 8.49 (s, 1H, NH). ¹³C NMR (101 MHz, CDCl₃) δ 16.1 (d, $J = 6.0$ Hz, OCH₂CH₃), 16.5 (d, $J = 6.0$ Hz, OCH₂CH₃), 55.7 (d, $J = 10.0$ Hz, C-3a), 56.7 (C-6a), 58.2 (d, $J = 15.0$ Hz, C-3), 63.4 (d, $J = 7.0$ Hz, OCH₂CH₃), 64.5 (d, $J = 7.5$ Hz, OCH₂CH₃), 70.4 (d, $J = 156.5$ Hz, C-1), 111.7 (2CHAR), 115.8 (C-*ipso*), 119.3 (CHAR), 120.1 (CHAR), 122.2 (CHAR), 122.7 (CHAR), 124.8 (CHAR), 125.1 (C-*ipso*), 127.0 (CHAR), 128.0 (d, $J = 5.0$ Hz, 2CHAR), 128.4 (d, $J = 3.0$ Hz, CHAR), 128.8 (d, $J = 8.5$ Hz, 2CHAR), 129.8 (CHAR), 132.4 (C-*ipso*), 134.4 (C-*ipso*), 134.7 (C-*ipso*), 137.0 (C-*ipso*), 172.4 (CO), 175.8 (CO). HRMS $C_{30}H_{29}ClN_3O_5P$ [M + H]⁺ 578.1606; found, 578.1605.

4.1.6. (2*RS*,3*RS*)-2-(1*H*-indol-3-yl)-*N*,5-diphenyl-3,4-dihydro-2*H*-pyrrole-3-carboxamide (PIP-BIN02)

A solution of BIN02 (240 mg, 0.44 mmol) in 0.05 M NaOH in THF/H₂O 2:1 (12 mL) was stirred at room temperature for 2.5 h. The mixture was concentrated *in vacuo*, water (2 mL) was added, and the pH of the mixture was acidified (pH = 3) with a 1 M solution of HCl. The reaction mixture was extracted with EtOAc, the organic phases were combined, dried, filtered, and concentrated to give a residue that was purified by column chromatography (CH₂Cl₂) to afford PIP-BIN02 (132 mg, 80 %) as a white solid. M.p. 148–149°C (EtOAc). IR 3344, 2989, 2926, 1712, 1379, 1229, 1180, 1017, 974, 741, 703 (ATR) cm⁻¹. ¹H NMR [500 MHz, DMSO] δ 3.27–3.36 (m, 1H, H-4), 3.47 (dt, $J = 9.5, 7.5$ Hz, 1H, H-3),

3.64 (ddd, $J = 17.0, 9.5, 2.0$ Hz, 1H, H-4), 5.68 (d, $J = 6.5$ Hz, 1H, H-2), 6.92 (ddd, $J = 8.0, 7.0, 1.0$ Hz, 1H, ArH), 7.01–7.11 (m, 2H, ArH), 7.25–7.34 (m, 3H, ArH), 7.39 (dd, $J = 16.5, 8.0$ Hz, 2H, ArH), 7.43–7.53 (m, 3H, ArH), 7.61 (dd, $J = 8.5, 1.0$ Hz, 2H, ArH), 7.92 (dd, $J = 8.0, 1.5$ Hz, 2H, ArH), 10.08 (s, 1H, NH), 10.97 (s, 1H, NH). ^{13}C NMR (126 MHz, $(\text{CD}_3)_2\text{SO}$) δ 40.4 (C-4), 50.6 (C-3), 73.5 (C-2), 111.0 (CHAr), 116.1 (C-*ipso*), 117.9 (CHAr), 118.4 (CHAr), 118.7 (2CHAr), 120.5 (CHAr), 121.8 (CHAr), 122.7 (CHAr), 125.4 (C-*ipso*), 127.2 (2CHAr), 128.0 (2CHAr), 128.1 (2CHAr), 130.0 (CHAr), 133.3 (C-*ipso*), 136.1 (C-*ipso*), 138.5 (C-*ipso*), 168.7 (CO), 172.3 (C-5). HRMS $\text{C}_{25}\text{H}_{22}\text{N}_3\text{O}$ [$\text{M} + \text{H}$] $^+$ 380.1754; found, 380.1757. Purity 100 % ($t_{\text{R}} = 3.35$ min).

4.1.7. Tetraethyl (1RS,3aSR,6aRS,1'SR,3'RS,3a'SR,6a'SR)-5,5'-diphenyl-4,4',6,6'-tetraoxo-1,1',2',3',3a,3a',4,4',5,5',6,6',6a,6a'-tetradecahydrobipyrrolo[3,4-c]pyrrole-1,1'-diphosphate (BxB03 and BxB04, Table 2, entry 3).

To a solution of **B02** (80 mg, 0.23 mmol) in anhydrous CH_2Cl_2 (1 mL) was added TMSCl (149 mg, 1.37 mmol). The mixture was stirred under microwave irradiation at 80 °C and 250 bar, for 30 min. The solvent was evaporated under reduced pressure and the residue was purified by silica gel flash chromatography (EtOAc/MeOH, 95:5) to afford **BxB03** (20 mg, 25 %), **BxB04** (34 mg, 43 %) and **B02** (13 mg, 16 %). **BxB03**: IR (NaCl) 3267, 2984, 1705, 1434 cm^{-1} . ^1H NMR (400 MHz, CDCl_3 , COSY, HETCOR) δ 1.33 (t, $J = 7.0$ Hz, 3H, OCH_2CH_3), 1.41 (dq, $J = 14.0, 7.0$ Hz, 9H, OCH_2CH_3), 2.74 (br s, 1H, NH), 3.69 (m, 1H, H-1'), 3.86 (ddd, $J = 18.5, 9.5, 7.5$ Hz, 1H, H-6a'), 4.10–4.41 (complex signal, 10H, OCH_2CH_3 , H-3a' and H-6a'), 4.62 (t, $J = 6.5$ Hz, 1H, H-3'), 4.82 (ddd, $J = 22.5, 4.0, 3.0$ Hz, 1H, H-1), 7.24 (d, $J = 1.5$ Hz, 1H, ArH), 7.27–7.31 (m, 2H, ArH), 7.37–7.51 (m, 7H, ArH), 7.81 (dd, $J = 4.0, 3.0$ Hz, 1H, H-3). ^{13}C NMR (101 MHz) δ 16.5 (m, OCH_2CH_3), 45.3 (C-3a'), 47.5 (C-6a'), 48.1 (d, $J = 7.0$ Hz, C-6a), 57.0 (d, $J = 175.0$ Hz, C-1'), 62.5 (d, $J = 14.5$ Hz, C-3'), 63.3 (d, $J = 7.0$ Hz, OCH_2CH_3), 63.6 (d, $J = 7.0$ Hz, OCH_2CH_3), 63.8 (d, $J = 7.5$ Hz, OCH_2CH_3), 64.5 (d, $J = 6.5$ Hz, OCH_2CH_3), 73.8 (d, $J = 159.5$ Hz, C-1), 74.7 (C-3a), 126.4 (2CHAr), 126.5 (2CHAr), 128.9 (CHAr), 129.2 (CHAr), 129.3 (2CHAr), 129.4 (2CHAr), 131.2 (C-*ipso*), 131.6 (C-*ipso*), 164.8 (d, $J = 14.0$ Hz, C-3), 174.0 (d, $J = 5.5$ Hz, CO), 174.7 (d, $J = 5.5$ Hz, CO), 175.2 (d, $J = 12.0$ Hz, CO), 175.7 (CO). HRMS $\text{C}_{32}\text{H}_{37}\text{N}_4\text{O}_{10}\text{P}_2$ [$\text{M} + \text{H}$] $^+$ 701.2136; found, 701.2126. **BxB04**: IR (NaCl) 3267, 2984, 1705, 1434 cm^{-1} . ^1H NMR (400 MHz, CDCl_3 , COSY, HETCOR) δ 1.26 (t, $J = 7.0$ Hz, 3H, OCH_2CH_3), 1.31–1.44 (m, 9H, OCH_2CH_3), 3.20 (br s, 1H, NH), 3.62 (m, 1H, H-1'), 3.81 (ddd, $J = 18.0, 10.0, 8.0$ Hz, 1H, H-6a'), 3.92 (dd, $J = 7.0, 5.0$ Hz, 1H, H-3'), 4.14 (m, 1H, H-6a'), 4.16–4.33 (m, 8H, OCH_2CH_3), 4.57 (dd, $J = 9.5, 7.5$ Hz, 1H, H-3a'), 4.89 (dt, $J = 22.0, 3.0$ Hz, 1H, H-1), 7.21–7.29 (m, 4H, ArH), 7.40 (m, 2H, ArH), 7.45 (m, 4H, ArH), 8.04 (dd, $J = 4.5, 3.0$ Hz, 1H, H-3). ^{13}C NMR (101 MHz) δ 16.5 (OCH_2CH_3), 16.61 (OCH_2CH_3), 46.8 (C-6a'), 47.4–47.5 (C-3a' and C-6a'), 56.6 (d, $J = 173.5$ Hz, C-1'), 63.1 (d, $J = 6.5$ Hz, OCH_2CH_3), 63.8 (d, $J = 16.5$ Hz, C-3'), 63.9 (d, $J = 6.5$ Hz, OCH_2CH_3), 64.3 (d, $J = 6.5$ Hz, OCH_2CH_3), 70.9 (C-3a), 74.7 (d, $J = 160.0$ Hz, C-1), 126.5 (2CHAr), 126.5 (2CHAr), 129.0 (CHAr), 129.2 (CHAr), 129.3 (2CHAr), 129.4 (2CHAr), 131.1 (C-*ipso*), 131.5 (C-*ipso*), 164.4 (d, $J = 13.0$ Hz, C-3), 173.7 (d, $J = 6.0$ Hz, CO), 174.5 (d, $J = 4.5$ Hz, CO), 174.7 (d, $J = 11.5$ Hz, CO), 175.6 (CO). HRMS $\text{C}_{32}\text{H}_{37}\text{N}_4\text{O}_{10}\text{P}_2$ [$\text{M} + \text{H}$] $^+$ 701.2136; found, 701.2131.

4.1.8. Tetraethyl (1RS,3aSR,6aRS,1'SR,3'RS,3a'SR,6a'SR)-5,5'-dimethyl-4,4',6,6'-tetraoxo-1,1',2',3',3a,3a',4,4',5,5',6,6',6a,6a'-tetradecahydrobipyrrolo[3,4-c]pyrrole-1,1'-diphosphate (BxB01 and BxB02, Table 2, entry 7).

To a solution of **B03** (100 mg, 0.346 mmol) in anhydrous CH_2Cl_2 (1 mL) was added TMSCl (225.61 mg, 2.08 mmol). The mixture was stirred under microwave irradiation at 80 °C and 250 bar for 30 min. The solvent was evaporated under reduced pressure and the residue was purified by silica gel flash chromatography (EtOAc/MeOH, 95:5) to afford **BxB01** (24 mg, 24 %), **BxB02** (34 mg, 34 %) and **B03** (3 mg, 3 %). **BxB01**: IR (NaCl) 3466, 2984, 1705, 1435 cm^{-1} . ^1H NMR (400 MHz,

CDCl_3 , COSY, HETCOR) δ 1.34 (dt, $J = 7.0, 14.0$ Hz, 9H, OCH_2CH_3), 1.42 (t, $J = 7.0$ Hz, 3H, OCH_2CH_3), 2.53 (br s, 1H, NH), 2.94 (s, 3H, NCH_3), 2.96 (s, 3H, NCH_3), 3.46 (m, 1H, H-1'), 3.61 (ddd, $J = 17.5, 9.0, 8.0$ Hz, 1H, H-6a'), 3.91 (dd, $J = 18.5, 4.0$ Hz, 1H, H-6a'), 3.97 (m, 1H, H-3a'), 4.21 (dtd, $J = 31.0, 14.5, 7.0$ Hz, 8H, OCH_2CH_3), 4.33 (m, 1H, H-3'), 4.64 (dt, $J = 22.5, 3.5$ Hz, 1H, H-1), 7.69 (m, 1H, H-3). ^{13}C NMR (101 MHz) δ 16.5 (d, $J = 6.0$ Hz, OCH_2CH_3), 16.6 (d, $J = 6.0$ Hz, OCH_2CH_3), 25.3 (NCH_3), 25.6 (NCH_3), 45.2 (C-6a), 47.3 (C-6a'), 47.9 (d, $J = 7.5$ Hz, C-3a'), 56.5 (d, $J = 174.5$ Hz, C-1'), 61.5 (d, $J = 15.5$ Hz, C-3'), 63.2 (d, $J = 7.0$ Hz, OCH_2CH_3), 63.5 (d, $J = 7.0$ Hz, OCH_2CH_3), 63.6 (d, $J = 7.5$ Hz, OCH_2CH_3), 64.4 (d, $J = 6.5$ Hz, OCH_2CH_3), 73.3 (d, $J = 160.0$ Hz, C-1), 74.7 (C-3a), 164.8 (d, $J = 13.5$ Hz, C-3), 174.8 (d, $J = 5.5$ Hz, CO), 175.6 (d, $J = 5.0$ Hz, CO), 176.1 (d, $J = 12.0$ Hz, CO), 176.6 (CO). HRMS $\text{C}_{22}\text{H}_{35}\text{N}_4\text{O}_{10}\text{P}_2$ [$\text{M} + \text{H}$] $^+$ 577.1823; found, 577.1820. **BxB02**: IR (NaCl) 3267, 2984, 1698, 1434 cm^{-1} . ^1H NMR (400 MHz, CDCl_3 , COSY, HETCOR) δ 1.32–1.39 (m, 9H, OCH_2CH_3), 1.42 (t, $J = 7.5$ Hz, 3H, OCH_2CH_3), 1.82 (br s, 1H, NH), 2.93 (br s, 1H, NCH_3), 2.94 (br s, 1H, NCH_3), 3.40 (ddd, $J = 8.0, 5.0, 3.5$ Hz, 1H, H-1'), 3.54–3.67 (complex signal, 2H, H-6a' and H-3a'), 4.02 (dd, $J = 18.0, 3.5$ Hz, 1H, H-6a'), 4.17–4.33 (complex signal, 9H, H-3' and OCH_2CH_3), 4.72 (ddd, $J = 22.0, 3.5, 2.5$ Hz, 1H, H-1), 7.92 (dd, $J = 4.5, 2.5$ Hz, 1H, H-3). ^{13}C NMR (101 MHz) δ 16.6 (d, $J = 5.5$ Hz, OCH_2CH_3), 16.7 (d, $J = 5.5$ Hz, OCH_2CH_3), 25.3 (NCH_3), 25.5 (NCH_3), 46.3 (d, $J = 8.0$ Hz, C-3a'), 47.5 (C-6a'), 47.4 (d, $J = 9.5$ Hz, C-6a), 56.2 (d, $J = 174.0$ Hz, C-1'), 63.1 (d, $J = 4.5$ Hz, OCH_2CH_3), 63.2 (d, $J = 16.0$ Hz, H-3'), 63.7 (d, $J = 7.0$ Hz, OCH_2CH_3), 63.8 (d, $J = 6.5$ Hz, OCH_2CH_3), 64.2 (d, $J = 6.5$ Hz, OCH_2CH_3), 71.1 (C-3a), 74.1 (d, $J = 160.5$ Hz, C-1), 164.4 (d, $J = 13.5$ Hz, C-3), 174.7 (d, $J = 5.5$ Hz, CO), 175.5 (d, $J = 3.5$ Hz, CO), 175.6 (d, $J = 12.0$ Hz, CO), 176.6 (CO). HRMS $\text{C}_{22}\text{H}_{35}\text{N}_4\text{O}_{10}\text{P}_2$ [$\text{M} + \text{H}$] $^+$ 577.1823; found, 577.1814.

4.1.9. Tetraethyl (1RS,3aSR,6aRS,1'SR,3'RS,3a'SR,6a'SR)-4,4',6,6'-tetraoxo-1',3,5,5'-tetraphenyl-1,1',2',3',3a,3a',4,4',5,5',6,6',6a,6a'-tetradecahydrobipyrrolo[3,4-c]pyrrole-1,1'-diphosphate (BxB05 and BxB06).

To a solution of **B16** (541 mg, 1.27 mmol) in anhydrous CH_2Cl_2 (7.5 mL) was added TMSCl (0.96 mL, 7.61 mmol). The mixture was stirred under microwave irradiation at 80 °C and 250 bar for 30 min. The solvent was evaporated under reduced pressure and the residue was purified by silica gel flash chromatography (EtOAc/MeOH, 95:5) to afford **BxB05** (292 mg, 27 %), **BxB06** (444 mg, 41 %) and **B16** (81 mg, 15 %). **BxB05**: IR (ATR) 3469, 2988, 2920, 1710, 1494, 1383, 1192, 1034, 1016, 963, 775, 732, 691 cm^{-1} . ^1H NMR (400 MHz, CDCl_3 , COSY, HETCOR) δ 1.21 (dt, $J = 9.5, 7.0$ Hz, 6H, OCH_2CH_3), 1.36 (t, $J = 7.0$ Hz, 3H, OCH_2CH_3), 1.45 (t, $J = 7.0$ Hz, 3H, OCH_2CH_3), 2.86 (d, $J = 5.5$ Hz, 1H, NH), 3.92–4.07 (m, 1H, OCH_2CH_3), 4.10–4.24 (m, 5H, OCH_2CH_3), 4.25–4.43 (complex signal, 5H, OCH_2CH_3 , H-6a, H-6a' and H-3a'), 5.28 (m, 1H, H-3'), 6.48 (ddd, $J = 17.0, 6.5, 3.0$ Hz, 4H, ArH), 7.27 (d, $J = 2.0$ Hz, 6H, ArH), 7.36 (t, $J = 5.0$ Hz, 6H, ArH), 7.52–7.63 (m, 2H, ArH), 7.67 (d, $J = 7.5$ Hz, 2H, ArH), 8.16 (d, $J = 3.5$ Hz, 1H, H-3). ^{13}C NMR (101 MHz) δ 16.4 (d, $J = 5.5$ Hz, $2\text{OCH}_2\text{CH}_3$), 16.6 (t, $J = 5.0$ Hz, $2\text{OCH}_2\text{CH}_3$), 49.1 (d, $J = 9.5$ Hz, C-3a'), 51.6 (C-6a), 53.8 (C-6a'), 60.6 (d, $J = 14.0$ Hz, C-3'), 63.5 (d, $J = 7.5$ Hz, OCH_2CH_3), 64.5 (t, $J = 7.0$ Hz, OCH_2CH_3), 64.8 (d, $J = 7.0$ Hz, OCH_2CH_3), 67.0 (d, $J = 172.0$ Hz, C-1'), 76.0 (C-3a), 85.5 (d, $J = 161.5$ Hz, C-1), 126.2 (2CHAr), 126.4 (CHAr), 127.4 (d, $J = 5.0$ Hz, 2CHAr), 128.2 (2CHAr), 128.5 (d, $J = 5.5$ Hz, 2CHAr), 128.8 (d, $J = 6.0$ Hz, 2CHAr), 128.9 (2CHAr), 129.0 (2CHAr), 129.1 (2CHAr), 129.2 (2CHAr), 131.2 (d, $J = 5.0$ Hz, 2C-*ipso*), 134.1 (d, $J = 4.5$ Hz, C-*ipso*), 135.8 (d, $J = 6.5$ Hz, C-*ipso*), 164.4 (d, $J = 14.0$ Hz, C-3), 171.5 (d, $J = 7.5$ Hz, CO), 172.3 (CO), 173.7 (d, $J = 4.5$ Hz, CO), 175.8 (CO). HRMS $\text{C}_{44}\text{H}_{47}\text{N}_4\text{O}_{10}\text{P}_2$ [$\text{M} + \text{H}$] $^+$ 853.2762; found, 853.2761. Purity 95.77 % ($t_{\text{R}} = 4.77$). **BxB06**: IR (ATR) 3247, 2976, 2932, 1704, 1497, 1383, 1365, 1252, 1227, 1026, 965, 796, 703 cm^{-1} . ^1H NMR (400 MHz, CDCl_3 , COSY, HETCOR) δ 1.19–1.33 (m, 12H, OCH_2CH_3), 3.42 (d, $J = 5.0$ Hz, 1H, NH), 4.04–4.16 (m, 3H, OCH_2CH_3), 4.16–4.30 (m, 5H, OCH_2CH_3), 4.30–4.43 (complex signal, 2H, H-6a and

H-6a'), 4.50 (t, $J = 5.5$ Hz, 1H, H-3'), 4.70 (dd, $J = 9.5, 5.0$ Hz, 1H, H-3a'), 6.56 (dd, $J = 6.5, 3.0$ Hz, 2H, ArH), 6.65 (dd, $J = 6.5, 3.0$ Hz, 2H, ArH), 7.29 (d, $J = 3.0$ Hz, 6H, ArH), 7.33–7.41 (m, 6H, ArH), 7.56–7.71 (m, 4H, ArH), 8.32 (d, $J = 4.0$ Hz, 1H, H-3). ^{13}C NMR (101 MHz) δ 16.4 (d, $J = 7.0$ Hz, $2\text{OCH}_2\text{CH}_3$), 16.6 (d, $J = 5.5$ Hz, $2\text{OCH}_2\text{CH}_3$), 49.5 (d, $J = 10.5$ Hz, C-3a'), 53.3 (d, $J = 2.5$ Hz, C-6a'), 53.9 (C-6a), 61.7 (d, $J = 15.0$ Hz, C-3'), 63.7 (d, $J = 7.5$ Hz, OCH_2CH_3), 64.2 (d, $J = 7.5$ Hz, OCH_2CH_3), 64.5 (d, $J = 7.0$ Hz, OCH_2CH_3), 65.3 (d, $J = 7.0$ Hz, OCH_2CH_3), 69.0 (d, $J = 168.0$ Hz, C-1'), 72.5 (C-3a), 86.6 (d, $J = 160.5$ Hz, C-1), 126.4 (d, $J = 18.0$ Hz, CHAr), 127.6 (d, $J = 4.5$ Hz, CHAr), 128.3 (CHAr), 128.5 (d, $J = 5.5$ Hz, CHAr), 128.8 (d, $J = 14.5$ Hz, CHAr), 129.1 (d, $J = 2.5$ Hz, CHAr), 131.3 (d, $J = 21.5$ Hz, 2C-*ipso*), 133.7 (d, $J = 3.5$ Hz, C-*ipso*), 135.1 (d, $J = 4.5$ Hz, C-*ipso*), 164.1 (d, $J = 13.5$ Hz, C-3), 171.1 (d, $J = 8.0$ Hz, CO), 172.6 (CO), 173.3 (d, $J = 4.5$ Hz, CO), 175.7 (CO). HRMS $\text{C}_{44}\text{H}_{47}\text{N}_4\text{O}_{10}\text{P}_2$ $[\text{M} + \text{H}]^+$ 853.2762; found, 853.2758.

4.2. X-ray crystallographic analysis

Crystals of **B24-red**, **BIN05**, **PIP-BIN02**, **BxB05** and **BxB06** were obtained from slow evaporation of methanol solutions. The single crystal X-Ray diffraction data set was collected at 294 K up to a max 2θ of ca. 57° on a Bruker Smart APEX II diffractometer, using monochromatic MoK α radiation $\lambda = 0.71073$ Å and 0.3° separation between frames. Data integration was performed using SAINT V6.45A and SORTAV (Blessing, 1995) in the diffractometer package. The crystal and collection data and structural refinement parameters are given in the Tables in the [supplementary data](#). The structures were solved by direct methods using SHELXT-2014 and Fourier's difference methods and refined by least squares on F2 using SHELXL-2014/7 inside the WinGX program environment [54,55]. Anisotropic displacement parameters were used for non-H atoms and the H-atoms were positioned in calculated positions and refined riding on their parent atoms. Atom coordinates are given and bond distances and hydrogen bonds. All this information is detailed in the Tables in the [supplementary data](#).

B24-red crystallized in the monoclinic $P2_1/a$ space group (Ortep in [Scheme 1](#)). The cyclohexane ring presents some orientational disorder (8 %) that has been modelled. The pyrrolidine ring presents an envelope conformation on N2, while the other pyrrolidine is flat. All the stereocenters (C1, C3a, C6a) exhibit the same quirality, either S or R, depending on the molecule in the crystal, as the space group is centrosymmetric. Molecules are connected through the N2-H2...O1 hydrogen bond (HB).

BIN05 crystallized in the triclinic $P-1$ space group (Ortep in [Scheme 2](#)). The pyrrolidine rings present envelope conformations on C1 and C2, respectively. The remaining rings appears as flat. The stereocenters C1, C2, C5, C6 exhibit quirality either S,R,R,R, respectively, or, R,S,S,S, respectively. Molecules are packed in dimers through the mutual N53-H53...O1 HB.

PIP-BIN02 crystallized in the monoclinic $C2/c$ space group with a water molecule (Ortep in [Scheme 3](#)). The rings are planar and the two stereocenters show identical quirality, either S,S or R,R. The water molecule binds to three molecules by the HB: N23-H23...O1, O1-H1...O6, O1-H2...N1.

BxB05 crystallized in the triclinic $P-1$ space group with two water molecules (Ortep in [Fig. 4](#)). In this case, the four pyrrolidine rings present twisted conformations on C1-C6a, on C3a-C6a, on N2'-C1' and on C6a'-C6', respectively. One pyrrolidine shows R,R,S quirality at the C1, C3a, C6a centers, while the other presents R,S,S quirality at the C1', C3a', C6a' sites. C3' exhibits a S quirkality. Due to the centrosymmetry of the space group, the crystal is racemic, and the fully inverted molecule is also equally present. The two water molecules bridge between themselves and with oxygens of the phosphonates through HB.

BxB06 crystallized in the monoclinic Cc space group with two molecules per asymmetric unit (Ortep in [Fig. 4](#)). Two pyrrolidines are twisted (on C1-C6a and on N2'-C1'), one is envelope on C6', while the

other appears as flat. The Flack parameter (0.04(12)) confirms the absolute configuration of the solved crystal structure, but it is observed that the two independent molecules exhibit inverse quirality at all quiral centers. Thus, also in these cases, both fully inverted molecules are equally present in the crystal. One pyrrolidine shows R,R,S quirality at the C1, C3a, C6a centers, while the other presents S,R,R quirality at the C1', C3a', C6a' sites. C3' exhibits a R quirkality. Both independent molecules are linked in pairs by several HB. The quirkality of the crystal is however an effect of the packing and not due the quirkality of the molecules themselves. In fact, this is not uncommon.

Crystallographic data for the reported structures has been deposited in the Cambridge Crystallographic Data Centre as supplementary publication, CCDC No. **B24-red** (2250189), **BIN05** (2250193), **PIP-BIN02** (2250192), **BxB05** (2250191) and **BxB06** (2250190). Copies of this information may be obtained free of charge from The Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK. Fax: þ44 1223 336 033. data_request@ccdc.cam.ac.uk. Web page: <https://www.ccdc.cam.ac.uk>.

4.3. Binding studies

4.3.1. Preparation of cellular membranes

Human brain samples were obtained at autopsy in the Basque Institute of Legal Medicine, Bilbao, Spain. Samples from the prefrontal cortex (Brodman's area 9) were dissected at the time of autopsy and immediately stored at -70°C until assay. The study was developed in compliance with policies of research and ethical review boards for postmortem brain studies.

To obtain cellular membranes (P2 fraction) the different samples were homogenized using an ultraturax in 10 volumes of homogenization buffer (0.25 M sucrose, 5 mM Tris-HCl, pH 7.4). The crude homogenate was centrifuged for 5 min at 1000 g (4°C) and the supernatant was centrifuged again for 10 min at 40,000 g (4°C). The resultant pellet was washed twice in 5 volumes of homogenization buffer and re-centrifuged in similar conditions. Protein content was measured according to the method of Bradford using BSA as standard.

4.3.2. Competition binding assays

The pharmacological activity of the compounds was evaluated through competition binding studies against the I₂-IR selective radioligand [^3H]2-BFI or the α_2 -adrenergic receptor selective radioligand [^3H]RX821002. Specific binding was measured in 0.25 mL aliquots (50 mM Tris-HCl, pH 7.5) containing 100 μg of membranes, which were incubated in 96-well plates either with [^3H]2-BFI (2 nM) for 45 min at 25°C or [^3H]RX821002 (1 nM) for 30 min at 25°C , in the absence or presence of the competing compounds (10^{-12} to 10^{-3} M, 10 concentrations).

Incubations were terminated by separating free ligand from bound ligand by rapid filtration under vacuum (1450 Filter Mate Harvester, PerkinElmer) through GF/C glass fiber filters. The filters were then rinsed three times with 300 μL of binding buffer, air-dried (120 min), and counted for radioactivity by liquid scintillation spectrometry using a MicroBeta TriLux counter (PerkinElmer). Specific binding was determined and plotted as a function of the compound concentration. Nonspecific binding was determined in the presence of idazoxan (10^{-5} M), a compound with well established affinity for I₂-IR and α_2 -adrenergic receptors, in [^3H]2-BFI and [^3H]RX821002 assays. To obtain the inhibition constant (K_i) analyses of competition experiments were performed by nonlinear regression using the GraphPad Prism program. K_i values were normalized to $\text{p}K_i$ values. I₂-IR/ α_2 selectivity index was calculated as the antilogarithm of the difference between $\text{p}K_i$ values for I₂-IR and $\text{p}K_i$ values for α_2 -AR.

4.4. Cellular models of Alzheimer's and Parkinson's diseases

4.4.1. In vitro model of neurodegeneration

4.4.1.1. Cell lines and treatments. The HT-22 mouse hippocampal neuronal cell line has been recognized as a valuable cell model for the study of neurotoxicity associated with AD. HT-22 cultures (Merck-Sigma Aldrich, SCC129) were maintained in DMEM-F12 (Gibco) containing 10 % (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin.

The SH-SY5Y cell line has been a valuable asset to help unravel the molecular complexity of PD. Cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, Ref.CRL-2266) and grown in RPMI medium (Gibco) supplemented with glutamine (2 mM), 10 % FBS and 1 % penicillin/streptomycin.

Cell lines were cultured under standard conditions (37 °C, 5 %CO₂). On attaining semiconfluence, cells were seeded on 96-well plates at 3x10⁴ per well and incubated for 24 h prior to treatment. Then, cells were treated with the indicated compounds for 24 h and viability was assessed using the MTT assay. None of the compounds tested were cytotoxic at those doses. Then, HT-22 and SH-SY5Y cultures were treated for 1 h with the compounds at 1 µM. This dose was chosen based on previous studies [28]. After that, glutamate (10 mM, Merck-Sigma) was added to the HT-22 cultures, and 6-hydroxydopamine (6-OHDA, 35 µM, Merck-Sigma) to the SH-SY5Y cultures, that were incubated for at least 16 h. As a final step, cultures were tested for viability and nitrite production.

4.4.1.2. Cell viability assay. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assay, according to manufacturer's instructions (Roche Diagnostic). Briefly, after exposure to glutamate or 6-OHDA cell culture medium was replaced with 100 µL of fresh culture medium added with MTT solution (0.5 mg/mL) for 1 h. Medium was removed and the resulting formazan crystals were dissolved in DMSO and the absorbance read at 595–650 nm in a spectrophotometer. All experiments were performed at least three times and the results were expressed as a percentage of the control.

4.4.2. In vitro model of neuroinflammation

4.4.2.1. Cell lines and treatments. The brain-derived rat C6 astroglia-like cell line (ATCC; CCL-107) was cultured in DMEM with 10 % FBS and 1 % penicillin/streptomycin in a humidified atmosphere containing 5 % CO₂ in air at 37 °C in an incubator. Cell line of murine BV2 microglia was maintained at 37 °C in DMEM added with 1 % of 100 U/mL of penicillin/streptomycin and 10 % fetal bovine serum in a humidified incubator containing 5 % CO₂. C6 and BV2 cells were cultured until 80–90 % confluent in 75 cm² flasks. To determine the inflammatory state, cells were further sub-cultured in 96-well plates at a density of 3x10⁴/well. Now, cultures were pre-treated for 1 h with the compounds **BIN02**, **BIN05** and **B06-red** at 1 µM. Then, lipopolysaccharide (10 µg/mL, Sigma) was added to the medium and cultures incubated for 24 h. Finally, nitrite production was evaluated.

4.4.2.2. Cell lines and treatments. Mouse primary astrocytes and microglia were harvested and cultured as previously described [56]. Briefly, after removal of the meninges the cerebral cortex was dissected, dissociated, and incubated with 0.25 % trypsin, EDTA at 37 °C for 1 h. Tissue was then centrifuged and the resulting pellet washed with Hanks' balanced salt solution (HBSS, Invitrogen). Cells were plated on non-coated flasks and incubated in standard conditions for at least 7 days. Now, each glial type was then isolated to create pure cultures. To that end, glial cells on culture flasks were agitated on an orbital shaker for 4 h at 240 rpm at 37 °C, the supernatant was collected, centrifuged,

and the cellular pellet containing the microglial cells resuspended in complete medium (HAMS/DMEM (1:1) containing 10 % FBS) and seeded on uncoated 96-well plates. Cells were allowed to adhere for 2 h, and the medium was removed to eliminate nonadherent oligodendrocytes. New fresh medium containing 10 ng/mL of GM-CSF was added. The remaining astroglial cells adhered to the flasks were then trypsinized, collected, centrifuged, and plated onto 96-well plates with complete medium. As determined by immunofluorescence with antibodies to Iba-1 (microglial marker) and GFAP (astrocyte marker), cultures obtained by this procedure were > 98 % pure. Afterwards, cells were treated with compounds (1 µM) for 1 h, followed by LPS (10 µg/mL) for 24 h. Then, nitrite production was measured.

4.4.2.3. Inflammation determination: Nitrites measurement. SH-SY5Y, HT-22, C6, BV2 and glial primary cultures seeded in 96-well plates were used. After treatments, cell culture supernatant (100 µL) was mixed to an equal volume of Griess reagent (1 % sulfanilamide and 0.1 % naphthyl ethylene diamine in 5 % phosphoric acid; Sigma-Aldrich) at room temperature for 15 min. Standard solution of sodium nitrite was used to determine nitrite concentrations. The absorbance was read at 492/540 nm using a microplate reader (Thermofischer). All experiments were performed at least three times.

4.5. In vivo *Caenorhabditis elegans* study

4.5.1. Study design

The overall objective of the study was to evaluate the neuro-protective effects of 2-BFI and new compounds **B06-red**, **BIN02**, **BIN05**. The first part of this study was to determine the beneficial effect of the selected compounds in the *C. elegans* through the thrashing assay. The sample size for the intervention was chosen following previous studies in our laboratory and using one of the available interactive tool (<https://www.biomath.info/power/index.html>). According to each drug assay, we used n = 120–150 or 50–70 as total for at least 3 replicates in *C. elegans*.

4.5.2. Worm strains, maintenance, and general methods

Strains used in this work and abbreviations are listed in the Table 7. All the strains were backcrossed to Bristol strain N2 (WT) five to twelve times. Standard methods were used for culturing and observing *C. elegans*, unless otherwise noted. Wild-type nematodes were propagated at 20 °C, while transgenic strains were maintained at 16 °C in a temperature-controlled incubator on solid nematode growth medium (NGM) seeded with *Escherichia coli* (*E. coli*) OP50 strain as food source. To obtain the age synchronized population of eggs, gravid adults were treated with alkaline hypochlorite solution (0.5 M NaOH, ~2.6 % NaClO) for 5–7 min. Fertilized eggs were suspended in S-medium for 12 h and L1 larvae were allowed to hatch overnight in the absence of food.

4.5.3. Compounds preparation and treatment

The compounds were dissolved in 100 % DMSO. Each concentration was then dissolved in MilliQ purified water to achieve a final concentration of 10 µM in 1 % DMSO in well. For the thrashing assay, treatments were carried out in liquid culture for 4 days at 20 °C. Each well contained a final volume of 60 µL, consisting of 25–30 animals in the L1 stage, compounds under study at the appropriate doses, and OP50 inactivated by freeze-thaw cycles and incubated in S-medium complete to a final optical density of 595 (OD₅₉₅) of 0.9–0.8 measured in the microplate reader.

Table 7

List of *C. elegans* strains used in this work.

Strain	Genotype	Source
CL2006	dvIs2 [pCL12(unc-54/human Abeta peptide 1–42 minigene) + rol-6(su1006)]	CGC

4.5.4. Thrashing assay

To assess the effect on locomotion through neurotransmitter imbalances, we employed the thrashing assay to quantify worm motility. For this, worms were placed in a liquid medium and the number of lateral swimming movements (thrashes sec^{-1}) was counted. A single thrash defined as a complete change in the direction of bending at the mid body (Fig. 9). Briefly, after chronic treatment in liquid culture, worms were transferred for 30 min to a fresh NGM plate. First, a small volume of M9 buffer was transferred to the glass slide. Next, 10 worms were randomly selected, allowed to stabilize in M9 buffer for 30 s, and each worm was analyzed separately or its motility over a 30-second period. Manual counting of thrashing was done. This assay was run per duplicate.

4.6. Statistical analysis

Figs. 6, 7 and 8 are expressed as the mean \pm SD of triplicate determinations. Experiments were repeated at least three times, yielding similar results. Data were first analysed using one-way ANOVA. Then, a significance level of $p < 0.05$ was applied to the post hoc statistical analyses (Tukey test). The SPSS statistical software package (version 20.0) for Windows (Chicago, IL, USA) was used.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by Ministerio de Ciencia, Innovación y Universidades, Agencia Estatal de Investigación (Spain, PID2019-107991RB-I00, PID2022-1380790B-I00), Basque Government (IT-1211-19 and 1512-22), Generalitat de Catalunya (GC) (2021 SGR 00357) and PDC2022-133441-I00 (MCIN/AEI/ 10.13039/501100011033 and by the “European Union NextGenerationEU/PRTR) and by UCM-Santander (PR44/21-29931 to J.A.M.-G.). The project leading to these results has received funding from “la Caixa” Foundation (ID 100010434) under agreement CI18-00002. This activity has received funding from the European Institute of Innovation and Technology (EIT). This body of the European Union receives support from the European Union’s Horizon 2020 research and innovation programme.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioorg.2023.106935>.

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