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ARTICLE TYPE

Synthesis and study of 2-Acetyl amino-3-[4-(2-amino-5-sulfo-phenylazo)-phenyl]-propionic acid: A new class of inhibitor for Hen egg white lysozyme amyloidogenesis

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The compounds capable of blocking the aggregation of amyloidogenic proteins may have therapeutic potentials. We report on the synthesis of 2-Acetyl amino-3-[4-(2-amino-5-sulfo-phenylazo)-phenyl]-propionic acid as HEWL (Hen egg white lysozyme) amyloid inhibitor starting from phenylalanine. The compound arrests the monomers and exhibits anti-aggregating activity. Moreover, the acetyl derivative **1** served as better inhibitor than the trifluoro acetyl derivative **2** against in vitro amyloid fibrillogenesis of HEWL model system.

Introduction

A significant number of fatal human diseases including Alzheimer's systemic amyloidoses, Parkinson's disease, Huntington's disease, type 2 diabetes, Creutzfeldt–Jakob disease and prion disease are associated with the deposition of protein fibrils in various organs.[1-7] These amyloidogenic diseases individually have their unique neuropathological, clinical and biochemical characteristics. Up to now, more than 40 different human proteins and peptides, including transthyretin, α-synuclein, β2-macroglobulin and β-amyloid have been isolated as the misfolded fibrillar components of disease related amyloid deposits.[8-12] The corresponding amyloidogenic precursor proteins associated with various diseases are not related to each other in sequence or in structure, but the amyloid fibrils formed share some common ultra structural and morphological characteristics. Despite extensive investigations on amyloid fibril formation, the details of aggregation process at atomic level are still unknown due to insolubility and noncrystallinity of the amyloid plaque. So, it is important to develop successful therapeutic strategies for inhibiting amyloid formation to prevent amyloid related diseases.

Hen egg-white lysozyme, an enzyme with 129 amino acids that lyses the cell walls of bacteria, has four disulfide bonds and adopts mainly helical conformation (~30% α-helix; ~6% β-sheet).[13] Though there is only ~40% sequence similarity, hen egg white lysozyme retain a high degree of structural homology with the human lysozyme which has been found to form amyloid fibrils that are responsible for hereditary non-neuropathic systemic amyloidosis.[14]

The protein aggregation into soluble oligomers, protofibrils, mature amyloid fibrils, or a combination of these is a requirement for toxicity. Hence, inhibition of amyloid aggregation may represent a possible therapeutic strategy for the prevention and treatment of amyloid diseases. Goda *et al.* has reported that hen egg white lysozyme forms amyloid fibrils in a highly concentrated ethanol solution.[15] They have also reported that addition of salt further promotes the association of the proteins. A number of diverse small molecules including synthetic peptides, other chemical compounds also have been synthesized to inhibit or reduce the aggregation of various proteins, particularly in relation to Aβ deposition, aggregation of lysozyme and transthyretin.[16-17] Ducruix *et al.* has reported that the cations and anions have different effects on the protein–protein interactions of HEWL.[18]

In this context, we have designed and synthesized 2-Acetyl amino-3-[4-(2-amino-5-sulfo-phenylazo)-phenyl]-propionic acid containing cationic and anionic parts in the same moity like Congo red to inhibit or reduce the aggregation of various proteins (Figure 1).[19] In this report, utilizing hen egg white lysozymes as a model system[20], attempts were made to investigate the anti-amyloidogenic and anti-aggregating activities of modified amino acids against the formation of aggregated species or amyloid fibrils of lysozyme.[20] Through UV-Visible, fluorescence and

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† Electronic Supplementary Information (ESI) available: Synthesis and characterization of compound **1** and **2**, ^{1}H NMR, ^{13}C NMR, ESI Table 1, Fugures ESI S1-2, Figure S1-S20. other electronic format see DOI: 10.1039/b000000x/

TCSPC spectroscopy interactions of HEWL monomers with compound **1** or **2** has been demonstrated. The anti amyloidogenic behavior of both the compounds were confirmed by Congo red, Thioflavin T binding assay, CD spectroscopy, FT-IR spectroscopy, atomic force microscopy and cross-polarized optical microscopic studies. Moreover, compound **1** (acetyl derivative) served as more effective inhibitor in comparison with compound **2** (trifluroacetyl derivative) against amyloid fibril formation of HEWL.

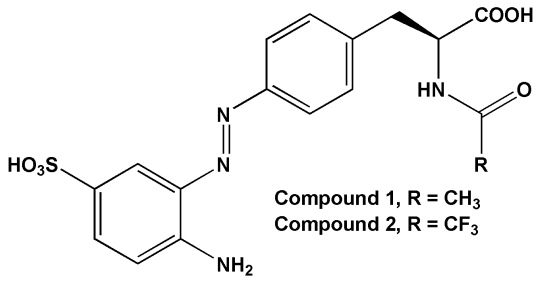


Fig. 1 The schematic presentation of reported inhibitors **1** and **2**.

Experimental section

Preparation of lysozyme solution

10 mL stock solution containing lysozyme at a concentration 2 mg/mL in double distilled water at pH 2.0 (by adding required amount of HCl) was prepared. In each of the three vials 1.8 mL of this stock solution was added. In the first vial 200 microlitre of acidic solvent was added. In the second and third vial 200 microlitre of compound **1** and **2** solution (2×10^{-4} M in same acidic water) were added seperately. These solution were incubated at 65ºC for 15 days. These solutions were used for different experiments.

NMR experiments

All NMR studies were carried out on Jeol JNM-ECS 400 MHz spectrometer at 298 K. Compound concentrations were in the range 1–10 mmol in $CDCl_3$ and $(CD_3)_2SO$.

FT-IR spectroscopy

All reported solid-state FT-IR spectra were obtained with a Perkin Elmer Spectrum RX1 spectrophotometer. The solutions were lyophilized and spectra were taken by KBr disk technique.

Mass spectrometry

Mass spectra were recorded on a Q-Tof Micro YA263 high-resolution (Waters Corporation) mass spectrometer by positive-mode electro spray ionization.

UV/Vis spectroscopy

UV/Vis absorption spectra were recorded on a UV/Vis spectrophotometer (Hitachi). For binding of HEWL monomers with compound **1** and **2** to 2 mL of HEWL solution (1×10^{-4} mg/mL) in acidic water (pH=2.0) 20 µL of both the compounds (2×10^{-4} M) were added gradually and spectra were recorded. For reference (blank) 2 mL of HEWL solution (1×10^{-4} mg/mL) at pH=2.0 has been used. For congo red binding assay 80µL of each growing solution were added to 2 mL of congo red solution (2.15×10^{-5} M) in 0.1 M sodium phosphate buffer pH = 7.4 and incubated for 20 minutes and data were collected.

Fluorescence spectroscopy

For quenching experiments fluorescence spectrum was recorded on a fluorescence spectrometer (Perkin Elmer) in aqueous medium (pH= 2.0) at excitation wavelength 293 nm (slit 5/5). To 2 mL of HEWL solution (1×10^{-4} mg/mL) 20 µL of both the compounds (2×10^{-4} M) were added individually and spectra were recorded. For reference (blank) 2 mL of HEWL solution (1×10^{-4} mg/mL) at pH=2.0 has been used.

For ThT binding study to 2.5 mL of ThT solution (2.5×10^{-5} M) in 0.1 M sodium phosphate buffer pH= 7.4) 40µL of each growing solution at different time interval were added and incubated for 20 min and data were collected. Each time freshly prepared ThT solution in sodium phosphate buffer has been used. Slit (5/10), Excitation wavelength= 410 nm

Circular Dichroic (CD) spectroscopy

CD study of hen egg white lysozyme fibrils with or without modified amino acids in aqueous medium (pH= 2.0) was carried out on a JASCO J-815-150S instrument at a temperature of 25°C. 100 microlitre of each solution (15 days incubation) was diluted to 500 microlitre with same acidic water (pH= 2.0) and spectra were recorded.

Atomic force microscopy

The morphology of the reported compounds were investigated by atomic force microscopy (AFM). A small amount of three solution incubated at different time were diluted with acidic water (pH= 2.0) and drop cashed on clean microscope cover glass and then dried by slow evaporation. The materials were then allowed to dry under vacuum at 30ºC for two days. Images were taken with an NTMDT instrument, model no. AP-0100 in semicontact-mode.

Time Correlated Single Photon Counting (TCSPC) spectroscopy

The fluorescence lifetimes were measured by the method of timecorrelated single photon counting using a picosecond spectrofluorometer from Horiba Jobin Yvon IBH. The instrument was equipped with FluoroHub single photon counting controller, Fluoro3PS precision photomultiplier power supply, and FCMCP-50SC MCP-PMT detection unit. A laser head or a nano- LED pulsed diode powered by a pulsed diode controller (IBH) was used as the excitation light source. The life time of free native HEWL was recorded at concentration (1×10^{-4} mg/mL in acidic water (pH=2.0). For complex 100µL of both the compounds (2×10^{-4} M) were added individually with free HEWL solution and data were recorded. The excitation was done at 280 nm wavelength and emission were recorded at 350 nm for all the samples. The typical response time of this laser head was <100 ps. To calculate the lifetime, the fluorescence decay curves were analyzed by an iterative fitting program provided by IBH. The fluorescence decay curves were fitted with the equation:

$$I(t) = \sum_{i=1}^{n} Aiexp(-\frac{t}{\tau i})$$

Where Ai and τi are the amplitude and lifetime of the ith fluorescence component respectively and n is the number of fluorescence exponentials required for the best nonlinear least squares (NLLS) fitting of the fluorescence decay curves.

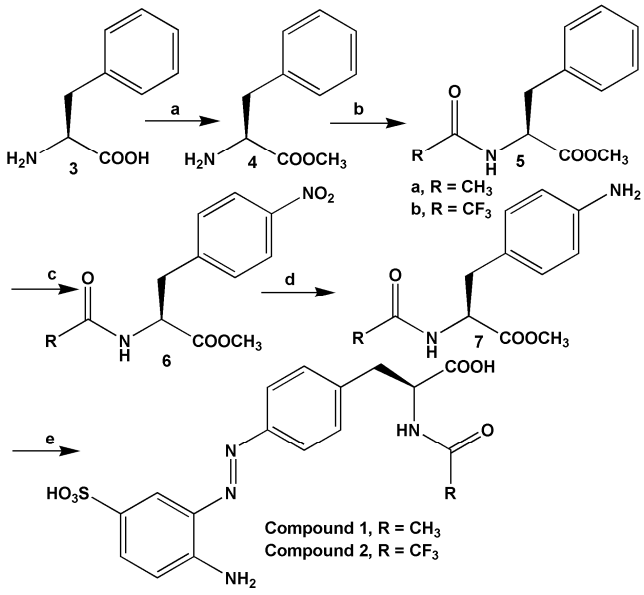
Congo red assay

An alkaline saturated Congo red solution was prepared. The

fibrils were stained by alkaline Congo red solution (80% methanol/20% glass distilled water containing 10 ml of 1% NaOH) for 2 min and then the excess stain (Congo red) was removed by rinsing the stained fibril with 80% methanol/20% glass distilled water solution for several times. The stained fibrils were dried under vacuum at room temperature for 24 h, then visualized at 40× magnification and birefringence was observed between crossed polarizers (Olympus optical microscope equipped with polarizer and CCD camera).

Results and Discussion

The compounds **1** and **2** were synthesized according to route depicted in Scheme 1. In the first step methyl ester protected phenylalanine **4** was obtained by treating phenylalanine with $SOCl_2$ and methanol. The amine group was protected by acetylation. The nitration followed by the reduction developed the amine **7**. The compounds **1** and **2** were obtained by diazo coupling reaction of **7** with sulfanilic acid. Considering the binding propensities of Congo red with amyloid fibril, the compounds were designed such that the compounds have some structural and functional similarity with Congo Red. The trifluoromethyl group has significant electronegativity and often used a bioisostere to create derivatives by replacing a methyl group. So, we have prepared a trifluoromethyl derivative **2** of compound **1**. The toxicity of the compounds **1** and **2** towards neuronal cells were investigated by MTT assays and the compounds failed to show any significant neuronal toxicity. The anti-aggregating activities of both the compounds were studied through different spectroscopic and microscopic techniques.



Scheme 1 Reagents and conditions: a) (i) $SOCl_2$, MeOH, 0°C, (ii) $NaHCO_3$, 83%; b) for R = CH_3, Ac_2O, pyridine, rt, 84%; for R = CF_3, CF_3COOEt, ET_3N, MeOH, rt,72%; (c) 1:1 conc. HNO_3: conc. H_2SO_4, 41%; d) [H], 51%; e) $NaNO_2$, dil. HCl, sulfanilic acid, 5°C, 90%.

Initially, the interaction of native hen egg white lysozyme with the two synthetic compounds was investigated by UV-Vis spectroscopy. From Fig. 2, the blue shift of maxima exhibits that the absorption of lysozyme gradually increases with the increase in concentration of the compounds **1** or **2**. The absorption maxima shifted from 277 nm to 258 nm upon addition of 100 µL of 2×10^{-4} M compound **1** where as the maxima shifted from 277 nm to 265 nm upon addition of same amount of compound **2** solution of same concentration. Moreover, there is a band at 380 nm (ESI Figure 1). This blue shift may be due to interaction of compound **1** or **2** with Tryp62 or Tryp108 of HEWL through various non-covalent interactions. There is a minor overlapping of absorption bands that may influence the HEWL maximum wavelength shift.

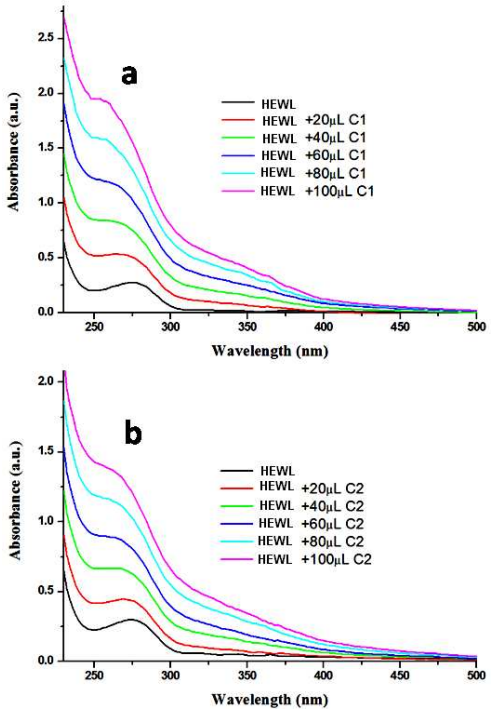


Fig. 2 UV-Visible spectra of lysozyme (1×10^{-4} mg/ mL) after addition of different amount of (a) Compound **1** (b) Compound **2** at pH= 2.0 Concentration of both the compounds were 2×10^{-4} M in aqueous solution at pH=2.0.

Fluorescence quenching measurements have been widely used to study the interaction of various organic compounds with hen egg white lysozyme and other proteins.[21-22] From the literature Tryp62 and Tryp108 are the most dominating fluorophores and positioned at the active sites which mainly bind with substrates. The Tryp28 and Tryp111 are present at the hydrophobic matrix region, which stabilize the protein structure.[23] Hence most of the outer substrates bind with Tryp62 or Tryp108 that results the change in its spectroscopic properties.[24] The fluorescence experiments have performed on hen egg lysozyme in presence of different amount of compound **1** or **2**. Fig. 3 shows that the fluorescence intensity of hen egg lysozyme gradually quenched by increasing amount of compound **1** or **2** with large red shift of fluorescence maxima. The maxima shifted from 350 nm to 385 nm on addition of 100 µL of 2x 10^{-4} M compound **1**. But in presence of same amount of compound **2** the maxima of lysozyme shifted from 350 nm to 374 nm only. The fluorescence quenching indicates strong interactions of

lysozyme with compound **1** and **2.** From UV-Visible and fluorescence spectra it may be conclude that compound **1** interact with HEWL more efficiently than compound **2**. We have also calculated binding constant for both the compounds using the equation

$$\log[F_0/F\text{-}1] = \log K + n \log[Q]$$

Where F_0 and F is the fluorescence intensity of fluorophore in the absence and in the presence of quencher, respectively. K, n, [Q]

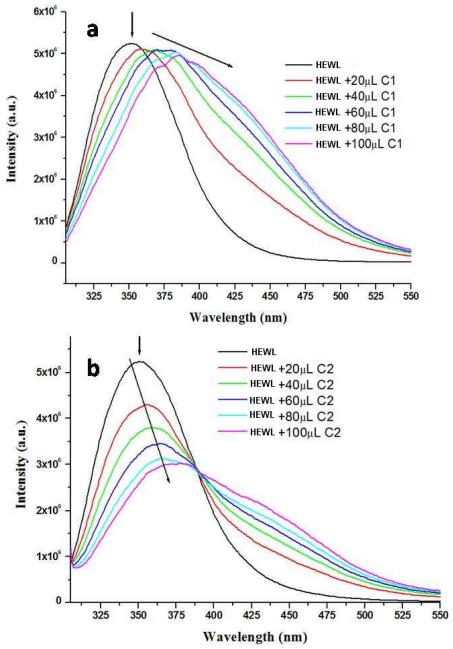


Fig. 3 Fluorescence spectra of HEWL (1×10^{-4} mg/ mL) after addition of different amount of (a) Compound **1** (b) Compound **2** [pH= 2.0] Concentration of both the compounds were 2×10^{-4} M in aqueous solution at pH=2.0, λ_{ex}= 293 nm.

are binding constant, number of binding site and concentration of compound **1** or **2**. From $\log[F_0/F\text{-}1]$ vs $\log[Q]$ curve (ESI Fig. 2) we have calculated binding constant for HEWL-compound **1** and HEWL-compound **2** complex. The binding constant for lysozyme-compound **1** complex is 7.32×10^5 where as for compound **2** it is 5.52 x 10^4 (ESI table 1). Hence binding affinity of compound **1** is stronger than that of compound **2**.

To confirm the intermolecular interaction of compound **1** and **2** with HEWL monomer time resolved fluorescence spectroscopy were done for HEWL, HEWL-compound **1** and HEWL-compound **2** complex using TCSPC (time-correlated single photon count) technique.[25] Fig. 4 shows the decay curves for HEWL monomer complex with both the compounds along with lysozyme monomer. All of the decay curves fitted well with biexponential decay pattern. The fitted decay parameters are listed in table 1.

Table 1: Nonlinear least squares (NLLS) fitting parameters obtained from fluorescence decay curves (Fig. 4)

Sample	τ_1 (ns)	A_1	τ_2(ns)	A_2	$\chi2$
HEWL	1.43	0.53	3.41	0.46	1.09
HEWL-compound **1**	1.44	0.72	5.29	0.27	1.18
HEWL-compound **2**	1.32	0.70	3.72	0.29	1.15

Comparing the data listed in the table 1, the lifetime of one component (τ_1) is almost same for all the three cases. But lifetime of other component (τ_2) increased upon complexation of both the compounds indicates formation of complex of HEWL with compound **1** and **2**.

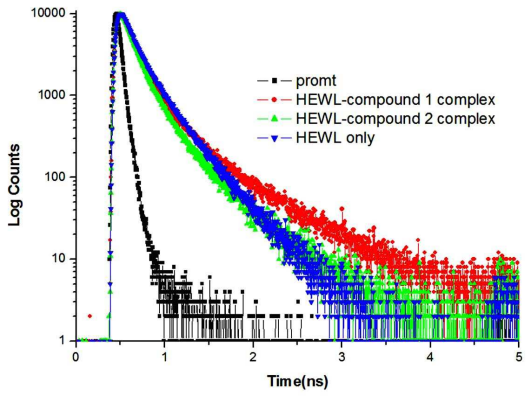


Fig. 4 Time dependent decay curves of hen egg white lysozyme (blue), lysozyme-compound **1** conjugate (red) and lysozyme-compound **2** conjugate (green). Black curve indicates promt i.e. instrumental response function (IRF).

To determine the inhibitory effect of these two compounds on HEWL fibrilization, HEWL was incubated in absence and presence of compound **1** or **2** at pH=2.0 at 65^0C for two weeks. The inhibitory effects were tested by Congo red and Thioflavin-T binding studies.[26] Fig. 5 shows that absorption of Congo red alone, in presence of HEWL, HEWL-Compound **1** and HEWL-Compound **2** complex after incubation for 2 weeks. From Fig. 5, with HEWL the intensity of Congo red increased and maxima shifted to higher wavelength with appearance of a new peak at 537 nm. For the HEWL-Compound **1** or **2** complexes, absorbance increases less than that of control with almost no change of peak position of Congo red. For HEWL-Compound **1** complex intensity of Congo red increase less than that of HEWL-Compound **2** complex.

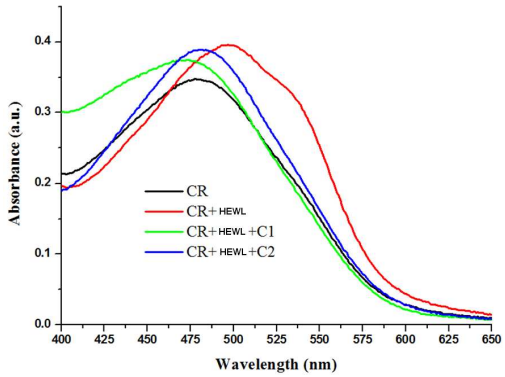


Fig. 5 UV-Visible spectra of Congo red (black), Congo red in presence of HEWL (red), HEWL-Compound **1** complex (green) and HEWL-

Compound **2** complex (blue). [Sodium phosphate buffer pH=7.4, Concentration of Congo red was 2.15 x 10^{-5} M, 80μL stock solution was added for each case].

The effect of these two compounds on the kinetics of HEWL amyloid formation was also investigated through monitoring maximal Thioflavin T emission intensity over the course of 4 days.[26c] Fig. 6 shows the relative intensity of Thioflavin T after addition of same amount of three incubated solutions at different time intervals. From Fig. 6, the fibrilization rate of HEWL decreases in presence of compound **1** or **2**. This bar diagram also indicates that inhibitory effect of compound **1** is more than that of compound **2.**

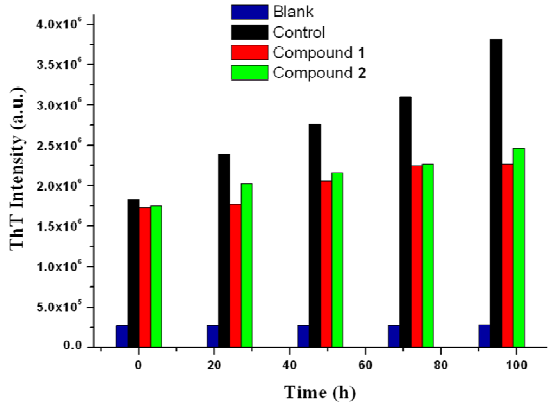


Fig. 6 ThT binding assay of (a) HEWL (black), (b) in presence of compound **1** (2×10^{-4} M) (red) and (c) in presence of compound **2** (2×10^{-4} M) (green) at different time intervals. ThT concentration 2.5 x 10^{-5} M in 0.1 M sodium phosphate buffer pH= 7.4, λ_{ex}= 410 nm.

CD is an excellent method of determining the backbone structural transition of proteins by interaction with other compounds. The far UV-circular dichroism spectra of hen egg white lysozyme amyloid fibril[26] in absence and in presence of compounds **1** or **2** after incubation at 65°C for 15 days is depicted in Fig. 7. The HEWL solution incubated for 15 days in absence of any inhibitors shows a net ellipticity transition from a random organization with a characteristic peak at 195-200 nm (and a positive transition about 230 nm) to an ordered beta-sheet-rich structure with a negative peak at 217 nm and a positive peak at 195 nm, typical of amyloid behavior.[27] But in presence of compound **1** HEWL solution shows the negative peaks at 206 nm, 212 nm and a shoulder at 224 nm indicates α-helical conformation. Compound **2** also shows similar result. Hence, both the compounds inhibit the β-sheet aggregation of HEWL.

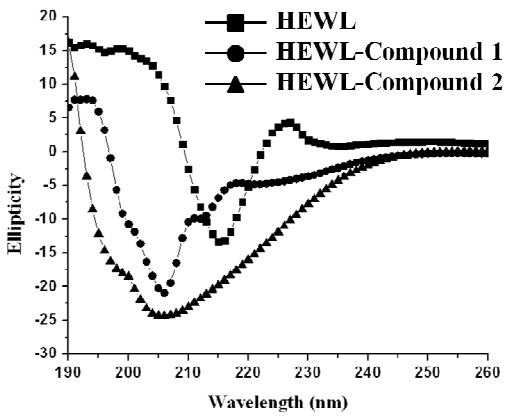


Fig. 7 Far UV-CD spectra of HEWL (squere), HEWL in presence of compound **1** (circle) and compound **2** (triangle) after incubation at pH=2.0 at 65°C for 15 days.

Furhter information on conformational preferences of hen egg white lysozyme amyloid fibril at different condition was obtained from FTIR studies.[28-29] Fig. 8 shows the FTIR spectra of HEWL alone, HEWL-compound **1**, HEWL-compound **2** complex incubated at pH=2.0 at 65°C for 15 days. FTIR spectra of lysozyme amyloid fibril (Figure 8a) have amide I bands at 1633 cm^{-1} and amide II band at 1503 cm^{-1} indicating presence of β sheet conformation in aggregated form.[30] But for HEWL-compound **1** complex (Fig. 8b) and HEWL-compound **2** (Fig. 8c) the amide I band occur at 1654 cm^{-1} and 1675 cm^{-1} and amide II band occur at 1539 cm^{-1} and 1540 cm^{-1} respectively which indicate α-helix or random coil conformation in the complex.

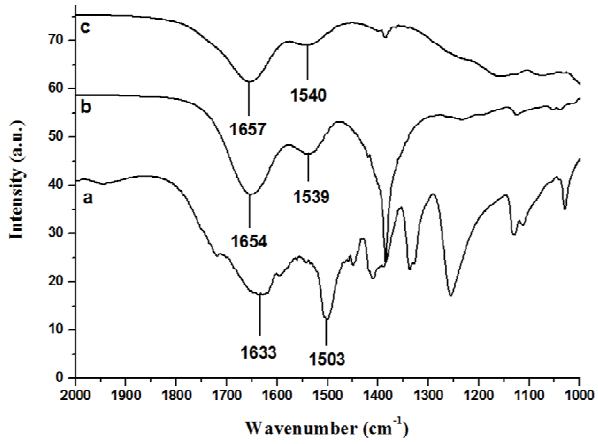


Fig. 8 FT-IR spectrum of (a) hen egg white lysozyme amyloid fibril, (b) lysozyme-compound **1** conjugate and (c) lysozyme compound **2** conjugate.

To further investigate the morphological change during fibrilization of hen egg white lysozyme in presence of compound **1** or **2**, the AFM studies have been performed.[31] To insight into fibrilization process during amyloid formation of hen egg white lysozyme , the AFM has been performed with sample having different incubation time. Figure 9a, 9b and 9c show the AFM images of HEWL amyloid obtained by incubation at 65°C at pH 2.0 for 2 days, 5 days and 15 days respectively in absence of compound **1** or **2**. From these micrographs, the monomer of

HEWL is gradually self-aggregated to form fiber like structure with increasing of incubation time. Fibriller aggregates have diameter range 50-80 nm. Fig. 9d and 9e show the morphology of HEWL aggregrate in presence of compound **1** and compound **2** respectively, after incubation under same condition for 15 days. HEWL-compound **1** or **2** conjugate exhibit granular aggregate with a diameter ca 100 nm (Figure 9d and 9e respectively).

The morphological resemblance between these hen egg white lysozyme-compound **1** or **2** conjugate and lysozyme amyloid stained by Congo red (CR) binding assay has been performed.[32-33] The aggregated fibrils obtained from the hen egg white lysozyme at pH 2 after incubation for 15 days, were stained by Congo red and were observed through cross polarizers. Figure 10a exhibits the green-gold birefringence of Congo red bound fibrils of lysozyme amyloid under cross polarizer.[34-35] But lysozyme-compound **1** or **2** conjugate exhibits scattered green-gold birefringence under cross polarizer (Figure 10b and 10c respectively).

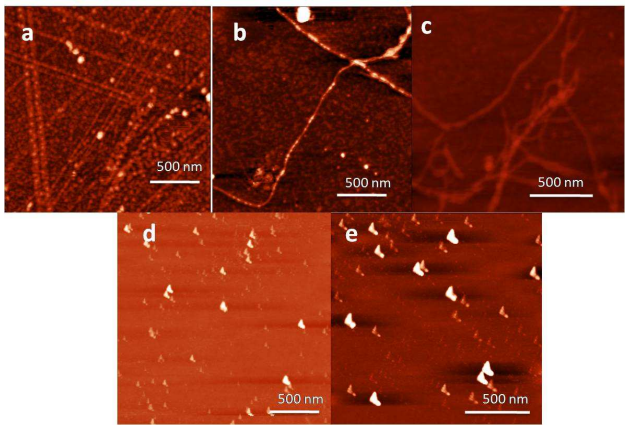


Fig. 9 AFM images of HWEL alone at incubation time of (a) 2 days (b) 5 days and (c) 15 days. (d) and (e) show AFM image of lysozyme-compound **1** conjugate and lysozyme-compound **2** conjugate after 15 days incubation under same conditions.

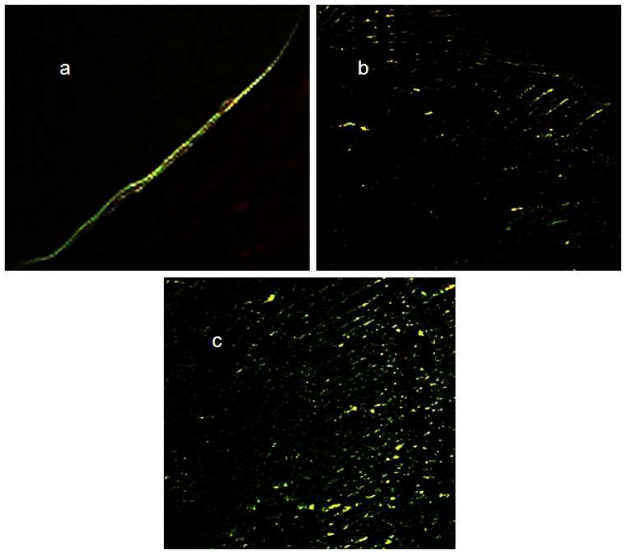


Fig. 10 (a) Congo red assay of hen egg white lysozyme fibriller aggregates showing green-gold birefringence under cross polarizer. (b) and (c) Scattered green-gold birefringence of (b) lysozyme-compound **1** conjugate and (c) lysozyme-compound **2** conjugate under cross polarizer.

Conclusions

In conclusion, the design, synthesis and amyloid inhibition propensities of 2-Acetyl amino-3-[4-(2-amino-5-sulfo-phenylazo)-phenyl]-propionic acid has been reported. The compounds have structural and functional similarity with Congo Red and contain cation and anions. The compound arrests the monomers and exhibits anti-aggregating activity against hen egg white lysozyme in vitro. Moreover, acetyl derivative served as more effective inhibitor in comparison with the trifluro acetyl derivative. This result shed some light on the rational design of effective therapeutic agent for amyloidogenic diseases and may foster the in vivo studies.

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References

1. S. Y. Tan and M. B. Pepys, *Histopathology*, 1994, **25**, 403–414.
2. R. W. Carrell and D. A. Lomas, *Lancet*, 1997, **350**, 134–138.
3. M. Sunde and C. C. F. Blake, *Quart. Rev. Biophys*., 1998, **31**, 1–39.
4. J. C. Rochet and P. T. Lansbury, *Curr. Opin. Struct. Biol*., 2000, **10**, 60–68.
5. A. L. Gharibyan, V. Zamotin, K. Yanamandra, O. S. Moskaleva, B. A. Margulis, I. A Kostanyan and L. A. Morozova-Roche, *J. Mol. Biol*., 2007, **365**, 1337–1349.
6. A. T. Petkova, R. D. Leapman, Z. Guo, W. M. Yau, M. P. Mattson and R. Tycko, *Science,* 2005, **307**, 262–265.
7. M. F Mossuto, A. Dhulesia, G. Devlin, E. Frare, J. R. Kumita, , P. P. D. Laureto, M. Dumoulin, A. Fontana, C. M. Dobson and X. Salvatella, *J. Mol. Biol*., 2010. **402**, 783–796.
8. C. M. Dobson, *Semin. Cell Dev. Biol*., 2004, **15**, 3-16.
9. J. W. Kelly, *Curr. Opin. Struct. Biol*., 1998, **8**, 101-106.
10. J. W. Kelly, *Proc. Natl. Acad. Sci. U.S.A*., 1998, **95,** 930-932.
11. P. T. Lansbury, Jr. *Proc. Natl. Acad. Sci. U.S.A.,* 1999, **96**, 3342-3344.
12. V. N. Uversky and A. L. Fink, Biochim. Biophys. Acta., 2004, **1698**, 131-153.
13. M. C. Vaney, S. Maignan, M. Ries-Kautt and A. Ducriux, *Acta Crystallogr. D: Biol. Crystallogr,* 1996, **52**, 505-517.
14. M. B. Pepys, P. N. Hawkins, D. R. Booth, D. M. Vigushin, G. A. Tennent, A. K. Soutar, N. Totty, O. Nguyen, C. C. F. Blake, T. Terry, G. Feest, A. M. Zalin and J. J. Hsuan. *Nature*, 1993, **362**, 553-557.
15. S. Goda, K. Takano, Y. Yamagata, R. Nagata, H. Akutsu, Maki, K. Namba, & K. Yutani, *Protein Sci*., 2000, **9**, 369–375.
16. (*a*) C. I. Stains, K. Mondal, and I. Ghosh, *ChemMedChem* 2007, **2**, 1674 – 1692 (*b*) F. G. DeFelice, J. C. Houzel, J. Garcia-Abreu, P. R. F. Louzada, R. C. Afonso, N. L. Meirelles, R. Lent, M. V. Neto and S. T. Ferreira, *FASEB J.,* 2001, **15**, 297–1299 (*c*) F. G. DeFelice, P. T. Velasco, M. P. Lambert, K. Viola, S. J. Fernandez, S. T. Ferreira

and W. L. Klein, *J. Biol. Chem.,* 2007, **282**, 11590–11601 (*d*) M. N. N. Vieira, J. D. Figueroa-Villar, M. N. L. Meirelles, S. T. Ferreira and F. G. De Felice, *Cell Biochem Biophys,* 2006, **44**, 549–553 (*e*) M. N. N. Vieira, L. Forny-Germano, L. M. Saraiva, A. Sebollela, A. M. B. Martinez, J-C Houzel, F. G. DeFelice and S. T. Ferreira (2007) *J. Neurochem* 2007, **103**, 736–748

17. (*a*) P. Raghu, G. B. Reddy and B. Sivakumar, *Arch. Biochem. Biophys.* 2002, **400**, 43–47 (*b*) Z. Gazova, A. Bellova, Z. Daxnerova, J. Imrich, P. Kristian, J. Tomascikova, J. Bagelova, D. Fedunova and M. Antalik, *Eur. Biophys. J.,* 2008 **37**, 1261–1270 (*c*) M. Pickhardt, J. Biernat, I. Khlistunova, Y-P. Wang, Z. Gazova, E-M. Mandelkow and E. Mandelkow, *Curr. Alzheimer Res.* 2007, **4**, 397–402 (*d*) M, Pickhardt. Z. Gazova, M. von Bergen, I. Khlistunova, Y. Wang, A. Hascher, E. M. Mandelkow, J. Biernat and E. Mandelkow, *J. Biol. Chem.* 2005, **280**, 3628–3635 (*e*) C. M. Wischik, P. C. Edwards, R. Y. K. Lai, M. Roth and C. R. Harrington, *PNAS,* 1996, **93**, 11213–11218 (*f*) C. Korth, B. C. H. May, F. E. Cohen and S. B. Prusiner PNAS, 2001, **98**, 9836–9841.
18. A. Ducruix, J. P. Guilloteau, M. Rie`s-Kautt and A. Tardieu, *J. Crystal Growth*, 1996, **168**, 28–39.
19. (*a*) T. Seki , H. Takahashi , K. Yamamoto , K. Ogawa , T. Onji, N. Adachi, S. Tanaka, I. Hide, N, Saito, N. Sakai, *J. Pharmacol Sci.* 2010, **114**, 206-16. (*b*) C. Wu, J. Scott, J. E. Shea, *Biophys. J.* 2012, **103**, 550-557.
20. (*a*) A. L. Gharibyan, V. Zamotin, K. Yanamandra, O. S. Moskaleva, B. A. Margulis, I. A. Kostanyan and L. A. Morozova-Roche, *J. Mol. Biol*., 2007, **365**, 1337–1349. (*b*) E. Frare, P. P. de Laureto, J. Zurdo, C. M. Dobson and A. Fontana, *J. Mol. Biol.*, 2004, **340**, 1153–1165.
21. (*a*) F-F. Chen, Y-N. Tang, S-L. Wang and H-W. Gao, *Amino Acids*, 2009, **36**, 399–407 (*b*) W. Wan, W. Min, J. Chen, X. Wu and Z. Hu, *Journal of Luminescence,* 2011, **131**, 820–824, (*c*) Q. Yue, L. Niu, X. Li, X. Shao, X. Xie and Z. Song, *J. Fluoresc.,* 2008, **18**, 11–15
22. (*a*) S. Bi, D. Song, Y. Tian, X. Zhou, Z. Liu and H. Zhang, *Spectrochim. Acta: Part A,* 2005, **61**, 629–636. (*b*) M. T. Shaikh, J. Seetharamappa, S. Ashoka and P. B. Kandagal, *Dyes Pigments,* 2007, **73**, 211–216. (*c*) X. Z. Feng, Z. Lin, L. J. Yang, C. Wang, C. Bai, *Talanta,* 1998, **47**, 1223–1229.
23. G. Paramaguru, A. Kathiravan, S. Selvaraj, P. Venuvanalingam and R. Renganathan, *Journal of Hazardous Materials,* 2010, **175**, 985–991.
24. R. S. Mulvey, R. J. Gaultieri, and S. Beychok, *Biochemistry*, 1973, **12**, 2683-2690.
25. E. Nishimoto, S. Yamashita, N. Yamashi and T. Imoto, *Biosci. Biotechnol. Biochem.* 1999, **63**, 329-336.
26. (*a*) D. Morshedi, N. Rezaei-Ghaleh, A. Ebrahim-Habibi, S. Ahmadian and M. Nemat-Gorgani, *FEBS Journal*, 2007, **274,** 6415–6425. (*b*) F. Tanaka, L. S. Forster, P. K. Pal, and J. A. Rupley, *The Journal of Biological Chemistry*, 1975, **250**, 6977-6982. (*c*) S. A. Hudson, H. Ecroyd, T. W. Kee, J. A. Carver, *FEBS J.* 2009, **276**, 5960–5972.
27. T. Knubovets, J. J. Osterhout, P. J. Connolly, A. M. Klibanov, *Proc. Natl. Acad. Sci. USA*. 1999, **96**, 1262–1267.
28. C. Toniolo and M. Palumbo, *Biopolymers*, 1977, **16**, 219-224.
29. V. Moretto, M. Crisma, G. M. Bonora, C. Toniolo, H. Balaram and P. Balaram, *Macromolecules*, 1989*,* **22**, 2939-2944.
30. (a) G. P. Dado and S. H. Gellman, *J. Am. Chem. Soc.*, 1994, **116**, 1054-1062. (*b*) S. E. Blondelle, B. Forood, R. A. Houghten & E. Peraz-Paya, *Biochemistry*, 1997, **36**, 8393-8400. (*c*) P. I. Haris and D. Chapman, *Biopolymers*, 1995, **37**, 251-263.
31. F. Yang Jr., M. Zhang, B-R. Zhou, J. Chen and Y. Liang, *J. Mol. Biol.*, 2006, **362**, 821–834.
32. (*a*) D. L. Taylor, R. D. Allen and E. P. Benditt, *J. Histochem. Cytochem.*, 1974, **22**, 1105-1112. (*b*) A. Banerjee, S. K. Maji, M. G. B. Drew, D. Haldar, A. K. Das and A. Banerjee, *Tetrahedron*, 2004, **60**, 5953-5954. (*c*) D. Haldar, M. G. B. Drew and A. Banerjee, *Tetrahedron*, 2006, **62**, 6370-6381.
33. P. Divry, *J. Belg. Neurol Psychiatr.,* 1927, **27**, 643.
34. M. Reches, Y. Porat and E. Gazit, *J. Biol. Chem*., 2002, **277**, 35475-35480.
35. Y. S. Kim, T. W. Randolph, M. C. Manning, F. J. Stevens and J. F. Carpenter, *J. Biol. Chem*., 2003, **278**, 10842-10850.