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Synthesis of tetrahydroxybiphenyls and tetrahydroxyterphenyls and their evaluation as amyloid-β aggregation inhibitors

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Abstract

3,3′,4,4′-tetrahydroxybiphenyl and three isomeric 3,3″,4,4″-tetrahydroxyterphenyls with varying geometries around the central phenyl ring have been synthesized and evaluated for their in vitro activity against aggregation of Alzheimer's amyloid-β peptide (Aβ). Results from Congo red spectral-shift assays reveal that all four compounds successfully inhibit association of Aβ monomers. For the tetrahydroxyterphenyls, efficacy varies with linker geometry: the *ortho*-arrangement affords the most successful inhibition and the *para*- geometry the least, perhaps due to differing abilities of these compounds to bind Aβ. Of the four small molecules studied, 3,3′, 4,4′-tetrahydroxybiphenyl is the most effective inhibitor, reducing Aβ aggregation by 50 percent when present in stoichiometric concentrations.

Keywords

Alzheimer's disease; amyloid-β aggregation inhibitors; hydroxyterphenyls; catechols

Alzheimer's disease (AD), the most common form of dementia,[1] is causally linked to the aggregation of amyloid-β peptide (Aβ), a peptide of 39–43 amino acids that is formed *via* proteolytic cleavage of the amyloid precursor protein (APP).[2] Aβ self-association results in a diverse array of oligomeric and fibrillar species; it is not yet clear which of these may be the pathogenic agent(s) in AD. Historically, research efforts focused on preventing the formation of insoluble Aβ fibrils found in the extracellular plaques characteristic of AD;[3, 4] however, discoveries in the last 10–15 years have shown that soluble oligomers are more neurotoxic than fibrils,[5] and that even the smallest Aβ assemblies, dimers and trimers, exhibit neurodegenerative effects.[6, 7] As such, there may be multiple Aβ targets to consider in the struggle toward AD prevention and treatment. The ability to rationally influence and control Aβ aggregation is central to this effort.

Numerous research groups have investigated the effects of a wide variety of compounds on Aβ association.[8–11] Among the many small molecules found to inhibit Aβ oligomerization and/or fibril formation are aminonaphthalene sulfonates,[12] benzofurans,[13] carbazole derivatives,[14, 15] coumarins,[16] *N*-phenyl anthranilic acids,[17] bis-styrylarene derivatives,[18, 19] nicotine,[20] bisphenol A derivatives,[21] and others.[22–25] In 2007, Reinke and Gestwicki

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investigated the effects of curcumin and related compounds on Aβ aggregation, finding that the most successful inhibitors of this type possess terminal aromatic rings containing hydrogen-bond donors and a relatively rigid central —"linker" region 8 – 16 Å in length.[26] This report, as well as the observations that small catechol derivatives[27] and other polyphenols[28] can inhibit Aβ fibril formation, led us to investigate the effect of tetrahydroxyterphenyls (Scheme 1) on the aggregation of Aβ monomers. These three compounds, PTT, MTT and OTT, have varying geometries around the linker phenyl ring, with the terminal rings attached at the *para*-, *meta*-, and *ortho*-positions, respectively. We reasoned that these compounds would exhibit an inhibitory effect on Aβ aggregation because they contain hydroxy-substituted aromatic rings connected by a rigid linker, which generally fit the requirements noted by Reinke and Gestwicki. Although the length of the phenyl linker (4.5 – 7.4 Å, depending on terphenyl geometry) is at the low end of the proposed optimum range, we felt these compounds were good candidates for study given their structural similarity to resveratrol, which Reinke and Gestwicki noted exhibits good activity despite its short linker length (4.4 Å).[26]

The target terphenyl-3,3″,4,4″-tetrols were synthesized as shown in Scheme 1. Microwave-promoted Suzuki-Miyaura coupling of 3,4-dimethoxyphenylboronic acid with an appropriate dibromobenzene (**1**) using ultra-low palladium concentrations[29] gave good yields of 3,3″,4,4″-tetramethoxyterphenyls (**2**). While the synthesis of **2a** could be completed using only 0.02 mol % Pd, to achieve complete conversion of **1b** and **1c** to **2b** and **2c** required a higher (though still "ultra-low") Pd loading (0.07 mol %), presumably due to steric effects. Cleavage of the methyl ethers with boron tribromide[30] led to satisfactory yields of the desired tetrols (**3**). To evaluate the need for the phenyl linker, biphenyl-3,3′, 4,4′-tetrol (BPT, **5**) was similarly prepared (Scheme 2).

All products were characterized by ^{1}H- and ^{13}C-NMR, IR, mp and HRMS (see Supplementary Data). The purities of the isolated tetrols were > 95% as measured by HPLC.

The Congo red spectral-shift assay was used to evaluate the efficacies of PTT, MTT, OTT and BPT as inhibitors of Aβ40 aggregation. Congo red (CR) binds to β-structured aggregates, resulting in a red-shift of its electronic absorption spectrum; quantification of this shift permits determination of the concentration of bound complex CR-Aβ, as described by Klunk and coworkers.[31, 32] By monitoring CR-Aβ concentration as a function of time, one can thus follow the course of Aβ40 aggregation. As Hudson *et al.* recently demonstrated, this approach is well suited to monitoring the effects of polyphenols, the addition of which can bias the results of the more common Thioflavin T (ThT) assay even when the added compound does not spectroscopically interfere in the region of ThT fluorescence.[33]

The following disaggregation protocol was employed to prepare Aβ40 monomers for aggregation assays. Lyophilized Aβ40 was allowed to come to room temperature and dissolved in hexafluoroisopropanol (HFIP) to a concentration of 5 mg/mL; the vial was sealed and allowed to stand overnight in the hood, after which the HFIP was evaporated under a stream of nitrogen for at least 1 hour. Following complete evaporation, the peptide film was dissolved in DMSO with thorough mixing to a concentration of 2 mM and aliquotted into non-siliconized microcentrifuge tubes. Aliquots were stored at −80 °C.

For Congo red assays, Aβ40 aliquots in DMSO were brought to room temperature and diluted to 50 μM in 10% DMSO (90% PBS, pH 7.4 by volume). Overall volumes varied from sample to sample to permit incorporation of inhibitors (0–500 μM, i.e., 0–10 X, from stock solutions in DMSO). Peptide samples with and without inhibitors were incubated and shaken at 37 °C and 750 or 1500 rpm to promote rapid aggregation, in a procedure adapted

from Goldsbury *et al.*[34] To determine the concentration of bound CR-Aβ complex, aliquots of peptide solutions were removed from the incubator and mixed with equal volumes of Congo red solution (50 μM in PBS, pH 7.4, prepared fresh daily) in a sub-micro cuvet. UV-visible spectra of these mixtures (with final Aβ and CR concentrations of 25 μM each) were measured; concentrations of bound complex ([CR-Aβ]) were determined based on absorbance values and extinction coefficients at 403 and 541 nm, according to Eqn 1,[32] for which the path length is 1 cm. To follow the time course of aggregation, the

$$[CR-A\beta]=\frac{A_{541}}{47,800}-\frac{A_{403}}{38,100} \quad (1)$$

concentration of CR-Aβ in a particular peptide sample (with or without inhibitor) was monitored over a period of hours and plotted as a function of time.

In the absence of added inhibitor, the peptide exhibits a brief lag period followed by a growth phase in which the concentration of bound CR-Aβ complex increases quasi-linearly; the CR-Aβ concentration plateaus as aggregation reaches equilibrium (Figure 1, filled squares). The addition of 10 equivalents of any terphenyltetrol significantly decreases the concentration of bound complex at equilibrium. For OTT and MTT, CR-Aβ concentrations at equilibrium are approximately the same as those evident at time zero; for PTT, the maximal CR-Aβ concentration is near one-half that of the control (Figure 1).

To quantify inhibitory efficacy more precisely, we determined IC_{50} values for the most successful terphenyltetrols, OTT and MTT. For comparison, we also examined the biphenyltetrol BPT, to probe the effects of removing the linker phenyl ring. A representative dose-response plot for BPT is shown in Figure 2. For each IC_{50} determination, the equilibrium CR-Aβ levels present at various inhibitor concentrations were measured in duplicate and compared to those of control (inhibitor-free) samples. A logistic sigmoidal fit was used to determine the IC_{50} characteristic of each run. Runs were performed in duplicate to determine the average IC_{50} values listed in Table 1. Of the two most promising terphenyltetrols, OTT exhibits greater efficacy than MTT (IC_{50} ~2.7 X versus 3.7 X); both of these out-perform PTT, which has an IC_{50} on the order of 10 X, as shown in Figure 1. Interestingly, the biphenyltetrol lacking the linker phenyl ring is the most successful inhibitor, with an IC_{50} near 1.0 X.

To confirm that the decreased CR-Aβ levels observed in the presence of these tetrols are, in fact, due to inhibition of Aβ aggregation, competitive binding studies were performed. For these measurements, 10 equivalents of inhibitor were added only after Aβ40 aggregation had reached equilibrium, and CR-Aβ concentrations were compared for inhibitor-free versus inhibitor-containing samples. For BPT and OTT, inhibitor addition did not affect the concentration of bound complex beyond the slight (2.6 %) decrease calculated to arise from the increased sample volume. These results indicate that BPT and OTT do not compete with CR to bind Aβ. As such, the decreased levels of bound CR-Aβ complex observed in the presence of these tetrols (*e.g.*, as shown in Figures 1 and 2) are confirmed to result from inhibition of Aβ aggregation, rather than displacement of bound CR dye. With the addition of 10 equivalents of MTT, the CR-Aβ concentration decreased by an average of 9 %. This decrease is slightly greater than the 2.6 % change expected for increased sample volume, and likely indicates a small degree of competition between MTT and CR to bind Aβ. However, this small decrease in CR-Aβ cannot explain the MTT results in Figure 1: although MTT may displace a small percentage of bound CR molecules, it also significantly inhibits Aβ aggregation.

Collectively, the results described herein demonstrate that the geometry around the linker phenyl ring significantly affects inhibitory efficacy in the terphenyltetrols OTT, MTT and PTT, perhaps because it impacts inhibitor binding to Aβ assemblies. Surprisingly, the biphenyltetrol BPT, which lacks the linker phenyl ring, is the most effective inhibitor of Aβ aggregation. Although this result may seem to contradict the linker requirements proposed by Reinke and Gestwicki,[26] BPT is more conformationally restricted than the inhibitors included in their study, such that the characteristics they associated with inhibitory efficacy may not apply in this case. Our finding that BPT inhibits Aβ aggregation by 50 % when present at stoichiometric levels renders it a promising architecture for further study. Future work will focus on designing and evaluating related inhibitor molecules and identifying the Aβ species they are targeting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

Aβ	amyloid-β peptide
AD	Alzheimer's disease
APP	amyloid precursor protein
PTT	*p*-terphenyl-3,3″,4,4″-tetrol
MTT	*m*-terphenyl-3,3″,4,4″-tetrol
OTT	*o*-terphenyl-3,3″,4,4″-tetrol
BPT	biphenyl-3,3′,4,4′-tetrol
Aβ40	amyloid-β(1–40)
CR	Congo red
CR-Aβ	Congo-red—amyloid-β bound complex
ThT	thioflavin T
HFIP	hexafluoroisopropanol

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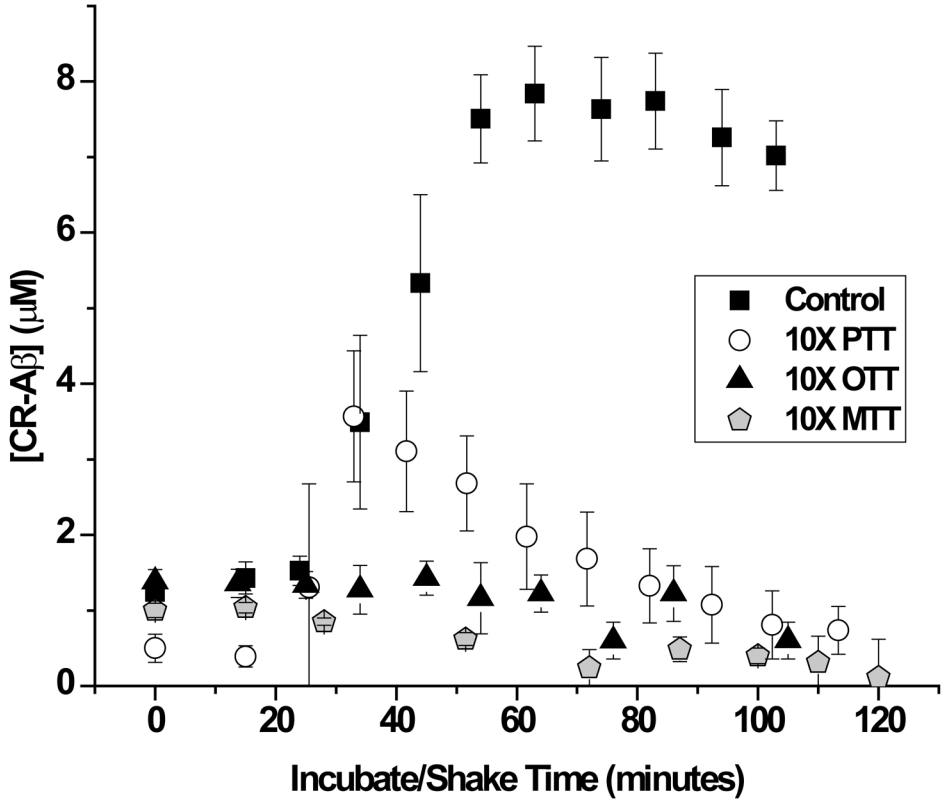


Figure 1.

Aggregation of Aβ40 monomers as monitored by Congo red spectral-shift assay. Peptide samples with and without inhibitor were incubated with agitation at 37 °C and 750 rpm prior to addition of Congo red; in the cuvet, each sample contained 25 μM Aβ. Terphenyltetrols PTT, MTT and OTT were tested at 10 X concentrations (i.e., 250 μM in the cuvet). Concentrations of CR-Aβ complex ([CR-Aβ]) were determined from UV-visible spectra according to Eqn 1.[32] Data points represent average values from at least three separate runs.

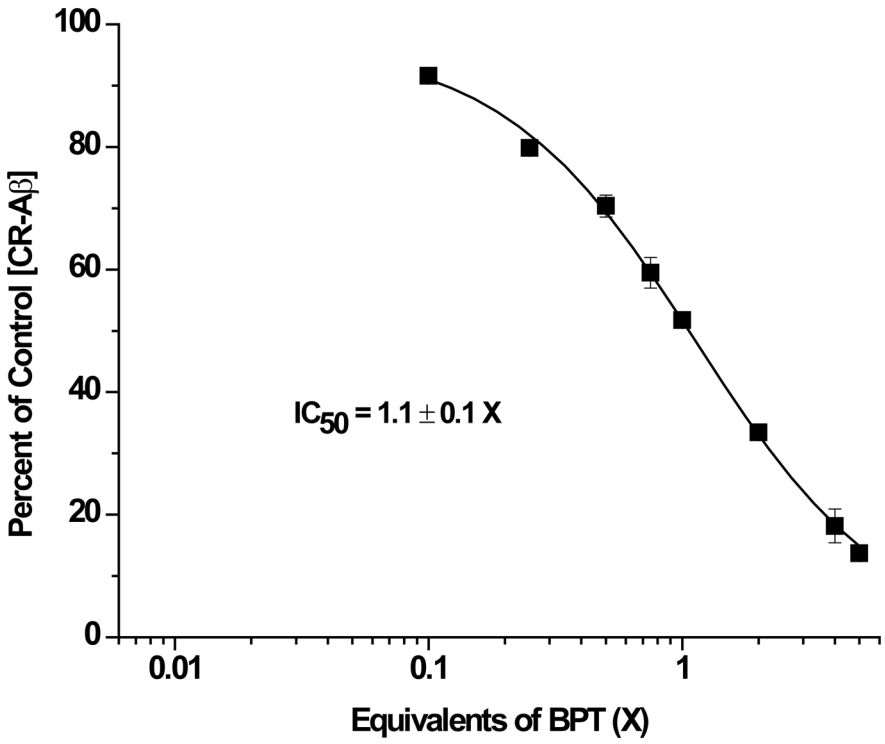
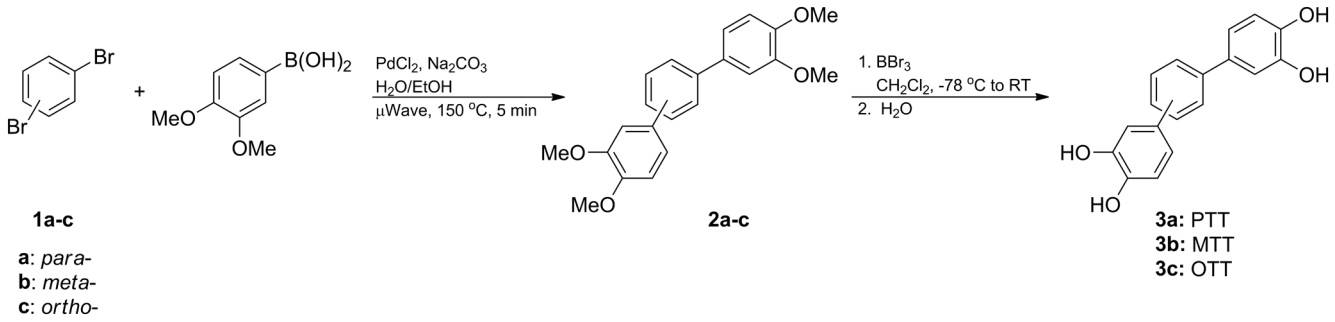


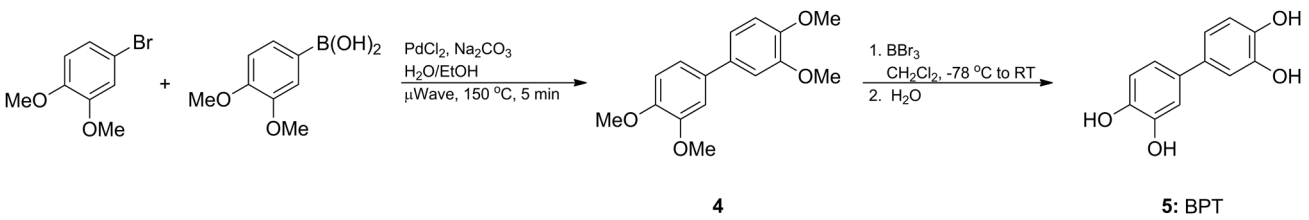
Figure 2.

Dose-response plot for determination of the IC_{50} for biphenyltetrol BPT monitored by Congo red spectral-shift assay. Peptide samples with and without inhibitor were incubated with agitation at 37 °C and 1500 rpm prior to addition of Congo red. Once Aβ aggregation was confirmed to reach equilibrium levels, CR-Aβ concentrations for samples containing various amounts of BPT were compared to those for inhibitor-free control samples (1X = 25 μM). All measurements for a particular IC_{50} determination were made in duplicate. A logistic sigmoidal fit was used to determine the IC_{50} for each experiment. IC_{50} values in Table 1 represent averages from two runs.



Scheme 1.

Synthesis of terphenyl-3,3″,4,4″-tetrols (**3**), PTT, MTT and OTT.



4 **5:** BPT

Scheme 2.
Synthesis of biphenyl-3′,3,4,4′-tetrol (**5**), BPT.

Table 1

IC_{50} values for terphenyl and biphenyl tetrols inhibiting Aβ40 aggregation.

Inhibitor	IC_{50} (Equivalents, X)[a]
BPT	1.0 ± 0.3
OTT	2.7 ± 0.3
MTT	3.7 ± 0.4

[a] Each IC_{50} value is an average determined from multiple experiments. 1X = 25 μM