β-KETOENOLE DYES: SYNTHESIS AND STUDY AS FLUORESCENT SENSORS FOR PROTEIN AMYLOID AGGREGATES

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β-ketoenole dyes: synthesis and study as fluorescent sensors for protein amyloid aggregates

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ABSTRACT

The series of the new β-ketoenole dyes ((2E,5Z,7E,9E)-6-hydroxy-2-(alkylamino)-10-phenyldeca-2,5,7,9-tetraen-4-ones) with variation of alkylamino tail groups was synthesized and studied as potential probes for the sensing of protein aggregates amyloid fibrils. The dyes are low fluorescent when free but able to increase their emission intensity in dozens of times in the presence of fibrillar insulin. The fluorescent response of the dye on fibrillar insulin strongly depends on the nature of the alkylamino tail group. For compounds with propylamino (dye 13...
and 2-hydroxyethylamino (dye 14) fragments the fluorescence intensity in the presence of fibrillar insulin exceeds that for the native one in 22 and 66 times correspondingly.

However dyes demonstrate from low or moderate exceed of the fluorescence intensity in the presence of aggregated lysozyme compared to native one (up to 8.7 times for the dye 53 bearing methyl ester tail group), due to their pronounced sensitivity to native lysozyme.

The dyes in complexes with insulin have rather height quantum yield up to 0.15, the large Stokes shifts values (about 100 nm and more), their binding constant values are about $10^5$ M$^{-1}$. The dye 14 allows fluorescent detection of the insulin amyloid fibrils in the concentration range 1-50 μg/ml. This causes an interest in the future study of the β-ketoenole as prospective fluorescent amyloid-sensitive molecules.

**KEYWORDS:** β-ketoenole dyes, fluorescent sensing, probes, amyloid fibrils, insulin, lysozime, proteins

1. INTRODUCTION

One of the most convenient methods for the analysis of biomolecules is the use of extrinsic fluorescent probes that noncovalently bind to them by electrostatic, van der Waals and hydrophobic interactions. The wide range of fluorescent molecules is developed for high efficient sensing, quantification and visualization of proteins and nucleic acids in *in vitro* and *in vivo* assays [1-4].
The spontaneous aggregation of proteins leading to formation of insoluble beta-pleated aggregates (amyloid fibrils) is among the actual targets in the biomedical researches, since these aggregates are connected with the range of harmful human diseases including neurodegenerative ones. This causes an interest in the development of new appropriate analytical tools to be used in the study of this process.

The extrinsic fluorescent probes are used for the detection and quantification of amyloid fibrils, monitoring of the kinetics of their formation and study of the factors and agents affecting these processes. For these purposes, amyloid sensitive fluorescent probes Thioflavin T and it’s derivatives are commonly applied. The histological dyes Congo Red and Chrysamine G are used for the staining and study of amyloid formations on tissues [5-7]. Recently we discovered and developed monomethine cyanines dyes as efficient fluorescent probes for the detection of protein β-pleated aggregates [8, 9]. Cyanines with high sensitivity to the amyloid fibrils and wide detection range (1.5-120 µg/ml for trimethine cyanine 7519 [10]) surpassing that of Thioflavine T were proposed. On the base of these cyanine dyes the inhibitory assay for the search of the compounds with anti-fibrillogenic activity was developed and applied. At the same time further search for the amyloid-sensitive probes with high fluorescent response to the fibrillar protein presence is still urgent.

One of the necessary requirements for the molecule to be applicable as the fluorescent probe for the amyloid formations detection is high affinity of the complex formation between this molecule and the amyloid fibril. As the most
probable mode of such complex formation, the insertion of the dye molecule into the
groove of the amyloid fibril is suggested [11]. As a result of such binding, the
fluorescent response is observed due to the quite rigid fixation of the dye molecule,
and the polarization of the absorbed light is caused by the same orientation the
bound dye molecules. Thus, in order to obtain an efficient response to the presence
of amyloid fibril, the molecule should have shape complimentary to that of the fibril
groove and the size fitting the fibril groove (about 6,5-7 Å) [12]. (Fig 1)

![Diagram](image1)

**Figure 1.** Scheme of the layout of the dye molecule in the fibrilar groove (left) [11]
and AFM image of the insulin amyloid fibrils (right) [13].

In the present work we firstly studied the β-ketoenole dyes as potential
fluorescent probes for the sensing of amyloid aggregates of proteins (Table 1). The
β-ketoenoles are the molecules of the elongated shape that is suggested as preferable
for fitting to the groove of the amyloid fibril; besides they have rather flexible
aliphatic chromophore chain providing the low intrinsic fluorescence intensity to the
unbound dye. Unlikely to the majority of the amyloid-sensitive dyes bearing either
positive (Thioflavin T, cyanine dyes) or negative (Congo Red) charge, the molecules of β-ketoenoles are uncharged.

With this aim the series of ((2E,5Z,7E,9E)-6-hydroxy-2-(alkylamino)-10-phenyldeca-2,5,7,9-tetraen-4-ones) dyes with variation of alkylamino substituents was synthesized and the fluorescent properties were characterized for the free dyes as well as in the presence of amyloidogenic proteins lysozyme and insulin in the native and aggregated form. The range of detection of the amyloid aggregates with the most efficient dye was determined. Besides, the fluorescent sensitivity of β-ketoenole dye to the serum albumin able to bind the variety of the small molecules was studied for the comparison.

*Table 1. Dye structures.*

| 13 | 14 |
|----|----|
| ![Dye 13](image1) | ![Dye 14](image2) |
| 47 | 50 |
| ![Dye 47](image3) | ![Dye 50](image4) |
| 51 | 52 |
| ![Dye 51](image5) | ![Dye 52](image6) |
2. MATERIALS AND METHODS

2.1. Synthesis and characterization of the dyes

The general procedure of ((2E,5Z,7E,9E)-6-hydroxy-2-(alkylamino)-10-phenyldeca-2,5,7,9-tetraen-4-ones) synthesis is as follows.

The dyes were prepared by mixing the 5mM 4-hydroxy-6-methyl-3-((2E,4E)-5-phenylpenta-2,4-dienoyl)-2H-pyran-2-one in 10 ml of DMF (described in [14]) with an excess of 10% amine. The mixture was heated at 100 °C for 30 minutes; during the reaction path it changed the color to red. The heating was stopped after the finishing of the carbon dioxide elimination. The cooled reaction mixture was precipitated by water. The formed product was washed twice with water and crystallized from DMF-ethanol mixture, filtered off, washed twice with ethanol on
the filter and dried. All obtained compounds are yellow-orange fine-crystalline substances.

Structures of investigated compounds were confirmed with $^1$H NMR and Anal. Calcd. (%).

(2E,5Z,7E,9E)-6-hydroxy-10-phenyl-2-(propylamino)deca-2,5,7,9-tetraen-4-one. 13. Yield 67%. Mp 255-259°C. $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 14.99 (1H, s), 10.43 (1H, s), 7.44 (2H, d, $J = 7.4$ Hz), 7.34 (2H, dd, $J = 13.4$, 6.2 Hz), 7.26 (1H, dd, $J = 9.8$, 4.7 Hz), 7.17 (1H, dd, $J = 15.0$, 10.8 Hz), 7.01 – 6.71 (2H, m), 6.01 (1H, d, $J = 15.1$ Hz), 5.20 (1H, s), 4.78 (1H, s), 3.24 (2H, dd, $J = 13.3$, 6.7 Hz), 1.98 (3H, s), 1.65 (2H, dd, $J = 14.4$, 7.2 Hz), 1.09 – 0.96 (3H, m). Found (%): C, 76.79; H, 7.74; N, 4.64; molecular formula C$_{19}$H$_{23}$NO$_2$ requires C, 76.73; H, 7.80; N, 4.71.

(2E,5Z,7E,9E)-6-hydroxy-2-((2-hydroxyethyl)amino)-10-phenyldeca-2,5,7,9-tetraen-4-one. 14. Yield 69%. Mp 273-275°C. $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 14.84 (1H, s), 10.44 (1H, s), 7.46 (2H, dd, $J = 12.9$, 7.2 Hz), 7.40 – 7.30 (2H, m), 7.30 – 7.22 (1H, m), 7.18 (1H, dd, $J = 15.0$, 10.8 Hz), 6.91 (1H, ddd, $J = 23.8$, 20.0, 13.2 Hz), 6.76 (1H, d, $J = 15.5$ Hz), 6.00 (1H, d, $J = 15.0$ Hz), 5.21 (1H, s), 4.83 (1H, s), 3.79 (2H, dt, $J = 10.7$, 5.2 Hz), 3.45 (2H, dt, $J = 11.4$, 5.6 Hz), 2.29 (1H, s), 2.01 (3H, s). Found (%): C, 72.14; H, 7.02; N, 4.63; molecular formula C$_{18}$H$_{21}$NO$_3$ requires C, 72.22; H, 7.07; N, 4.68.

(2E,5Z,7E,9E)-2-(allylamino)-6-hydroxy-10-phenyldeca-2,5,7,9-tetraen-4-one. 47. Yield 44%. M143-144°C. $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$ 15.13 (1H, s), 10.30 (1H, t, $J = 6.0$ Hz), 7.52 (2H, d, $J = 7.7$ Hz), 7.36 (3H, t, $J = 7.5$ Hz), 7.28 (1H, t, $J =$
7.3 Hz), 7.06 (2H, dd, $J = 9.2$, 4.8 Hz), 6.89 (1H, d, $J = 14.2$ Hz), 6.15 (1H, dd, $J = 14.0$, 6.4 Hz), 6.02 – 5.86 (1H, m ), 5.34 (2H, s), 4.89 (1H, s ), 3.97 (2H, t, $J = 5.6$ Hz), 1.98 (3H, s). Found (%): C, 77.11; H, 7.12; N, 4.70; molecular formula C$_{19}$H$_{21}$NO$_2$ requires C, 77.26; H, 7.17; N, 4.74.

(2E,5Z,7E,9E)-2-(3-(dimethylamino)propylamino)-6-hydroxy-10-phenyldeca-2,5,7,9-tetraen-4-one. 50. Yield 40%. Mp 121-123°C. $^1$H NMR (DMSO, 400 MHz) $\delta$ 15.19 (1H, s), 10.32 (1H, s), 7.52 (2H, d, $J = 7.5$ Hz), 7.36 (2H, t, $J = 7.5$ Hz), 7.28 (1H, t, $J = 7.3$ Hz), 7.11 – 6.98 (2H, m ), 6.88 (1H, dd, $J = 15.4$, 6.4 Hz), 6.15 (1H, d, $J = 14.0$ Hz), 5.31 (1H, s ), 4.82 (1H, s ), 3.32 (2H, dd, $J = 13.0$, 6.3 Hz), 2.25 (2H, t, $J = 6.8$ Hz), 2.13 (6H, s), 1.99 (3H, s ), 1.75 – 1.57 (2H, m ). Found (%): C, 74.02; H, 8.24; N, 8.19; molecular formula C$_{21}$H$_{28}$N$_2$O$_2$ requires C, 74.08; H, 8.29; N, 8.23.

(2E,5Z,7E,9E)-2-(sec-butylamino)-6-hydroxy-10-phenyldeca-2,5,7,9-tetraen-4-one. 51. Yield 36%. Mp 159-162°C. $^1$H NMR (DMSO, 400 MHz) $\delta$ 10.30 (1H, d, $J = 9.4$ Hz), 7.52 (2H, d, $J = 7.5$ Hz), 7.36 (2H, t, $J = 7.5$ Hz), 7.28 (1H, t, $J = 7.2$ Hz), 7.04 (2H, dt, $J = 14.3$, 7.4 Hz), 6.88 (1H, d, $J = 14.7$ Hz), 6.23 – 6.08 (1H, m ), 5.31 (1H, s ), 4.81 (1H, s ), 3.68 – 3.53 (1H, m ), 2.01 (3H, s ), 1.50 (2H, dt, $J = 14.0$, 6.9 Hz), 1.16 (3H, d, $J = 6.4$ Hz), 0.89 (3H, t, $J = 7.3$ Hz). Found (%): C, 77.22; H, 7.97; N, 4.46; molecular formula C$_{20}$H$_{25}$NO$_2$ requires C, 77.14; H, 8.09; N, 4.50.

(2E,5Z,7E,9E)-6-hydroxy-2-(isobutylamino)-10-phenyldeca-2,5,7,9-tetraen-4-one. 52. Yield 45%. Mp 170-173°C. $^1$H NMR (DMSO, 400 MHz) $\delta$ 10.44 (1H, t, $J = 5.9$ Hz), 7.52 (2H, d, $J = 7.4$ Hz), 7.37 (2H, dd, $J = 14.1$, 6.9 Hz),
7.28 (1H, t, J = 7.3 Hz), 7.12 – 6.98 (2H, m), 6.89 (1H, t, J = 11.3 Hz), 6.27 – 6.03 (1H, m), 5.32 (1H, s), 4.85 (1H, s), 3.15 (2H, t, J = 6.4 Hz), 1.99 (3H, s), 1.78 (1H, d, J = 6.4 Hz), 0.94 (6H, d, J = 6.7 Hz). Found (%): C, 76.96; H, 8.01; N, 4.39; molecular formula C_{20}H_{25}NO_{2} requires C, 77.14; H, 8.09; N, 4.50.

(2E,5Z,7E,9E)-6-hydroxy-2-((2-methoxyethyl)amino)-10-phenyldeca-2,5,7,9-tetraen-4-one. 53. Yield 65%. Mp 153-155°C. \(^1\)H NMR (DMSO, 400 MHz) \(\delta\) 15.15 (1H, s), 10.30 (1H, t, J = 5.8 Hz), 7.52 (2H, d, J = 7.4 Hz), 7.37 (2H, t, J = 7.5 Hz), 7.28 (1H, t, J = 7.3 Hz), 7.14 – 6.96 (2H, m), 6.98 – 6.80 (1H, m), 6.16 (1H, dd, J = 13.4, 6.8 Hz), 5.32 (1H, s), 4.85 (1H, s), 3.49 (4H, d, J = 6.6 Hz), 3.30 (3H, s), 2.00 (3H, s). Found (%): C, 72.89; H, 7.34; N, 4.49; molecular formula C_{19}H_{23}NO_{3} requires C, 72.82; H, 7.40; N, 4.47.

(2E,5Z,7E,9E)-6-hydroxy-2-((3-methoxypropyl)amino)-10-phenyldeca-2,5,7,9-tetraen-4-one. 54. Yield 57%. Mp 106-107°C. \(^1\)H NMR (DMSO, 400 MHz) \(\delta\) 15.18 (1H, s), 10.30 (1H, t, J = 5.8 Hz), 7.59 – 7.47 (2H, m), 7.36 (2H, t, J = 7.5 Hz), 7.28 (1H, t, J = 7.3 Hz), 7.15 – 6.97 (2H, m), 6.89 (1H, dd, J = 14.3, 7.6 Hz), 6.21 – 6.01 (1H, m), 5.31 (1H, s), 4.85 (1H, d, J = 9.5 Hz), 3.38 (4H, dd, J = 10.8, 4.7 Hz), 3.25 (3H, d, J = 2.4 Hz), 1.99 (3H, s), 1.78 (2H, dd, J = 12.7, 6.2 Hz). Found (%): C, 73.45; H, 7.65; N, 4.25; molecular formula C_{20}H_{25}NO_{3} requires C, 73.37; H, 7.70; N, 4.28.

(2E,5Z,7E,9E)-2-(heptylamino)-6-hydroxy-10-phenyldeca-2,5,7,9-tetraen-4-one. 88. Yield 62%. Mp 132-134°C. \(^1\)H NMR (DMSO, 300 MHz) \(\delta\) 15.16 (1H, s), 10.35 (1H, s), 7.56 (2H, d, J = 7.3 Hz), 7.40 (3H, t, J = 7.4 Hz), 7.31 (1H, t, J = 7.3
Hz), 7.09 (2H, dt, $J = 16.4, 11.3$ Hz), 6.91 (1H, d, $J = 14.4$ Hz), 6.27 – 6.07 (1H, m), 5.34 (1H, s), 4.87 (1H, s), 3.36 – 3.24 (2H, m), 2.02 (2H, s), 1.56 (2H, m), 1.31 (8H, m, $J = 4.6$ Hz), 0.89 (3H, m, $J = 7.1$ Hz). Found (%): C, 77.95; H, 8.68; N, 3.92; molecular formula $C_{23}H_{31}NO_2$ requires C, 78.15; H, 8.84; N, 3.96.

2.2. Insulin and lysozyme aggregates formation

Human insulin (Private Joint Stock Company «On the production of insulin «Indar», Ukraine) was dissolved at 340 $\mu$M concentration in 0,1 mM HCl solution (pH 2). Fibrils were formed by incubating the protein solution in a thermomixer incubator at 65 °C for about 5 h. To prepare lysozyme aggregates, lysozyme of hen egg white (Sigma-Aldrich Co. USA) was dissolved at 1 mM concentration in 0,1 mM HCl solution (pH 2) and incubated in a thermomixer incubator at 65 °C for 24 hours.

2.3. Preparation of dye solutions and protein solutions

$\beta$-ketoenole stock solutions were prepared by dissolving the weighted amount of the dyes at 2 mM concentration in DMSO or DMF. Working solutions of free dyes were prepared by dilution of the dye stock solutions in 50 mM Tris-HCl buffer (pH 7.9) to the concentration 2 $\mu$M. The working solutions of dye-proteins complexes were prepared by adding to the dye 2 $\mu$M solution the aliquot of monomer or fibrillar protein stock solution, the protein concentration in the working solution was thus 3,4 $\mu$M for insulin and 4 $\mu$M for lysozyme. Bovine serum albumin (BSA) was obtained from Sigma-Aldrich Co. USA. To prepare the dye-BSA working solution, to the 0.2mg/ml (3 $\mu$M) BSA solution in 50 mM Tris-HCl buffer
(pH 7.9) the aliquot of the dye stock solution was added, the dye concentration in the working solution was thus 2 µM.

2.4. Spectral measurements

Fluorescence excitation and emission spectra were registered using the fluorescent spectrophotometer Cary Eclipse (Varian, Austria). Fluorescence emission was excited at the maximum wavelength of excitation spectrum of corresponding dye solution. The quantum yield value of the dyes 14 and 50 (2 µM) in the presence of fibrillar insulin (13.6 µM) was determined using Rhodamine 6G solution in ethanol as the reference (quantum yield value 0.95) [15]. All the spectral-luminescent characteristics of unbound dyes in aqueous buffer were studied at room temperature.

2.5. Estimation of equilibrium constants of the dye-to-fibril binding

To estimate the equilibrium constant of the dyes 14 and 50 binding to fibrillar insulin, fluorescent titration of the dyes (2 µM) upon addition of 0-45 µM of fibrillar insulin was performed. Taking into account only the points for the concentrations 2 M of protein molecules and higher, we could consider the concentration of the binding sites to be much higher than this of the dyes and thus the concentration of the free protein to be roughly equal to its total concentration. Under this assumption, the equation for the equilibrium constant K of dye-fibril binding could be written as:

$$\frac{C_d}{C_{bd}} = 1 + (K \times C_F)^{-1}$$  (1)

where $C_d$, $C_{bd}$ and $C_F$ are total dye, fibril-bound dye and fibrillar protein concentrations respectively.
Further, let us consider the totally unbound and totally bound with fibrils dye solution to have the fluorescence intensity $I_0$ and $I_{\text{max}}$ respectively. In this case the measured dye fluorescence intensity $I$ at the fibrillar protein concentration $C_F$ could be written as $I = I_0 \times (C_d - C_{bd})/C_d + I_{\text{max}} \times C_{bd}/C_d$, that can be transformed into:

$$C_d/C_{bd} = (I_{\text{max}} - I_0)/(I - I_0)$$

(2)

Together with (1), (2) gives

$$I - I_0 = A \times K \times C_F/(1 + K \times C_F)$$

(3)

A being the denotation for $(I_{\text{max}} - I_0)$ difference. Thus the experimental dependence of $I - I_0$ on $C_F$ was approximated with the equation (3), $A$ and $K$ being obtained as approximation parameters. Accounting for several assumptions made, the obtained $K$ value could be regarded as a rough estimation of the binding constant value rather than its precise value. Besides it should be reminded that the estimated binding constant is only an apparent value calculated with respect to protein globule concentration and not this of the binding sites that is unknown; actually the estimation of binding constant with respect to protein globule concentration is common for the ligand-fibril binding studies.

2.6. Computer simulations of the dye 14 dimensions

To estimate the dimensions of the dye 14, geometry optimization of the dye structure was first performed using the PM3 method from the HyperChem 6.03 program package. Further the isosurface with the total charge density 0.002 that characterizes the molecular dimensions was built; linear dimensions i.e. length, height and width of the obtained isosurface were then estimated.
3. RESULTS AND DISCUSSION

3.1. Synthesis of the β-ketoenole dyes

In present work we obtained series of new compounds by the pyran ring opening reaction of 4-hydroxy-6-methyl-3 - ((2E, 4E) -5-phenylpenta-2,4-dienoyl) -2H-pyran-2-one with primary aliphatic amines. The mechanism of this reaction was studied in [14, 16], it occurs through the nucleophilic attack of the 6-carbon atom of the pyran cycle leading to its next opening and decarboxilation. We have found that on the first step of this reaction the interaction of 4-hydroxy-6-methyl-3 - ((2E, 4E) -5-phenylpenta-2,4-dienoyl) -2H-pyran-2-one with amines (Scheme 1) leads to the formation of the corresponding salt. When heated, it is dissociated and at the same time the amino group attacks a carbon atom of the methyl group in the pyran ring, causing its opening and subsequent decarboxylation [14, 16]. In the case of n-alkyl amines, formation of the corresponding salts, ring opening and decarboxylation occurs quite easily, but iso-amines react considerably worse and require higher reaction temperature.

Scheme. 1. Synthesis of the studied β-ketoenole dyes.
In the case of the tert-butylamine, the reaction stopped at the stage of the salt formation, opening of the pyran ring does not occur (Fig. 1), the behavior of α-phenyl ethyl amine is similar. This observation indirectly confirms the reaction mechanism proposed in [14, 16].

Such molecules could be divided into two parts: hydrophobic ones containing phenyl moiety and polymethine chain, and hydrophilic molecules containing ketoenol fragment and tail alkylamino group. For related compounds the existence of the set of tautomeric forms of the ketoenol fragment was established by NMR [16]. The compounds described here are also able to the formation of the tautomers. In their NMR spectra in addition to the main groups of signals there are the minor signals with similar morphology corresponding to the presence of 3-6% of admixtures. According to data of LC/MS the molecular ions of these admixtures have the same molecular weight as that of the base compound that also confirms the presence of the tautomeric forms of compounds (data not presented).

3.2. Spectral properties of dyes in buffer and in the presence of native proteins

The fluorescent characteristics of the 9 β-ketoenole compounds in buffer solution and in the presence of insulin, lysozyme and BSA in their native form are presented in Table 2.

The excitation maxima for the studied dyes in the free state are located in the range 414-428 nm; emission maxima are in the range 523–560 nm, except for the
dye 88 (emission maximum at 604 nm) and the dye 52 possessing long-wavelength emission maximum (at 606 nm) together with the short-wavelength one (545 nm). The addition of insulin, lysozyme and BSA could cause the shift of the excitation and emission spectrum maxima both to longer or shorter wavelengths for up to 35 nm. Excitation maxima in the presence of proteins are places in the range 408-435 nm respectively, while positions of the emission maxima are at 522-571 nm. Exceptions are the dye 88 with long alkyl tail that has emission bands with maxima in the range 604-611 nm respectively. For the dyes 52 (free) and the dye 47 (in the presence of insulin) emission spectra contain two bands, the long-wavelength one for the both dyes has the maximum at 606-607 nm. The large values of the shift between the excitation and emission maxima for free dyes and the dyes in the presence of proteins (98-190 nm) should be mentioned.
Table 2.

Spectral-luminescent properties of β-ketoeneole dyes in the presence of the native proteins.

| Name | free dye | with insulin | with lysozyme | with BSA |
|------|----------|--------------|---------------|----------|
|      | \( \lambda_{\text{ex}} \), nm | \( \lambda_{\text{em}} \), nm | \( I_0 \), a.u. | \( \lambda_{\text{ex}} \), nm | \( \lambda_{\text{em}} \), nm | \( I^M \), a.u. | \( I^M/I_0 \) | \( \lambda_{\text{ex}} \), nm | \( \lambda_{\text{em}} \), nm | \( I^M \), a.u. | \( I^M/I_0 \) |
| 13   | 427      | 542          | 1.7           | 413      | 542          | 3.7           | 2.2           | 427      | 525          | 17.7         | 10.4         | 427      | 536          | 1           | 0.6           |
| 14   | 426      | 539          | 1.3           | 426      | 539          | 1.6           | 1.2           | 426      | 540          | 10           | 7.7          | 410      | 540          | 7           | 5.4           |
| 47   | 415      | 545          | 2.6           | 415      | 433 / 607    | 4.7 / 7.3     | 1.8 / 2.8     | 410      | 541          | 1.9           | 7.3          | 415      | 530          | 5           | 1.9           |
| 50   | 414      | 523          | 0.8           | 427      | 526          | 2             | 2.5           | 427      | 526          | 7.7           | 9.6          | 418      | 523          | 0.8         | 1             |
| 51   | 417      | 554          | 5.5           | 415      | 539          | 6.8           | 1.2           | 415      | 543          | 10           | 1.8          | 417      | 550          | 3.8         | 0.7           |
|   | 52  | 414 | 545 / 606 | 7.5 / 15 | 415 | 553 | 6.2 | 0.8* | 415 | 571 | 9.3 | 1.2* | 408 | 555 | 5   | 0.7* |
|---|-----|-----|-----------|----------|-----|-----|-----|------|-----|-----|-----|------|-----|-----|-----|------|
|53 | 415 | 535 | 0.7       | 427      | 522 | 4.3 | 6.1 | 423  | 525 | 2.6 | 3.7 | 415  | 535 | 4   | 5.7 |
|54 | 428 | 560 | 3.6       | 420      | 536 | 4.9 | 1.4 | 427  | 525 | 17.7| 4.9 | 428  | 531 | 2   | 0.6 |
|88 | 414 | 604 | 0.7       | 416      | 604 | 0.7 | 1   | 410  | 611 | 3.2 | 4.6 | 435  | 611 | 1.5 | 2.1 |

$\lambda_{ex}, \lambda_{em}$ – maximum wavelength of fluorescence excitation and emission spectra;

$I_0 (I^M)$ - fluorescence emission intensity of the dye in free state (in the presence of native protein);

a.u. - arbitrary units.

* – $I_0$ was this at the wavelength 545 nm
As it was suggested, the fluorescence intensity of the free $\beta$-ketoenole dyes is low 0.7-7.5 a.u. The enhancement of the dyes fluorescence intensity in the presence of the native proteins does not exceed 10 times, and formed protein-dye complexes are of the quite low intensity. The dyes 51 and 52 with the branched alkyl substituent slightly change their emission upon the addition of proteins. Other dyes demonstrate the quite pronounced emission increase (in 3.7-10 times) in the presence of lysozyme. The dye 14 having the tail alkyl hyrdoxy group gives fluorescent response to the presence of both lysozyme the albumin. At the same time, the compound 53 bearing the methyl ester as tail group possesses better sensitivity to insulin and BSA than to lysozyme.

3.3. Spectral properties of dyes in the presence of fibrillar proteins
Insertion of the dye molecule into the groove of the amyloid fibril is suggested to be the most possible model of the dye-fibril binding. With the help of the computer simulation, the dimensions of the dye 14 were estimated to be about 23Å×7.8Å×4.8Å, that is characteristic for the dyes of the studied series. Thus the studied dyes fit to the fibrillar groove formed by the β-pleated structure of the fibril, the width of which is believed to be equal to 6.5-7Å [12]. Thus, we can expect the formation of the dye-fibril fluorescent complex due to the fixation of the dye molecules in the fibril groove.

The fluorescent characteristics of the 9 β-ketoenole compounds in the presence of fibrillar aggregates of insulin and lysozyme are presented in Table 3.

For the majority of the studied β-ketoenole dyes, the addition of the fibrillar insulin results in the long-wavelength shift of the excitation maximum wavelength for up to 22 nm (except of the dye 88 with the 3 nm short-wavelength shift). The short-wavelength shifts of the emission maximum wavelength for up to 26 nm were also observed for the majority of the dyes except 50 (almost no change), 88 (7-nm long-wavelength shift) and 54 (two maxima shifted to the short- and long-wavelength region with respect to the free dye were observed).

Table 3.

Spectral-luminescent properties of β-ketoenole dyes in the presence of the aggregated proteins

| Name | with fibrilar insulin | with fibrilar lysozyme |
|------|-----------------------|------------------------|
|      |                       |                        |
|    | $\lambda_{\text{ex}}, \text{nm}$ | $\lambda_{\text{em}}, \text{nm}$ | $I^F, \text{a.u.}$ | $I^F/I^M$ | $\lambda_{\text{ex}}, \text{nm}$ | $\lambda_{\text{em}}, \text{nm}$ | $I^F, \text{a.u.}$ | $I^F/I^M$ |
|---|---|---|---|---|---|---|---|---|
| 13 | 441 | 528 | 80 | 21.6 | 441 | 517 | 10 | 0.6 |
| 14 | 430 | 531 | 97 | 60.6 | 427 | 540 | 37 | 3.7 |
| 47 | 436 | 528 | 49.7 | 6.8* | 426 | 532 | 5.9 | 0.3 |
| 50 | 436 | 524 | 25 | 12.5 | 426 | 522 | 27.7 | 3.6 |
| 51 | 437 | 528 | 20.9 | 3.1 | 415 | 543 | 8.5 | 0.8 |
| 52 | 435 | 535 | 17 | 2.7 | 408 | 555 | 9.2 | 1.0 |
| 53 | 434 | 525 / 591 | 22 | 5.1 | 440 | 526 | 22.7 | 8.7 |
| 54 | 436 | 545 / 588 | 33.7 / 39.6 | 6.9 / 8.1 | 441 | 517 | 10 | 0.6 |
| 88 | 411 | 611 | 1.9 | 2.7 | 410 | 611 | 3.5 | 1.1 |

$\lambda_{\text{ex}}, \lambda_{\text{em}}$ - maxima of fluorescence excitation and emission spectra;

$I^F$ - emission intensity of dye in the presence of fibrillar form of the insulin and lysozyme;

$I^F/I^M$ - emission increasing of the dye in the presence of corresponding proteins;

a.u. - arbitrary units;

* - $I^M$ was this at the wavelength 607 nm

The presence of fibrillar insulin leads to the enhancement of the dye fluorescence intensity ($I^F$) as compared to that of both free dye ($I_0$) and the dye in the
presence of native insulin (I^M); the highest values of the I^F/I^M ratio were observed for
the dyes 13 and 14 containing “short” unbranched propylamino and
hydroxyethylamino groups as substituents (22 and 61 times respectively). The rather
high fluorescent response with I^F/I^M between 7 and 13 times was also observed for
the dyes 47, 50 and 54 containing allylamino, dimethylaminopropylamino and
methoxypropylamino substituents. The other dyes did not demonstrate significant
I^F/I^M values. It should be also mentioned that the fluorescence quantum yield value
for the dyes 14 and 50 in the presence if fibrillar insulin is rather high and equals to
0.12 and 0.15 respectively.

For the dyes 14 and 50 the titration with fibrillar insulin was performed; the
dependence of the dye fluorescence intensity on the concentration of the added
fibrils allowed us to estimate the equilibrium constant of the dye to fibril binding.
This estimation gave the values of the binding constant K = (2.7±0.9)×10^5 M\(^{-1}\) for
the dye 14 and (2.5±1.4)×10^5 M\(^{-1}\) for the dye 50, being thus of the same order of
magnitude.

As for the fibrillar lysozyme, its presence leads to the long-wavelength shift of
the excitation spectrum maximum wavelength up to 25 nm, except for the dyes 51,
52 and 88 that slightly shift their maxima to the short-wavelength shift region. At the
same time, for the emission maximum the wavelength shift was predominantly to
the short-wavelength region (up to 43 nm for the dye 54), exceptions being the dyes
14, 52 and 88 (long-wavelength shifts for up to 10 nm).
As for the emission intensity, only for three dyes 14, 50 and 53 the fluorescence sensitivity to the fibrillar lysozyme overpasses that to native protein. The $I^F/I^M$ values for these three dyes are not significant: the highest value is for the dye 53 (8.7 times), while for the dyes 14 and 50 it is about 3.7. Other compounds possess the fluorescent sensitivity to lysozyme aggregates equal to or lower than this for the native protein.

It should be mentioned that the insignificant $I^F/I^M$ value observed for the most insulin-sensitive dye 14, as well as for several other dyes in the presence of fibrillar lysozyme is partially connected with the fluorescent sensitivity of these dyes to the native lysozyme ($I^M$ value).

The binding to native lysozyme leading to the fixation of the fluorescent conformation of the molecule and thus to the fluorescent response occurs for the majority of the studied dyes. That could be explained by the high content of the charges amino acids in the structure of this protein (8 negatively charged residues and 18 positively charged ones) and thus attributed to the electrostatic interaction. Generally the β-ketoenole molecules are uncharged, so its affinity to the charged amino acid residues would be higher in the case of the molecule’s transition to the zwitterionic tautomer form (Fig. 3).

![Zwitterionic tautomer form of β-ketoenole dye](image)

*Fig. 3. Zwitterionic tautomer form of β-ketoenole dye*
It was observed that some of β-ketoenoles are even more sensitive to the native lysozyme than to its well-structured beta-pleated aggregates that formed the binding places in the fibrillar grooves. Thus we could suggest that electrostatic interaction with charged groups mainly drive the interaction of the dye molecules to the lysozyme. The reorganization of the protein structure to beta-pleated aggregates does not lead to the essential increase in the number of tightly bound and thus fluorescent molecules, and can even decrease their number.

3.4. Application of dye 14 for fibrillar insulin detection: detection limit and linear detection range

To examine the applicability of the dye 14 bearing 2-hydroxyethyl group as amyloid-sensitive probe for the quantification of amyloid insulin, we performed titration of the 2 μM dyes solutions with increasing amounts of the aggregated protein (Fig. 4). The lower limit for the fibrillar insulin detection by the dye 14 was determined as equal to the insulin concentration leading to the two times dye fluorescence intensity increase. The upper limit of the detection range was determined as the maximum concentration where the dependence of dye fluorescence intensity on the fibrils concentration is still linear. Thus the 2μM concentration of the dye 14 allows fluorescent detection of the insulin amyloid fibrils in the concentration range 0.2-9 μM (1-50 μg/ml) (R = 0.97703; Fig. 4); the upper limit of the fibrillar insulin detection by the dye 14 thus exceeds that for the amyloid-specific dye Thioflavin T (0.5-25 μg/ml) [17].
It should be mentioned also that the large shifts between the excitation and emission maxima for the fibril-bound dye (about 100 nm), good fluorescence intensity increase, rather high quantum yield and sufficient detection range make the dye 14 promising as probe for fluorescent detection of the fibrillar aggregates.

4. CONCLUSION

The series of β-ketoenole dyes was firstly synthesized and characterized, their fluorescent properties as potential probes for the sensing of amyloid aggregates of proteins were studied.

These dyes are low fluorescent when free, but have the large shift between excitation and emission maxima reaching 190 nm. The majority of dyes possess the insignificant sensitivity to the native proteins insulin and bovine serum albumin,
while their fluorescent response on the presence of native lysozyme is more pronounced (emission intensity increase up to 10 times was observed). This could be explained by the high content of the charged aminoacids in the protein and allows suggesting about high impact of the electrostatic interaction into this binding.

The sensitivity of these dyes to fibrillar proteins depends strongly of the nature of their functional substitutens. The most intensive fluorescent response on the presence of amyloid fibrils of insulin is observed for dyes 13 and 14 that carry “short” propylamino and or 2-hydroxyethylamino groups (fluorescence intensity in the presence of fibrils exceeds that for the native proteins in 22 and 60 times respectively). For the dyes containing 2-hydroxyethylamino (14) and dimethylaminopropylamino groups (50), the values of equilibrium constant of binding to fibrillar insulin were found to be $(2.7 \pm 0.9) \times 10^5 \text{ M}^{-1}$ and $(2.5 \pm 1.4) \times 10^5 \text{ M}^{-1}$ respectively. At the same time, the studied dyes demonstrate significantly lower fluorescent sensitivity to fibrillar lysozyme as compared to fibrillar insulin.

The dye 14 demonstrates the good fluorescent response and linear range (1-50 μg/ml) of amyloid fibril detection, large value of the shift between excitation and emission maxima (about 100 nm), its quantum yield in the complex with fibrillar insulin reaches 0.15. We suggest this dye as prospective fluorescent molecule for the sensing of β-pleated protein aggregates and investigation of the aggregation reaction.

Thus we suggest the β-ketoenole dyes as prospective fluorescent molecules for the design on their base of the probes for the detection of the β-pleated protein aggregates and investigation of the aggregation reaction.
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