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Dihydroxybenzoic Acid Isomers Differentially Dissociate Soluble Biotinyl-Aβ(1−42) Oligomers

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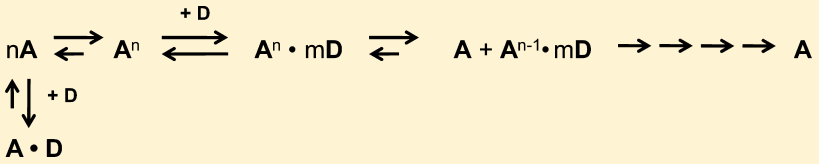

ABSTRACT: Polyphenolic compounds including a number of natural products such as resveratrol, curcumin, catechin derivatives, and nordihydroguaiaretic acid have effects on the assembly of Aβ fibrils and oligomers as well as on fibril morphology. Based on a lead structure obtained from a screen of a small molecule diversity library, simple benzoic acid derivatives distinguished by the number and position of hydroxyls on the aromatic ring displayed different abilities to dissociate preformed biotinyl-Aβ(1−42) oligomers. The 2,3-, 2,5-, and 3,4-dihydroxybenzoic acid (DHBA) isomers were active oligomer dissociators. The remaining DHBA isomers and the monohydroxy and unsubstituted benzoic acids were inactive and did not compete with the active compounds to block oligomer dissociation. None of the compounds blocked oligomer assembly, indicating that they do not interact with monomeric Aβ to shift the oligomer−monomer equilibrium. Dissociating activity was not associated with quinone redox cycling capacity of the compounds. Gallic acid (3,4,5-trihydroxybenzoic acid) stabilized biotinyl-Aβ(1−42) oligomers against intrinsic dissociation and blocked the effects of the active dissociators, independent of the concentration of dissociator. A model for the mechanism of action of the DHBA dissociators proposes that these compounds destabilize oligomer structure promoting progressive monomer dissociation rather than fissioning oligomers into smaller, but still macromolecular, species. Gallic acid blocks dissociation by stabilizing oligomers against this process.

Assembly of Aβ peptides and other amyloidogenic peptides and proteins into soluble nonfibrillar structures produces a species highly toxic to cells.[1−4] Aβ oligomers are proposed as a primary etiologic agent in Alzheimer's disease initiating a cascade of events by binding to synapses, dysregulating multiple aspects of synaptic function, and disrupting long-term potentiation which is postulated to form the basis of learning and memory.[5−8] Efforts at ameliorating toxic Aβ effects by inhibiting assembly of amyloid fibrils with small molecules have shifted to the assembly of the more diffusible and acutely toxic soluble Aβ oligomers. Thus far, literature reports of screens for oligomer assembly inhibitors have been limited.[9−14] An adaptation of our previously reported assay system for screening chemical libraries for Aβ oligomer assembly inhibitors using a biotinylated Aβ(1−42) peptide, neutravidin-coated ELISA plates for capture, and streptavidin-HRP detection in an oligomer-specific configuration[15,16] readily measures dissociation of preformed bio42 oligomers.

A small molecule structural diversity compound library (ApexScreen 5040 from TimTec) was screened for structures that (1) blocked soluble Aβ peptide oligomer assembly from synthetic biotinyl-Aβ(1−42) (bio42) monomers or (2) dissociated preformed bio42 oligomers. In both cases, a NeutrAvidin (NA)-coated and blocked 96-well ELISA plate was used to capture the N-terminally labeled bio42, followed by streptavidin-horseradish peroxidase to selectively detect multimeric bio42.[15] Several structural classes of compounds with the ability to inhibit bio42 oligomer assembly were defined. The properties of one class, a substituted 8-hydroxyquinoline series of assembly inhibitor compounds, have been described.[16] Here we describe the oligomer dissociating activity of a series of hydroxybenzoic acid isomers from commercial sources selected on the basis of an active dihydroxybenzoic acid (DHBA) derivative from the screen. These simple structures differ in the number and position of phenolic hydroxyls which profoundly affected their potency and kinetics of dissociation. None of these compounds block bio42 oligomer assembly, indicating that they likely target a different aspect of oligomer structure than assembly inhibitors such as the 8-hydroxyquinolines.[16]

Interestingly, the most potent and rapidly acting compound against oligomers, 2, 5-dihydroxybenzoic acid (gentisic acid), had no effect on preformed Aβ(1−40) amyloid fibrils, while 3,4,5-trihydroxybenzoic acid (gallic acid) rapidly decreased fibril-dependent thioflavine T fluorescence without competing for thioflavine T binding. Gallic acid did not dissociate but instead stabilized preformed bio42 soluble oligomers against

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both intrinsic dissociation and DHBA compound-induced dissociation.

Superdex 75 size exclusion chromatography and glutaraldehyde cross-linking analysis of the bio42 oligomer dissociation reaction failed to detect intermediate size oligomeric bio42 species less than the 70−100 kDa exclusion limit of Superdex 75. These observations support a model in which the DHBA dissociators bind to bio42 oligomers and allow individual peptide monomers to dissociate rather than fission oligomers into smaller assemblies.

MATERIALS AND METHODS

The benzoic acid derivatives, resveratrol, nordihydroguaiaretic acid, Tween 20 (ultrapure), Triton X-100, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), BSA (Fraction V), PMSF, trifluoroacetic acid, DMSO, tetramethylbenzidine (free base), *N*,*N*-dimethylacetamide, Sephadex G-75 (medium), 70% glutaraldehyde (Fluka, EM grade), and 30% w/w H_2O_2 were purchased from Sigma-Aldrich (St. Louis, MO). Nitroblue tetrazolium was from MP Biomedicals, curcumin from Cayman Chemicals (Ann Arbor, MI), and (−)-epigallocatechingallate (EGCG) from Enzo Life Sciences (Plymouth Meeting, PA). *N*-α-Biotinyl-Aβ(1−42) (bio42) (catalog number 23523; lot numbers 20240, 29672, and 56610) was purchased from Anaspec (San Jose, CA). Monoclonal Aβ (unmodified and biotinylated) antibodies to the human sequence Aβ-peptide, 6E10 (a.a. 3−8) and 4G8 (a.a. 17−24) were purchased from Covance Laboratories (Princeton, NJ). HRP-labeled streptavidin and HRP-goat antimouse secondary antibody were purchased from Rockland, Inc. (Gilbertsville, PA). NeutrAvidin was obtained from Pierce (Rockford, IL). High binding ELISA plates were obtained from Costar (product number 9018) (Cambridge, MA). 12% NuPAGE gels and Mes SDS running buffer were from Invitrogen. [Life Technologies] (Grand Island, NY). Nitrocellulose (0.2 μm pore size) was obtained from BioRad (Hercules, CA).

Preparation of Preformed Biotinyl-Aβ(1−42) (Bio42) Oligomers. An aliquot of N-terminal biotinyl-Aβ(1−42) peptide stored at −75 °C in HFIP solution was disaggregated as described[16] to convert any assembly intermediates into monomeric peptide. Briefly, the HFIP was removed from the desired amount of peptide in a polypropylene tube with a gentle stream of inert gas, dissolved in a minimal volume of neat trifluoroacetic acid (TFA), incubated at room temperature for 10 min, and then removing the TFA with an inert gas stream. 25 μL of HFIP was added to redissolve the peptide film and was then removed along with any residual TFA with an inert gas stream. DMSO was added to the film to produce an 8 μg bio42/mL stock solution. After 10 min the disaggregated peptide in DMSO was diluted 50-fold into oligomer-forming buffer (OFB, 20 mM sodium phosphate, 145 mM NaCl, pH 7.5) in a polypropylene container to 0.16 μg/mL (33.7 nM expressed as monomeric peptide) and mixed thoroughly. After 1 h at room temperature an equal volume of OFB containing 0.6% v/v Tween 20 was added to stop the assembly reaction and stabilize the oligomers. This 16.8 nM mixture of oligomeric and monomeric bio42 peptide (bio42 oligomers) was either used immediately or frozen in aliquots at −75 °C in polypropylene tubes for storage up to 6 months. Oligomers prepared by this method are >70 kDa, and their 100−600 kDa size distribution by size exclusion chromatography[17] is similar to that of Aβ oligomers from AD brain.[18]

Dissociation of Preformed Bio42 Oligomers. 25 μL of 16.8 nM preformed bio42 oligomers in OFB + 0.3% Tween 20 was placed in the bottom of wells in a polypropylene (wide well) 96-well plate (0.5 mL well capacity (Fisher 12565502) and 125 μL of OFB containing the desired concentration of compound and 1% DMSO added. The plate was sealed with a plastic sheet (Nunc 236366) and incubated at room temperature with shaking at 150 rpm for 16−18 h. Tween 20 was included in the incubation to prevent oligomer reformation and to improve recovery at the low final peptide concentration of 2.8 nM. The presence of Tween 20 is not required for dissociation by compounds but significantly improves the reproducibility of the assay.

After incubation, the plate was centrifuged at 1000*g* for 10 min at room temperature to collect condensation from the plastic seal area covering the wells. The amount of bio42 oligomers remaining was measured by transferring 100 μL from each well to an NA-coated (50 ng/well) well of an ELISA plate, incubating with shaking for 2 h, and detecting with SA-HRP.[15]

Time Course of Bio42 Oligomer Dissociation. A dose response of 2,3-, 2,5-, and 3,4-DHBA compounds was prepared in quadruplicate in one plate with preformed 2.8 nM bio42 oligomers, using one plate for each time point. At each time point (0, 2, 4, 6, 8, 12, and 24 h), a plate was frozen at −20 °C. After the last time point was collected and frozen, all plates were thawed and the oligomer content measured. Control experiments indicated that the freezing and storage did not affect the oligomer signal.

Superdex 75 Size Exclusion Chromatography. A time course (0, 2, 5, and 8 h) of dissociation of preformed bio42 oligomers (30 nM total Aβ) was carried out in OFB + 0.1% Tween 20 in the presence of 5 μM 2,5-dihydroxybenzoic acid or 1% DMSO as a solvent vehicle control at room temperature in 0.65 mL low binding 0.65 mL) Eppendorf tubes (250 μL per tube). At each time point a 25 μL aliquot was removed for glutaraldehyde cross-linking, and the remaining sample was frozen at −20 °C. After thawing at room temperature, 200 μL was injected onto a 10/30 HR Superdex 75 column equilibrated with OFB + 0.1% Tween 20 operating at 0.5 mL/min. Fractions (0.2 mL) were collected in a 96-well polypropylene plate (Costar 3365), and frozen at −20 °C for analysis by ELISA. All chromatography runs were on the same day.

Aliquots of column fractions were applied to NA-coated plates (50 ng/well) blocked with OFB + 0.1% Tween 20, incubated for 2 h, and washed. The plates were sequentially incubated for 1 h with 50 μL/well of 0.5 μg/mL 4G8 monoclonal antibody in OFB + 0.1% Tween 20 + 2 mg/mL BSA (Calbiochem), washed, and then incubated for 1 h with 50 μL of 1:10000 goat−anti-mouse IgG-HRP (Rockland). After washing, HRP activity was detected with tetramethylbenzidine.[15] All ELISAs were performed on the same day.

Glutaraldehyde Cross-Linking of Bio42 Oligomers. 25 μL samples containing 30 nM bio42 were incubated for 5 min at room temperature in 0.65 mL low binding 0.65 mL Eppendorf tubes with 0.0125% glutaraldehyde (final concentration), quenched by the addition of Tris-HCl, pH 7.5 to 40 mM, and NuPAGE sample buffer (4×) added. The samples were heated for 5 min at 90 °C and then either frozen or separated immediately on 12% NuPAGE gels in Mes running buffer. The gels were transferred to 0.2 μm pore size nitrocellulose for 70 min at 30 V constant voltage in a Novex blotter in NuPAGE transfer buffer + 10% methanol. The blots

were boiled in OFB for 5 min and then blocked in 3% w/v Blotto (Santa Cruz) in OFB. Detection was with either streptavidin conjugated to an 800 nm fluor or 4G8/goat−anti-mouse conjugated to an 800 nm fluor. Quantification was with an Odyssey imager (LI-COR Biosciences, Lincoln, NE).

Proteolytic Sensitivity of Bio42 Oligomers. 25 μL of 16.8 nM preformed bio42 oligomers was placed in the bottom of wells in a polypropylene 96-well plate (0.5 mL well capacity) and 125 μL of OFB containing 10 μM compound in OFB + 0.1% Tween 20 added for 30 min at room temperature. 15 μL of a 2-fold dilution series of trypsin in OFB + 0.1% Tween 20 was added to each well followed by shaking at 150 rpm for an additional 30 min at room temperature. 1 μL of freshly dissolved 20 mg/mL phenylmethylsulfonyl fluoride in DMSO was added to each well to inhibit further proteolysis at a 120 μg/mL final concentration. 100 μL of the reaction in each well was transferred to a NA-coated and blocked ELISA plate and incubated with shaking for 2 h. The oligomer signal remaining was detected with SA-HRP as for the dissociation reaction.

Redox Cycling of Quinone Precursors. The redox cycling capacity of compounds was measured with a colorimetric assay system with alkaline glycine as an electron donor adapted from Flückiger et al.[19] Compounds were diluted from stock solutions in DMSO to give 0.3125−20 pmol per microliter in distilled water and a residual 1% v/v of DMSO. 25 μL of each dilution of compound was incubated in a well of a clear polystyrene 96-well round-bottom plate (Costar 3798) with 200 μL of a reaction mixture consisting of 10 mM sodium tetraborate, 0.7 mg/mL borohydride-treated and dialyzed BSA, and 0.2 mM nitroblue tetrazolium chloride (NBT) in 2 M potassium glycinate buffer, pH 10, for 1 h in the dark at room temperature. The BSA-stabilized NBT formazan complex was measured at 530 nm in a Biotec Synergy HT plate reader.

RESULTS

2,5-Dihydroxybenzoic acid dose dependently reduced the amount of oligomeric bio42 detected by the single-site assay format which produces a signal only when multiple biotins are present in the multimeric species captured by the biotin-binding protein NeutrAvidin.[15] Monomeric protein is not detected by the streptavidin-HRP because the single biotin in the biotinyl-Aβ(1−42) monomer is captured by the NeutrAvidin on the plate. The biotin/avidin system is often used in screening of chemical compounds because of the high affinity of the interaction and because only biotin-related compounds interfere significantly. Verification of conversion of oligomeric bio42 to monomeric peptide and the selectivity of the single-site assay configuration for oligomers was provided by Sephadex G75 size exclusion chromatography (Figure 1). As oligomers dissociated, the void volume oligomer peak (>70 kDa) decreased in both the oligomer-specific (Figure 1a) and total Aβ (Figure 1b) profiles and the signal for total Aβ in the monomer region of the profile increased.

Dose−response curves (0−20 μM) were generated for all six of the dihydroxy positional isomers, the three monohydroxy isomers, benzoic acid, and 3,4,5-trihydroxybenzoic acid. Figure 2 shows dose−response curves at 18 h for the active compounds. The structures for the active compounds are shown in Chart 1. The 2,3-, 2,5-, and 3,4-isomers of dihydroxybenzoic acid showed consistent bio42 oligomer dissociating activity at 16−18 h to different degrees and with different kinetics. By contrast, 3,4,5-trihydroxybenzoate (gallic acid), the three monohydroxy benzoic acids (2-, 3-, and 4-hydroxybenzoic acid), and benzoic acid did not dissociate bio42 oligomers under these conditions. None of these compounds inhibited bio42 oligomer assembly at up to 20 μM. The relative potencies of the active compounds were maintained throughout the time course of bio42 oligomer dissociation (Figure 3a), and the dissociation rate ($t_{1/2}$) was dependent on compound concentration (Figure 3b). The EC_{50}'s of the 2,3- and 2,5-DHBA compounds reached a minimum and plateaued after 10−12 h, while the plateau with 3,4-DHBA was reached after 4 h (Figure 3c).

The effect of oligomer concentration on the potency (EC_{50}) of DHBA isomers (Figure 4) reveals a possible difference between DHBA isomers in their mechanism of dissociation. In

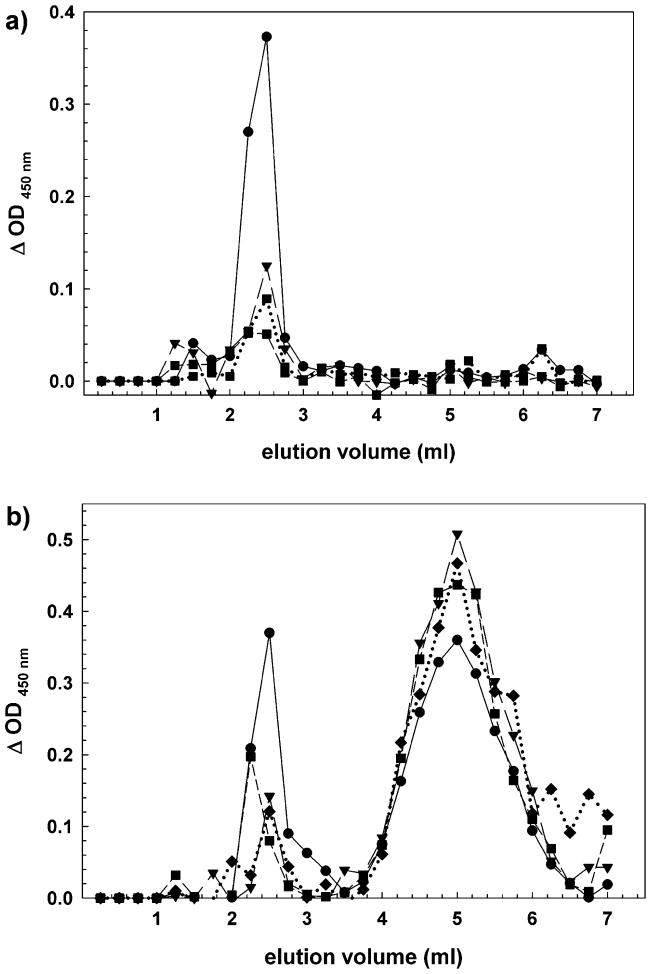

Figure 1. 2,5-Dihydroxybenzoic acid dissociates bio42 oligomers. Size exclusion chromatography of bio42 oligomers on Sephadex G75 (medium) in OFB + 0.1% Tween 20 before and after treatment of 16.8 nM bio42 oligomers (concentration as monomer) for 16 h with no compound (closed circle), 10 μM 2,3-DHBA (closed inverted triangle), 10 μM 2,5-DHBA (closed square), or 10 μM 3,4-DHBA (closed diamond). Oligomers eluted between 2.25 and 2.75 mL; monomers eluted between 4 and 6 mL. (a) Oligomer-specific assay: biotinylated monomers and oligomers are captured in the wells of a neutravidin (NA)-coated plate. Only oligomers are detected with SA-HRP because the single N-terminal biotin on monomeric peptide is bound by the NA. % no compounds = 1% DMSO solvent control. (b) Total bio42 Aβ assay: biotinylated monomers and oligomers are captured on an NA plate as with the oligomer-specific format in (a). Both oligomers and monomers are detected with an antibody (4G8, a.a. 17−24) to the middle of the Aβ peptide which is accessible in both NA-bound biotinylated oligomers and monomers.

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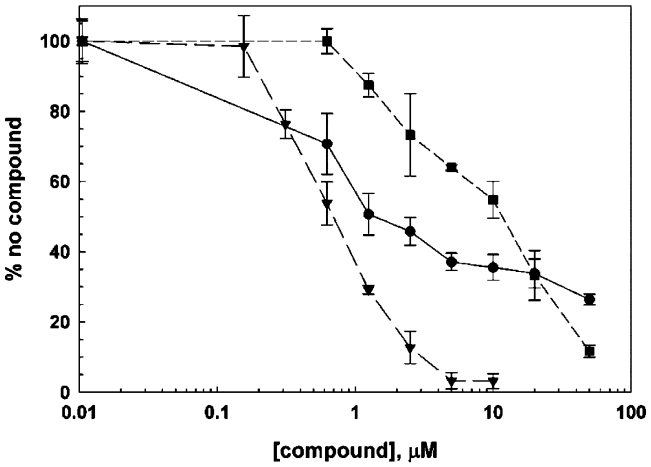
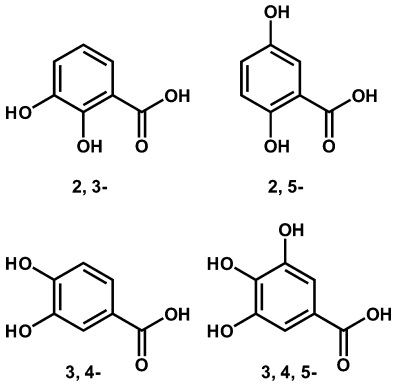


Figure 2. Dose response of DHBA isomer dissociation of bio42 oligomers. Preformed bio42 oligomers (2.8 nM) are incubated overnight at room temperature in the presence of the indicated concentrations of compounds and assayed for remaining oligomers as described in the Materials and Methods section.

Chart 1. Structures of Oligomer-Dissociating Dihydroxybenzoic Acid Isomers



all cases the concentration of DHBA isomer is in excess of the Aβ concentration (expressed as monomer). For the 2,5- and 3,4-isomers the difference is reflected in the lack of a significant shift in potency of the compounds as the oligomer (and total Aβ) concentration is increased. However, the 2,3-DHBA potency shows a steep dependence on oligomer concentration. At high oligomer concentration (~100 nM total Aβ), all of the compounds require a higher concentration of compound to dissociate 50% of oligomers, perhaps indicating a change in the way compounds interact with the oligomers.

A measure of Aβ oligomer and amyloid fibril structural integrity is their resistance to proteolytic degradation. Perturbation of oligomer structure by compound binding could expose protease-sensitive sites on bio42 oligomers or produce trypsin-sensitive intermediates. Monomeric peptide is readily fragmented by low concentrations of a variety of proteases. Treatment of oligomers with dissociating compounds at 10 μM for 1 h followed by trypsin digestion did not affect the sensitivity of bio42 oligomers to trypsin compared to the DMSO (no compound) control (data not shown). This suggests that treatment with the active DHBA isomers does not significantly perturb oligomer structure prior to dissociating the oligomers.

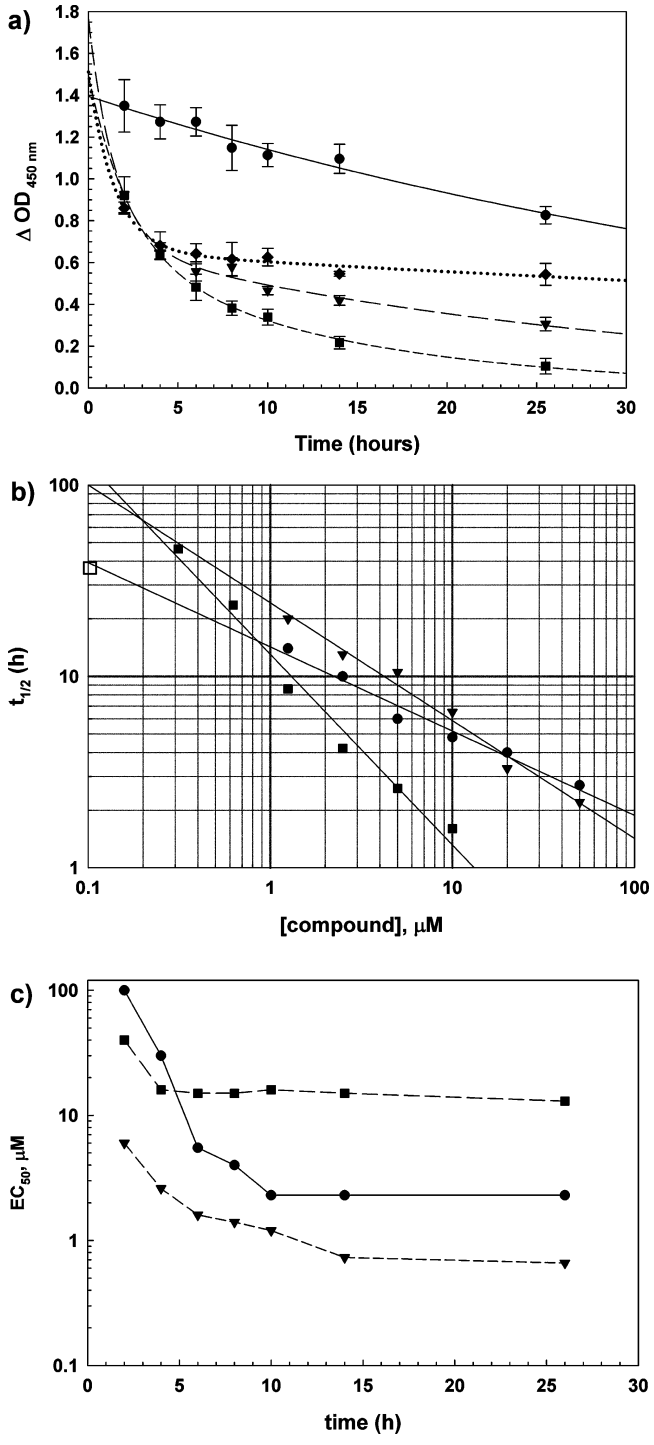


Figure 3. Effect of DHBA isomers on the time course of bio42 oligomer dissociation. (a) Time course of dissociation: the amount of oligomers remaining after incubation for different times with a range of concentrations of 2,3-, 2,5-, and 3,4-DHBA was determined as described in the Materials and Methods section. The time point includes the 2 h incubation of oligomers with compound while binding to the NA plate. No compound (large open square); 2,3-DHBA (closed inverted triangles); 2,5-DHBA (closed squares); 3,4-DHBA (closed diamonds). The smooth curves are calculated fits: no compound yields a single-exponential decay; compounds produce double-exponential decay. (b) DHBA concentration dependence of the half-time for dissociation of bio42 oligomers. The half-times for dissociation plotted against concentration for the three dissociating DHBA compounds show similar slopes for 2,3-DHBA (closed circles) and 2,5-DHBA (closed inverted triangles) but is different for 3,4-

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Figure 3. continued

DHBA (closed squares). The open square is the half-time for the no compound vehicle (1% DMSO) control. The lines are linear least-squares fit to the data for individual compounds. (c) Time dependence of DHBA EC_{50} for bio42 oligomer dissociation. The apparent EC_{50}'s for dissociation decrease with incubation time to a plateau for each compound. 2,3-DHBA (closed circles) and 2,5-DHBA (closed inverted triangles) reach a minimum within 10−12 h while 3,4-DHBA (closed squares) is unchanged after 4 h.

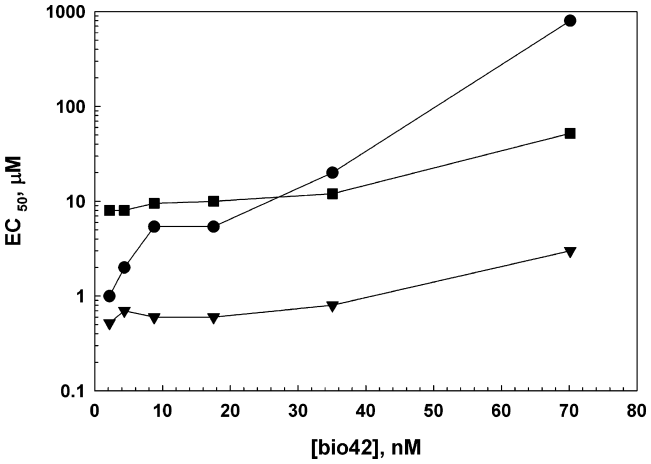

Figure 4. Bio42 oligomer concentration dependence of dissociating compound potency. The effectiveness (EC_{50}) of the three dissociating DHBA compounds was compared at different concentrations of bio42 oligomers. Oligomers were determined as described in Materials and Methods after appropriate dilution. 2,3-DHBA (closed circles); 2,5-DHBA (closed inverted triangles); 3,4-DHBA (closed squares).

DHBA-like compounds were tested for their ability to antagonize bio42 oligomer dissociation by 2,3-, 2,5-, and 3,4-DHBA. Non-oligomer-dissociating DHBA isomers, benzoic acid, and the monohydroxybenzoic acid isomers at 10 μM failed to shift the dose−response curves of dissociation by the active DHBA isomers (data not shown). By themselves, non-dissociating compounds neither enhanced dissociation nor stabilized preformed oligomers over a range of compound concentrations, suggesting that the non-oligomer-dissociating compounds either do not bind to oligomers or bind at a different site. Thus, the orientation of the hydroxyl groups of the dihydrobenzoic acids is important in the dissociation mechanism.

Gallic Acid Stabilization of Bio42 Oligomers. By contrast to the non-oligomer-dissociating DHBA isomers, benzoic acid, and the monohydroxybenzoic acid isomers, gallic acid (3,4,5-trihydroxybenzoic acid) preserved the oligomer signal, blocking the basal (solvent control, no compound added) intrinsic rate of bio42 oligomer dissociation. Since gallic acid[20] and gallic acid-containing polyphenols such as (−)-epigallocatechin gallate (EGCG) have been reported to disrupt Aβ fibril structure[21] and remodel amyloidogenic polypeptides into unstructured nontoxic oligomeric species,[22,23] this was not an unexpected outcome. Consistent with the previously noted fibril remodeling, we confirmed that incubation of gallic acid with preformed Aβ(1−40) amyloid fibrils produced a time- and dose-dependent decrease in fibril-induced thioflavine T fluorescence which was not due to gallic acid competition with the fluorophore. None of the other benzoic acid-related compounds, including the oligomer dissociators, had any effect on ThT fluorescence of fibrils even after several hours of incubation.

Exposure of preformed bio42 oligomers under the conditions of this assay (2.8 nM bio42 peptide in the presence of 0.1% Tween 20 for 16−18 h at room temperature) to 10 μM gallic acid did not lead to a decrease in the monomer or oligomer signal, nor was there a change in the apparent size distribution of oligomers due to the presence of gallic acid as assessed by chromatography on Sephadex G75, Sephacryl S-300, or Sepharose 6B. Apparent stabilization of oligomers is therefore not an artifact due to increased clumping of the peptide which was observed with fibrils.[22]

Antagonism of 2,3-, 2,5-, and 3,4-DHBA Bio42 Oligomer Dissociation by Gallic Acid. Gallic acid also stabilized bio42 oligomers against dissociation by 2,3-, 2,5-, and 3,4-DHBA in a dose-dependent fashion. Increasing concentrations of gallic acid progressively shifted the concentration of dissociator required to achieve 50% oligomer dissociation to higher concentrations (Figure 5). The amount of gallic acid required to neutralize the effect of the different dissociators was similar (~5 μM) even though the EC_{50}'s for the dissociators varies from 0.7 to 20 μM. A similar concentration of gallic acid was required to stabilize the oligomer against intrinsic dissociation. This suggests that despite the structural similarity, the binding site for gallic acid that stabilizes bio42 oligomers against dissociation by DHBA derivatives is distinct from the site of action of the DHBA dissociating compounds.

Potential Chemical Modification of Aβ by DHBA Derivatives. A potential mechanism for phenolic perturbation of Aβ fibrils and oligomers is chemical oxidative modification of peptide amino acid residues caused by redox cycling catalyzed by quinone adducts formed by some compounds like EGCG.[22] The benzoic acid derivatives and several known redox-active phenolic compounds that inhibit Aβ fibril assembly were tested *in vitro* for their intrinsic ability at alkaline pH to reduce nitroblue tetrazolium in the presence of glycine as a source of reducing electrons to its intensely colored formazan.[19] Figure 6 indicates the relative reactivity of 500 pmol of each compound at 60 min of incubation. Only the benzoic acid derivatives with vicinal hydroxyls, 2,3-, 3,4-, and 3,4,5-substitution, generated significant signal. Several other polyphenols noted for their antioxidant activity were also redox active in this assay, including resveratrol, curcumin, and nordihydroguaiaretic acid (Chart 2). The most active oligomer dissociation-inducing DHBA derivative is the 2,5- isomer, yet it is <10% as redox active as the 2,3- and 3,4-isomers. Gallic acid (3,4,5-isomer) is highly redox active, yet it has no dissociating effect on preformed bio42 oligomers, although it does decrease ThT fluorescence of Aβ(1−40) fibrils. Hence, the potential for redox activity is not a prerequisite for oligomer dissociation, although it may be a contributing factor for some compounds.

2,5-Dihydroxybenzoic Acid Dissociates Oligomers but Not by Fragmentation. The production of small bio42 oligomers as reaction intermediates in the dissociation of the large >100 kDa bio42 oligomers by 2,5-dihydroxybenzoic acid (gentisic acid) was investigated by size exclusion chromatography (stable intermediates) and by glutaraldehyde cross-linking (metastable intermediates) . Superdex 75 analysis of dissociation reactions at different times following addition of either solvent control or 5 μM 2,5-DHBA failed to detect intermediates <70 kDa, the exclusion limit for Superdex 75 (Figure 7). Figure 7a shows the elution profile of bio42 with a characteristic oligomer peak in the column void volume (MW

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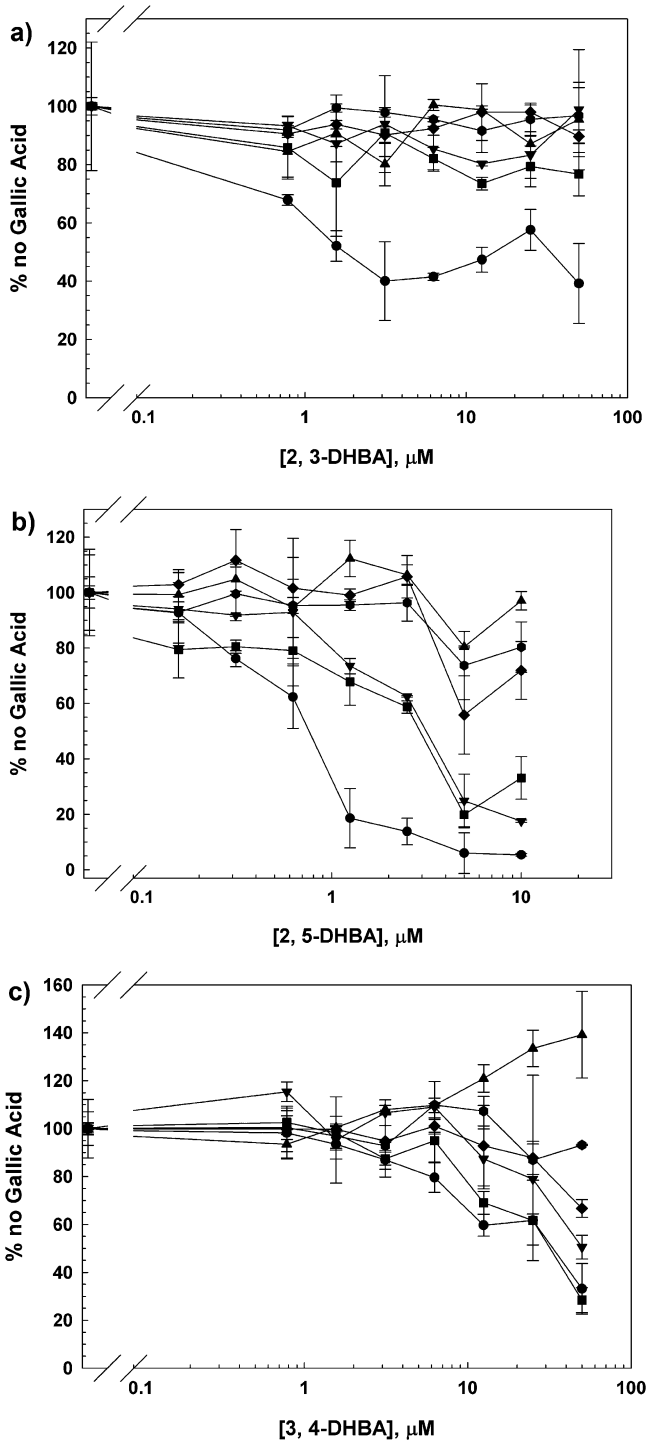


Figure 5. Gallic acid (3,4,5-trihydroxybenzoic acid) antagonizes bio42 oligomer dissociation by DHBA isomers. Preformed bio42 oligomers (2.8 nM) were incubated with a dose–response of 2,3-DHBA (a), 2,5-DHBA (b), and 3,4-DHBA (c) titrated at each DHBA concentration with 0 (closed circles), 0.625 μM (closed inverted triangles), 1.25 μM (closed squares), 2.5 μM (diamond), 5 μM (hexagon), and 10 μM (triangle) gallic acid, incubated for 16 h and the oligomers determined as described in Materials and Methods. % no gallic acid = 1% DMSO solvent control.

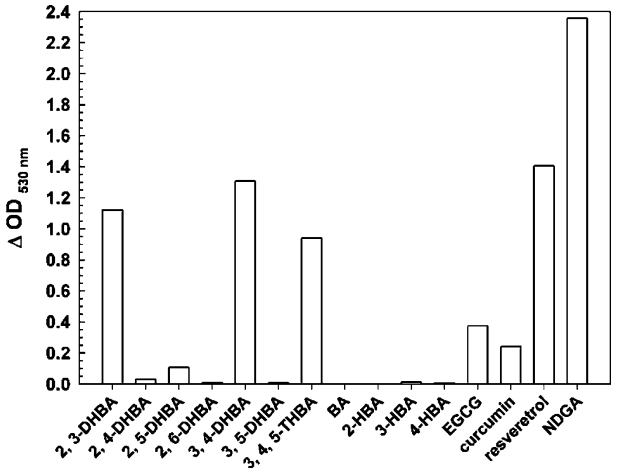
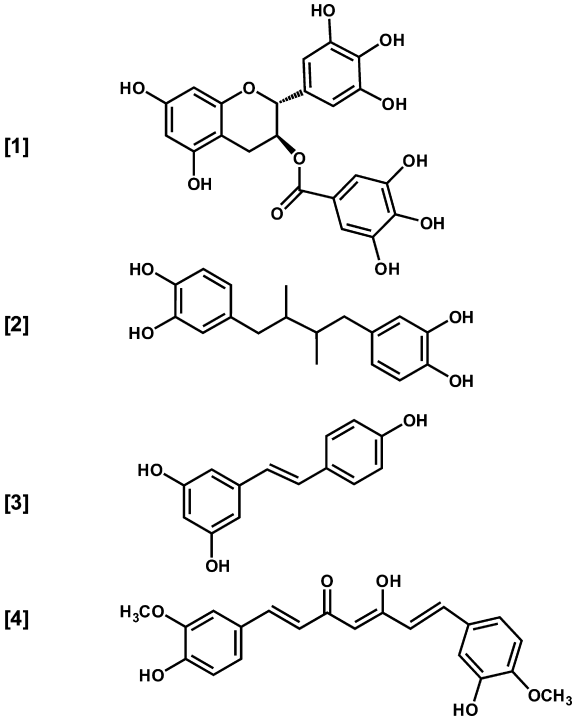


Figure 6. Redox activity of phenolic compounds. The nitroblue tetrazolium reduction activity of benzoic acid derivatives and natural product polyphenols was measured as described in Materials and Methods. DHBA isomers as indicated; 3,4,5-THBA = 3,4,5-trihydroxybenzoic acid (gallic acid); BA = benzoic acid; 2-BA = 2-hydroxybenzoic acid; 3-BA = 3-hydroxybenzoic acid; 4-BA = 4-hydroxybenzoic acid; EGCG = (−)-epigallocatechin gallate; NDGA = nordihydroguaiaretic acid.

Chart 2. Structures of Natural Product Phenolics[a]



[a][1]: (−)-epigallocatechin gallate (EGCG). [2]: nordihydroguaiaretic acid (NDGA). [3]: resveratrol. [4] curcumin (enol-form).

>70 kDa, arrow) and a larger peak in the included volume of the column containing monomeric bio42 and comigrating dimer (due to the hydrodynamics of the unstructured peptide). Only two time points (0 and 5 h) are shown for clarity. No intermediate sizes were observed at any time point. Integration of the bio42 content of the oligomer (fractions 37–50) and monomer (fractions 66–78) peaks provides an estimate of the quantity of the two species. Figure 7b shows that in the presence of 5 μM 2,5-DHBA the oligomer content decreases while the monomer content increases with time. The amount of dimer species that can be trapped by cross-linking from bio42 oligomers decreases with time of treatment (Figure 7c), and the

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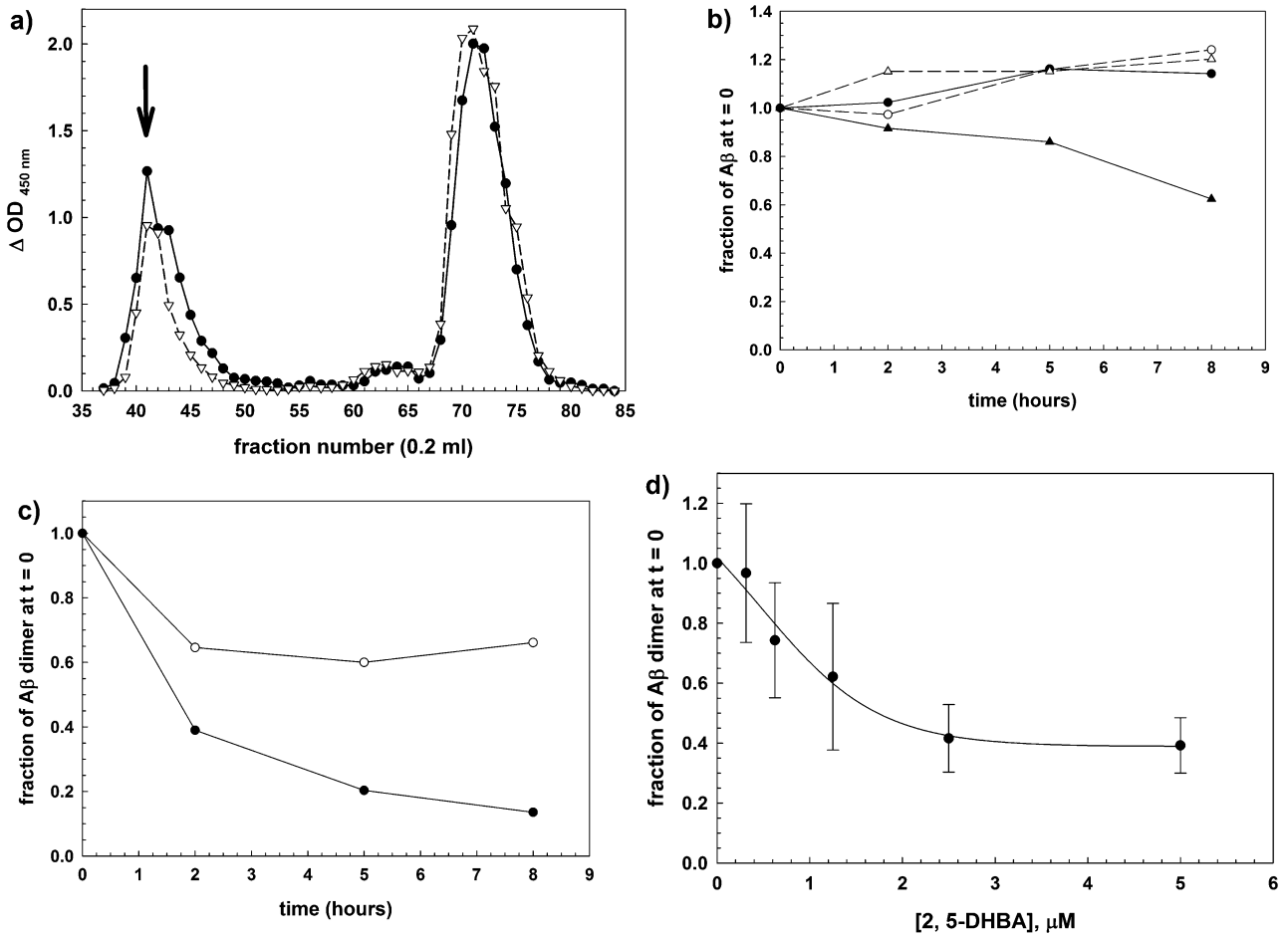

Figure 7. 2,5-DHBA does not fragment bio42 oligomers into intermediate size species. (a) Superdex 75 profile of Aβ immunoreactivity treated for 0 or 5 h with 5 μM 2,5-DHBA. After treating preformed bio42 oligomers (30 nM total bio42 with 2,5-DHBA, the reaction mixture was chromatographed and fractions assayed for bio42 content as described in Materials and Methods. Closed circle and solid line = zero time; open inverted triangle and dashed line = 5 h; arrow = column void volume and oligomer peak. Profiles were also run with samples treated for 2 and 8 h. (b) Treatment of bio42 oligomers with 2,5-DHBA reduces the amount of oligomeric bio42 and increases the amount of monomeric bio42. Superdex 75 column profiles of bio42 content were run as in (a) and the bio42 content of the oligomeric void volume (fractions 37−50) and monomer (fractions 66−78) summed to estimate the amount of oligomers and monomers present after 0, 2, 5, and 8 h of treatment with DMSO (open symbols and dashed lines) or 5 μM 2, 5-DHBA (closed symbols and solid lines). The oligomer fraction is denoted by triangles and monomer fraction by circles. (c) Glutaraldehyde cross-linking of bio42 oligomers treated with 2,5-DHBA shows decreasing cross-linked dimer content with time. Oligomer samples from the time course of 2,5-DHBA treatment were cross-linked with glutaraldehyde, electrophoresed, and dimer content quantified by SDS-PAGE as described in Materials and Methods. Open circles = DMSO; closed circles = 5 μM 2,5-DHBA. (d) Glutaraldehyde cross-linking of bio42 oligomers treated with 2,5-DHBA shows decreasing cross-linked dimer content with increasing 2,5-DHBA concentration. Oligomer samples treated with a series of 2,5-DHBA concentrations for 16 h were cross-linked with glutaraldehyde, electrophoresed, and dimer content quantified by SDS-PAGE as described in Materials and Methods.

decrease is dose-dependent (Figure 7d). The DMSO solvent controls show only a slow intrinsic dissociation of oligomers.

DISCUSSION

A number of potential mechanisms could account for the dissociation of bio42 oligomers by the active DHBA compounds. Stabilization or sequestration of monomeric peptide by monomeric or micellar compound is unlikely since none of the DHBA isomers affect assembly of oligomers, even at high concentration. A mechanism involving the fission of the 100−600 kDa bio42 oligomer assemblies in the presence of DHBA dissociators is also unlikely as only oligomer and monomer (possibly dimer since they cochromatograph by gel filtration) were observed by size exclusion chromatography. Intermediates <70 kDa that were stable to chromatography were not detected, and glutaraldehyde cross-linking of treated oligomers revealed time (Figure 7c) and concentration (Figure 7d) dependent decreases of dimeric species trapped from oligomers.

A more plausible explanation is that the DHBA isomers bind to and destabilize oligomers, increasing the rate at which Aβ monomers dissociate from the multimeric complex of peptides. The kinetics of the process also support this possibility as the time course of intrinsic oligomer dissociation (no compound present) is fit by a monoexponential function with a $t_{1/2} \sim 30$ h at 22 °C. In the presence of dissociating DHBA isomers the process becomes biexponential with the second, faster rate dependent on the concentration of DHBA isomer (Figure 3).

Gentisic acid (2,5-DHBA) is a simple phenolic compound, yet it is an effective dissociator of bio42 oligomers. It was a significantly more potent and rapid acting dissociator of bio42 oligomers than the 2,3- and 3,4-isomers, producing significant

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dissociation of preformed oligomers during the 2 h binding to the NA-coated ELISA plate. The difference in slopes of the 2,5-DHBA dissociation dose−response curves and the slopes of the $t_{1/2}$ with dose for the other two active DHBAs (Figure 3b) suggests a potential difference in mechanism of action. Both 2,3- and 3,4-DHBA were redox active *in vitro*, suggesting that the vicinal hydroxyls on the benzoic acid ring were prone to quinone formation. Since 2,5-DHBA was only weakly redox active under the same conditions, quinone-mediated oxidative reactions could be one potential mechanism for oligomer dissociation, but not the only one, at least *in vitro*. By contrast, 3,4,5-trihydroxybenzoic acid, gallic acid, was not only strongly redox active, as was the more complex gallate-containing polyphenol EGCG. Neither agent dissociated oligomers. Gallic acid stabilized bio42 oligomers against dissociation by all three active DHBA dissociators. Since one of the reported properties of EGCG is to remodel fibrils into nontoxic oligomers, gallic acid may also convert toxic Aβ oligomers into nontoxic species. It thus appears that oligomer dissociation and detoxification may occur in different ways.

More detailed studies of compound interaction with oligomers will be required to fully elucidate the catalysis of Aβ oligomer disassembly. An advantage of the DHBA compounds is that they have relatively simple structures yet are reasonably potent and soluble compared to the other complex polyhydroxy natural products that have been observed to have effects on Aβ fibril and oligomer structure. Their effect on biological oligomers will also be interesting since cell culture-produced biological Aβ oligomers and Aβ oligomers isolated from transgenic mice and from AD brain[24] are considerably more stable to disassembly than those formed from synthetic peptide.

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ABBREVIATIONS

HFIP, 1,1,1,3,3,3-hexafluoroisopropanol; TFA, trifluoroacetic acid; PMSF, phenylmethylsulfonyl fluoride.

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