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A Robust and Scalable High-Throughput Compatible Assay for Screening Amyloid- β -Binding Compounds

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Abstract

A robust fluorescent readout assay using topologically-sensitive dyes improves the screening of novel amyloid-binding molecules. One of the key components that make this assay more realistic is the use of endogenous amyloid obtained from 5XFAD mouse brains. The assay conditions were optimized for high throughput screening operation with Z-prime values >0.6. Using a combination of library of 3,500 compounds including known drugs, natural-derived molecules and random organic molecules, 8 unique molecules were identified as potential amyloid-binding agents.

Keywords

Alzheimer's disease; amyloid; high throughput screening

INTRODUCTION

With the prevalence of Alzheimer's disease (AD) projected to rise dramatically in the coming decades [1], there is an increasing urgency to develop novel therapies for the prevention and treatment of this debilitating disease [2, 3]. Currently, the mechanism that

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SUPPLEMENTARY MATERIAL

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regulates neuronal degeneration in AD remains unknown; however, the cytopathological hallmarks of AD appear to be the formation of amyloid- β ($A\beta$) plaques between the neurons, which leads ultimately to profound neuron toxicity and atrophy [4]. According to the $A\beta$ cascade hypothesis, failure to eliminate soluble $A\beta$ from the brain resulted in $A\beta$ aggregation, which alters homeostasis and neuronal environment resulting in cognitive decline and dementia [5]. Therefore, if $A\beta$ plaques were an underlying mechanism that causes dementia, preventing their formation or dismantling existing plaques, specifically at the early onset of the disease, would be an ultimate goal to prevent AD. Given the critical unmet medical needs in AD, there has been considerable interest in the identification of novel $A\beta$ binding/inhibitor molecules. Rational design of $A\beta$ inhibitors is hampered due to the lack of high-resolution structures of $A\beta$ plaques. A large number of reports in the literature describe a range of intermediates along the $A\beta$ aggregation pathway that have been implicated as potentially toxic species [6-10]. However, at present no detailed structural information is available for $A\beta$ fibrils, as knowing which parts of $A\beta$ are important for filament formation is relevant for the development of drugs, owing to their short-lived intermediates and their insoluble nature. As a result, there is still enormous uncertainty over the question whether rationally structural-based drug design of $A\beta$ inhibitors is an effective approach to identify $A\beta$ -binding inhibitors. One of the alternative approaches focuses on high throughput screening (HTS) [11]. In fact, the flexibility of HTS has allowed numerous and disparate areas of biology to engage with an equally diverse palate of chemistry [12]. Here, we describe the development of an HTS assay to screen for $A\beta$ -binding molecules called high-throughput amyloid thioflavin competitive binding optical assay (HATCO) to identify $A\beta$ -binding molecules. The assay relies on the fluorescence (FL) readout of thioflavin-T; according to the quantum mechanic theory, this class of FL dyes possesses substantial conformation freedom while remaining in free solution, and thus rapidly quenches excited states generated by photon excitation and exhibits a relatively low FL signal [13]. However, upon binding to $A\beta$ plaques, the conformation freedom is dramatically reduced. This increased structural rigidity decreases the vibrational and rotational processes, which results in a decreased radiation decay rate in both ground and excited states [13], cumulatively, this phenomenon contributes to the observed increase in the FL quantum yield when bound to $A\beta$ plaques as compared to the unbound state [14]. A schematic depiction for the HATCO assay is shown in Fig. 1, particularly for the case of thioflavin-T, when not bound to $A\beta$ plaques, the dye emits a weak FL signal at a $\lambda_{\max}$ of approximately 440–445 nm. However, when bound to $A\beta$ plaque, its FL increases dramatically and results in a strong FL signal with a characteristically shifted $\lambda_{\max}$ to approximately 485 nm [15]. This dynamic reporter has been exploited to monitor fibrillary kinetics in real time, and produced a single time-point readouts [16-18]. LeVine et al. first reported the use of this technique for screening $A\beta$ -binding compounds [17]. Klunk et al. employed this dynamic reporter to develop quantitative methods to determine the properties of $A\beta$ peptide aggregation [19, 20], since then, a number of high-throughput methods have been derived to screen for compounds able to interfere with $A\beta$ aggregation [21-23].

To date, all of the screening assays discussed above have been limited to screening a small number of molecules, and many, if not all, still employed synthetic $A\beta$ peptides as a

surrogate for endogenous A β in the brain during the assay. The rationale for this approach is the ease of using synthetic peptides, which can be stored as lyophilized powders and the process of amyloidogenesis can be achieved by simply dissolving the peptides in conditioned buffers. Simple as this system seems, it is nevertheless often influenced by the tendency of different batches of peptides to vary in their secondary structure and aggregation state [24]. The formation of plaques using peptides is a complex process, which is based on a continuous process involving protein misfolding, association and conformational rearrangements, and all depend on time, solvents and handling procedures. As a result, this error-prone process has a proclivity for inconsistent outcomes, hit compounds identified from such operations do not always have the same effect *in vivo*. Taken together, these limitations support the use of reliable source of A β plaques to ensure the reproducibility of the assay. In this work, we report for the first time the optimization of the HATCO assay using endogenous source of A β obtained from the brains of 5XFAD mice for a large-scale HTS operation. The APP/PS1 double transgenic mouse model coexpressed five familial AD (FAD) and additively increase A β ₄₂ production [25]. This animal model express A β at 1.5 months, and at the age of 6 months, massive levels of A β could be found in the subiculum, CA regions, and the cortex [25]. For HTS assays, all of the brain lysate was obtained from 8-month-old 5XFAD mice, and the control brain lysate was generated from age-matched wild-type (wt) mice. Since the library of screened compounds was selected based on ideal logP values for blood-brain barrier (BBB) penetration, but not on structural priority similar to known A β -binding molecules, we expect that the HATCO assay will enhance the likelihood to identify novel A β -binding molecules with diverse and untapped regions of chemical structures, thus offering the promise of advancing the chemical genetics for AD therapeutic drugs.

METHODS

Black 384-well low-flange, flat-bottom assay plates were obtained from Corning (New York). Brain lysates of wt and 5XFAD mice were prepared freshly from isolated brains. Thioflavin-T and DMSO were obtained from Sigma Aldrich (St Louis, MO). All other reagents/solvents were of analytical grade, and used as received from commercial source without further purifications.

The 5XFAD mice were maintained at Vanderbilt University under standard conditions, in a 12 h light/dark cycle and with free access to food and water. The 5XFAD mice overexpress both mutant human APP and PS1 express high APP levels correlating with high burden and accelerated accumulation of the A β . A colony of 5XFAD transgenic mice obtained from Jackson Laboratories was maintained by crossing 5XFAD mice with a wt C57BL/6J strain. The mice were genotyped by a standard polymerase chain reaction using DNA isolated from tail tips with the following primers: PSEN1 forward, 5'-TCATGACTATCCTCCTGGTGG-3' and reverse, 5'-CGTTATAGGTTTTAAACACTTCCCC-3'. For APP, forward, 5'-AGGACTGACCACTCGACCAG-3' and reverse, 5'-CGGGGGTCTAGTTCTGCAT-3'. We also genotyped mice for the presence of retinal degeneration Pde6brd1 mutation using forward, 5'-AAGCTAGCTGCAGTAACGCCATTT-3' and reverse, 5'-ACCTGCATGTGAACCCAGTATTCTATC-3'. After polymerase chain reaction

amplification, the DNA product of each reaction was analyzed by size fractionation through a 1% agarose gel; with Pde6b mutant = 560 bp, APP transgene = 377 bp and PSEN1 transgene = 608 bp.

Animal experiments were conducted per the guidelines established by Vanderbilt University's Institutional Animal Care and Use Committee. At the end of the study, animals were euthanized by cervical dislocation after sedated with isoflurane. Clinical signs were used to check after animal euthanasia including heartbeats, toe-pinching for reflection. Further, if animals show signs of illness (weight loss, food withdrawal, or infection) they will be sacrificed before the endpoints. All experimental procedures in this study were approved by the Vanderbilt University IACUC panel.

Brain lysate preparation

Excised midbrains of 5XFAD (8-month-old) or wt mice (age-matched) were homogenized for 5 min in 200 μ L of buffer comprised of 21.4 g sucrose, 5mL of 1Mtris base, and 0.5mL of 0.5 MEDTA/250mL DPBS using the T-25 basic Ultra-Turrax homogenizer. Additional 100 μ L aliquots of this buffer were then added to the homogenized sample and rehomogenized for 2 min until a total added volume of 800 μ L was attained. The sample was diluted to 4mL total volume using another buffer comprised of 5% BSA, and 0.03% Tween 20 in DPBS solution. Roche Complete protease inhibitor tablets were added to both buffers immediately prior to use (1 tablet/10mL buffer). The sample was centrifuged at 16,000 g for 30 min at 4°C. Aliquots of the supernatant were stored at -80°C until use.

HATCO HTS assay

To translate the assay initially performed on a cuvette to a 384-well format, the general protocol includes the following steps: A fresh stock solution of thioflavin-T was prepared in distilled and deionized water after passing through a micro filter. The concentration of total protein was measured according to Beer's law with an extinction coefficient of 26,620M⁻¹cm⁻¹ at 416 nm. The stock solution was stored in darkness using aluminum foil and kept at 4°C throughout the assay. Unless stated, each assaying well contained 10 μ M of thioflavin-T, 5 mg/mL of freshly isolated and homogenized brain lysate (5 μ L) obtained from 5XFAD mice and with 30 μ M of screening compounds. During the assay development, the positive control was used at a concentration of 100 μ M to achieve its maximal effect. For the negative control, the wells contained the same components minus positive control. The protein concentration of brain lysate throughout the study was normalized. After equilibration for 10min at room temperature, the FL signal of the sample was measured using a Biotek Synergy NEO plate reader at an emission lambda max of 485 nm using an excitation of 465 nm and the signals of the different conditions were analyzed. The final volume per well was 50 μ L. All assays were performed using a single dose of screening compounds in triplicate.

Once the preliminary hit compounds were identified, a cross-screening protocol was employed to validate the hits with duplicated concentrations for each hit. Combined with the data in the first run, each candidate has three screening values, which enable for statistical analysis. The best fit compounds will be selected for the next screening using nine

concentrations each to determine the tentative IC₅₀ values, adapted within the HATCO assay. In this data range, the original test concentration was also included in the analysis. In its finalized 384-well format, this procedure involves two phases. In the first phase of the cross-screening procedure, the absorbance profile of each hit compound was evaluated over the 250–900 nm wavelength range in a triplicate fashion using the Synergy Neo Multi-Microplate reader (BioTek). Scatterplots of absorbance as a function of wavelength were then used to qualitatively assess whether the analyzed hit compound significantly absorb at the relevant wavelengths. For example, as the way HATCO assay was designed, a reduction of the emission of thioflavin-T at 485 nm, due to displacement by the small molecule, was the primary indicator of A β -binding potential of the small molecule in the assay. If the hit compounds absorbing at this wavelength, they should be considered false positives. To quantitatively cross-screen for false positives secondary to interference of thioflavin-T absorbance or emission at 410 nm and 485 nm, respectively, a separate experimental design was employed. In this second phase of cross-screening, 125 nL of each hit compound (10milliM) was plated with 25 μ L of thioflavin-T (10 μ M) in triplicate fashion. As a control, 25 μ L of 10 μ M thioflavin-T was plated in the presence of 125 nL of DMSO. Again, the Synergy Neo Multi-Mode Microplate reader (BioTek) was employed to measure thioflavin-T's emission at 485 nm (excitation 410 nm). Statistical determination of false-positives was accomplished via a Paired 2-tailed Student T-test comparing of the mean fluorescence for the DMSO-treated control wells to that of wells containing thioflavin-T and the positive hit compound. In this work, a *p*-value <0.05 was used as the threshold statistic for classification as a false positive hit.

Compound library selection

We screened approximately 3500 compounds selected from NIH Clinical Collection I & II. Many of these small molecules have been used in humans for different purposes. Another library where compounds were selected from is Spectrum collection. This library has a wide range of analogs of biologically active and structurally diverse compounds. Those compounds have known properties and they can be used for prioritization in the screening tier, the compounds including 50% drug components, 30% natural product, and 20% other bioactive components.

All compounds were screened on 11 plates. The Z-prime value was calculated for each plate as a measure of assay quality. Outliers were selected by determining which wells exhibited FL values that fell outside of three standard deviations of the means of the FL value of all test compounds on individual plates.

Automatic dispenser and liquid

In a 384-well plate format using fluorescent readout, precise distribution of minutes amount of dye via robotic system as well as participating reagents and solvents is crucial to maintain the quality and reproducibility of the assay. Buffers and solvents were distributed across the plate via the ECHO liquid handling system, while thioflavin-T aliquots and 5XFAD brain lysate were dispensed using the Bravo system. All tip-based dispensers used tip-touch procedure to eliminate the possibility of reagent adhesion to the pipette tips. Each well is thoroughly mixed ten times by the automated liquid handling algorithm to increase

equilibrium. To reduce variability between samples secondary to variations in loading, deep-well source plates of thioflavin-T and 5XFAD lysate were generated using the COMBI liquid handling system. It is noteworthy that prior to loading the source plate using the COMBI liquid handling system, all reagents were vortexed for 10 min and sonicated 15 min at 4°C. While the tested compound library was dispensed using the ECHO liquid handling system. The positive control was designed as the observed FL signal in the presence of the promethazine, an A β inhibitor that we reported in the past [26] while negative control is associated with the observed FL signal in the absence of promethazine.

Estimation of the assay quality

Before screening a large library of compounds, pilot screens were used to assess the quality of the assay to predict whether the design is suitable for use in a full-scale and high-throughput manner. The Z-prime value incorporates both the dynamic range of the assay as well as well-to-well variability [27]. The Z-prime value is defined in terms of four parameters: the means (μ) and standard deviations (σ) of both the positive (p) and negative (n) controls (μ_p , σ_p , and μ_n , σ_n).

$$Z - \text{prime} = \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

where the means (μ) and standard deviations (σ) of both the positive (p) and negative (n) controls (μ_p , σ_p , and μ_n , σ_n). Z-prime values can be categorized into three groups corresponding to useless, marginal and excellent assays. A hypothetical ideal assay carries a Z-prime value of 1, the maximum Z-prime mathematically possible, whereas a Z-prime value less than 0 indicates that there is too much overlap between positive and negative controls for the assay to be useful. More commonly seen are assays characterized by Z-prime in the range of 0 to 1. In this range, an assay characterized by a Z-prime between 0–0.5 is considered marginally useful while a Z-prime above 0.5 qualifies an assay to be considered excellent.

RESULTS

The quality of brain lysate as a source of A β

The HATCO assay relies on specific interaction between thioflavin-T and A β present in the 5XFAD brain lysate resulting in a significantly dynamic signal change. Questions remain over whether small amount of brain lysate has enough A β to activate the signal or whether other proteins in the brain may also have affinity for thioflavin-T, thus producing false-positive signals. To answer these fundamental questions, serial dilutions of 5XFAD and wt brain lysates were incubated with thioflavin-T; as depicted graphically in Fig. 2, the FL at 485 nm emanated from thioflavin-T among the dilutions of 5XFAD are consistently higher than found in wt counterparts. Further, the signal was reciprocal to the concentration of brain lysate. It is noteworthy that the concentration of the brain lysate in each assay was quantified by BCA assay and distributed equally as designated in each well during the assay. During the validation process, over 50 different batches of mouse brain lysates have been prepared,

tested, and confirmed this observation, demonstrating the robustness, reliability and reproducibility of using 5XFAD brain lysate.

Solvent effect

During the course of work, we realized that although DMSO is a great solvent for amphiphilic molecules, which have structurally diverse functional groups, the major concern regarding the use of DMSO is the potential perturbation of the inter-molecular binding since it is an amphiphile. To address this concern, the proportion of DMSO was incrementally increased in the HATCO assay from 2.5% to 50% of the assays total volume. For example, in a 2.5% total volume condition, 625 nanoliters of DMSO was added to 20 μ L of 5XFAD lysate (5 mg/mL) and 5 μ L of thioflavin-T (10 μ M). Similarly for the 50% total volume DMSO condition, 12.5 μ L of DMSO was added to the same volumes of A μ -containing lysate and thioflavin-T. As graphically illustrated in Fig. 3, the inclusion of DMSO in the assay attenuates thioflavin-T fluorescence at 485 nm in a dose-dependent manner. Negligible loss of A μ -specific thioflavin-T signal at 485 nm up to 5% total volume of DMSO, with a dose-dependent decrease in signal at increasing higher concentrations. Cumulatively, the result suggests that the HATCO assay is compatible with the use of DMSO as a solvent up to a threshold tolerance 5% of the assay's total volume.

The HATCO assay can be optimized for HTS operation

The quality of the HATCO assay was assessed using the Z' value via triplicate 384-well plates in order to predict if it is suitable for an HTS setting. In this assay, all components of the assay were scaled down in term of volume and the precision optimized using the BRAVO liquid handling system; all dispensing operations were double-checked to ensure no presence of microdroplets on the pipette tips that might resulting in some outliers among the sample leading to an apparent unfavorable Z-factor. All wells were added with equal amount of brain lysate (5mg/mL; 20 μ L) followed by thioflavin-T (10 μ M, 5 μ L). In a typical "min-max" experiment in a checkerboard pattern, the wells correspond to "min" value contained only Thioflavin-T (control, in Fig. 4). The "max" wells contained brain lysates and Thioflavin-T. One of the first tasks is to determine the optimal incubation time and we found that after incubation, we not only can get ideal "min-max" values, but the *p* values are also remarkable at Z'-prime values >0.6 and a coefficient of variation ~ 4%. In this scale screening of 3,500 compounds using eleven 384-well plates, in which come overall average Z-prime value for 11 plates was 0.6215.

HATCO assay reveals novel A β -binding compounds

Using the optimized assay conditions, we screened a library of 3,500 structurally diverse compounds with diverse chemical constituents and classifications, including of which 50% are FDA-approved drugs, 30% natural products, and 20% random bioactively organic compounds. Through the assay, 44 hit compounds were identified in the primary screen (Supplementary Figure 1).

The screening assay was repeated for those hit compounds. In contrast to the previous experiment in which only a single well was allocated for each molecule, each of the hit compound was tested in duplicate in this cross-validation assay. As expected, the outcome

highlighted the reliability of the assay, 35 of 44 originally identified hit compounds exhibited similar properties as observed in the primary screen that they fit the classification of A β -binding molecules. Nevertheless, 20 compounds failed to reach statistical significance when the assay was repeated for these 35 compounds in a triplicate, due to exhibition of low percent of inhibition and displayed below the threshold with p values >0.5 and thus they were eliminated from further consideration.

HATCO competitive binding study

After primary and cross-screening experiments, only 15 compounds were qualified for the next screening operation involving competition binding assays, in which many and various concentrations of a putative hit compound ranging from 15–300 nM was tested as described in the primary assay, albeit in a duplicate fashion using the 384-well format. The addition of increasing concentrations of identified lead compounds to a mixture of fixed amount of brain lysate and thioflavin-T resulted in a dose-dependent decrease in fluorescence. To facilitate the generation of concentration-response curves, fluorescence at each data point was averaged on a compound-by-compound basis and plotted as a function of concentration. As expected, plotting of the resultant fluorescence data as a function of concentration in a logarithmic fashion revealed the characteristic sigmoidal curve. From this curve, IC₅₀ values for each compound were calculated by fitting the data to a 4-parameter logistical model in the GraphPad software package. Overall, 8 compounds were indentified as having potentials for use as A β -binding agents (Table 1).

DISCUSSION

Since the arrival of the A β cascade hypothesis as a potential mechanism of AD proposed over 26 years ago [28], only a small number of A β -binding molecules were identified. Some have been tested in clinical trials as contrast agents, while others were solely used *in vitro* due to poor BBB penetration. Since then, additional new A β -binding molecules were derived, albeit based on structural assimilation deduced from known compounds. This practice greatly limits our search for other chemical structures other than a few familiar A β binding motifs, via aromatic pi-stacking [29], or chemical structures, such as naphthalene [30] or benzothiazole [31]. The random screening of a large library of compounds with ideal BBB penetrant characteristics using endogenous A β source from the brain lysate will not only facilitate the identification of novel chemical motifs, but it also maximizes the chance for the hit compounds to be translated for *in vivo* applications. The work will overcome the common reproducibility issues of generating A β plaques using peptides *in vitro* via nucleation-polymerization process [32], which is stochastic in character and strongly affected by nonspecific interaction.

The HATCO HTS assay successfully identified a number of new and known compounds, which have diverse chemical structures with A β -binding capability. Specifically, the unique ring structures of these molecules distinguish themselves from furan, benzoquinone, pyridine or stilbene rings, usually found in most A β -binding molecules. The ability of the hit compounds to inhibit thioflavin-T from binding to A β resulted in significantly attenuated FL signal as defined in the HATCO assay was confirmed based on repeated triplicate assays. As

expected, the IC₅₀ values obtained from this assay are unsuitable for immediate future therapeutic plans, but rather, the potential lead compounds require further structure activity relationship (SAR) studies for optimization. Interestingly, the assay also showed that resveratrol, a chemical found in red wine also exhibits A β binding capability in a concentration-dependent manner (Supplementary Figure 2); the FL signal of thioflavin-T at 485 nm was reduced 4.4%, 25.9% and 51% at 100, 500, and 2000 μ M, respectively. This observation is in line with previously reported data demonstrating the role of resveratrol in modulating A β levels in patients with AD [33-36]. Although, it has modest IC₅₀ values (data not shown), the benefit of consuming natural products through healthy diets gains more ground in the campaign to prevent AD. Another notable message about this work is that the screened compounds have been preselected with ideal logp values thus identifying hits are BBB penetrants suitable for immediate *in vivo* assessment. Among the hit compounds, so far, we have only tested promethazine further *in vivo* experiments to demonstrate that the agent identified from the HATCO assay could cross the BBB and retained in the amyloid-burdened brain compared to normal brain and that its distribution within the brain corroborates with that of amyloid plaques [26]. The rest of the hit compounds will be assessed in the same manner in a near future.

In conclusion, the data in this work suggest that the HATCO assay is simple though robust and reliable. Once the condition is optimized, it can be used for screening a large library of compounds. To our knowledge, screening compounds for the brain, as shown in this experiment, is more challenging than others because one would have to face the solubility issues. We hope this assay will continue to yield more A β -binding molecules and eventually lead to the development of a new generation of therapy/diagnostic agents for AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Topologically activated property of Thioflavin-T. A) insignificant FL signal in free solution. B) Enhanced FL signal upon binding to Aβ plaques from 5XFAD brain lysate. C) An inhibitor competes with Thioflavin-T for Aβ resulted in reduced FL signal.

Fig. 2.
The FL signal intensity of Thioflavin-T was enhanced in proportion to the increasing concentration of 5XFAD brain lysate.

Fig. 3. Solvent effects of the HATCO assay. DMSO was included in the assay from 2.5%–50% of total volume. For each of these conditions, a FL profile secondary to excitation at 410 nm was collected on the sample across an emission range of 430–550 nm.

	MAX (5XFAD brain lysate)	MIN (wt brain lysate)	Percent coefficient of variation
Average	2098.73	1069.44	0.04
Standard deviation	85.02	46.68	0.04
Z'-factor			> 0.6215

Fig. 4. Scatter plot of the emission at 485 nm of Thioflavin-T measured from 384-well plate loaded with Thioflavin-T alone or Thioflavin-T with 5XFAD brain lysate corresponding to “min-max” conditions, respectively in a checkerboard pattern. The Z-prime values were assessed along with variations.

Table 1A β -binding molecules identified from HATCO assay after screening 3,500 compounds

Chemical Structure	Chemical Name	IC ₅₀ (μ M)	MW (g/mol)	CLogP
2-Hydroxy-3,5-dinitro-benzoic acid (5-nitro-furan-2-ylmethylene)-hydrazide	61.23	365.21	2.40
3-Phenylazo-pyridine-2,6-diamine (Phenazopyridine)	74.29	213.24	2.05
2, 4a, 6a, 9, 10, 12b, 14a-Heptamethyl-11-oxo-1, 2, 3, 4, 4a, 5, 6, 6a, 11, 12b, 13, 14, 14a, 14b-tetradecahydronicene-2-carbaldehyde	108.52	481.69	6.82
2, 3, 5, 6-Tetrachloro-[1, 4]benzoquinone (Chloranil)	90.85	245.88	3.3
2-Hydroxy-5-[4-(pyridine-2-ylsulfamoyl)-phenylazo]-benzoic acid (Azulfidine)	68.07	398.39	3.88
1, 6, 7, 1', 6', 7'-Hexahydroxy-5, 5'-diisopropyl-3, 3'-dimethyl-[2, 2']binaphthalenyl-8, 8'-dicarbaldehyde (Gossypol)	84.17	518.55	5.36
(E)-5-(4-hydroxystyryl)benzene-1, 3-diol (Resveratrol)	a	228.25	2.83
N, N-dimethyl-1-(10H-phenothiazin-10-yl)propan-2-amine (Promethazine)	53.63	284.42	4.89